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Neurodegeneration of the human globus pallidus in Huntington’s disease

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ABSTRACT

Huntington’s disease (HD) is characterised by variable symptoms including motor impairment, chorea, cognitive and psychiatric changes. The most pronounced and well documented neuropathology occurs within the striatum of the basal ganglia. However, there are no studies which have used detailed stereology-based research to characterise neurodegeneration of a principle target of striatal outflow, the globus pallidus (GP). This thesis extends the basal ganglia pathology studies to the external (GPe), internal (GPi), and ventral (VP) portions of the GP, to determine the extent of pallidal involvement in the pathogenesis of HD. To undertake this study, unbiased stereological quantification methods were used to investigate the overall volumetric changes, the extent of Nissl-positive pallidal neuron loss, and parvalbumin-positive pallidal soma volume changes in the GPe, GPi and VP of 8 HD and 8 control cases of the post-mortem human brain. The resulting findings were compared with HD striatal neuropathological grade, and symptom heterogeneity including quantitative symptom scores of motor impairment, chorea, cognition and mood. The results revealed substantial heterogeneity of neurodegeneration in each pallidal region in HD cases compared to control cases. The GPe is the most vulnerable to HD, highlighted by a striking reduction in overall GPe volume (54%), pallidal neuron number (59%), and pallidal cell soma volume (35%). In comparison, the GPi is less vulnerable to HD, with a smaller reduction in overall GPI volume (40%), a lesser degree of pallidal neuron loss compared to the GPe (19%), and a minor reduction in pallidal cell soma volume (21%). Furthermore, this study reports the first evidence that the VP is also affected in HD, with a reduction in overall VP volume (31%) and pallidal neuron number (24%). The correlation between the extent of GPe and GPi neurodegeneration with striatal neuropathology confirms and expands on previous studies showing early striatal enkephalinergic projection loss to the GPe, and later striatal substance-P projection loss to the GPi. Comparison of pallidal degeneration with symptom scores revealed a correlation between GPe neurodegeneration and the severity of motor impairment. GPi neurodegeneration was shown to correlate with scores relating to irritability and anxiety, signifying an additional role in limbic and mood related pathways. In the case of the VP, its correlation with Mini-Mental State Examination score and motor impairment reinforces the role of the VP in cognitive and motor networks. Our results suggest that the neurodegeneration of GPe, GPi and VP have different impacts on the motor, limbic, and cognitive functions of the basal ganglia, providing a novel perspective for understanding basal ganglia circuit dysfunction and clinical heterogeneity in HD.
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CHAPTER 1: GENERAL INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of CAG trinucleotide repeats in the HD gene on chromosome 4, which encodes a mutant protein called huntingtin (MacDonald et al., 1993). The most pronounced and well characterised neuropathology in HD occurs within the striatum of the basal ganglia, in which there is gross atrophy. This is principally due to the well documented loss of medium spiny GABAergic projection neurons in the striatum, which follows a dorsal-ventral gradient (Vonsattel et al., 1985, Vonsattel et al., 2008). Efferent fibres from the dorsal striatum converge towards the globus pallidus (GP), where they constitute a massive fibre system that synapse and traverse through the external (GPe) segment (via the indirect motor circuit) and internal (GPi) segment (via both the indirect and direct motor circuits). Furthermore, the ventral pallidum (VP) was first identified in 1975 as a primary output for the ventral striatum, and is an important component of the limbic circuit.

Evidence for the role of the GPe and GPi in HD have come from immunohistochemical studies of striatal projections to these regions, and neuroimaging studies of the pallidum. Of the three major output pathways of the striatum, the enkephalin-containing indirect pathway projecting to the GPe is involved predominantly in early pathological grades of the disease, based on the loss of enkephalin immunoreactivity from striatal efferent terminals in the GPe (Reiner et al., 1988, Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009). The loss of substance-P-containing direct pathway striatal projections to the GPi proceeds far more gradually in comparison (Deng et al., 2004). These results have been confirmed by a study from our group (Allen et al., 2009). Clear hypotheses have been raised to relate the early loss of enkephalinergic indirect pathway neurons to the symptomatology of HD (Albin et al., 1989, Deng et al., 2004). Loss of the indirect pathway to the GPe, with sparing of the direct pathway, could result in imbalance of these pallidal circuits, favouring decreased GABA in the motor thalamus of the direct pathway, with loss of surround inhibition generated by the indirect pathway. This is postulated to result in the appearance of uncontrolled, chorea movements, a characteristic feature of HD (Albin et al., 1989, Hedreen and Folstein, 1995, Deng et al., 2004). Considerable loss of the substance-P striato-GPi direct pathway projection neurons may contribute to the dystonia in later HD, while the near complete loss of this projection system by grade 4 is associated with akinesia in terminal HD (Albin et al.,
1989, Deng et al., 2004). Neuroimaging studies have reported severe atrophy of the globus pallidus in HD patients (Aylward et al., 1997, Rosas et al., 2003, Fennema-Notestine et al., 2004, Douaud et al., 2006). However, in vivo imaging studies have not specifically isolated the GPe, GPi and VP separately, possibly due to the limitations in spatial resolution with imaging techniques such as MRI, in addition to difficulties with regional delineation without boundary markers. These studies suggest that the pattern of striatal degeneration, and subsequent differential vulnerability of striatal medium spiny neuron projections to degeneration, may have a differential impact on their output targets. Since the main output targets are the pallidal subdivisions, it is important that a detailed anatomical investigation into the impact of striatal degeneration on each pallidal region is carried out.

Despite the extensive literature examining the implications of HD on the striatum, very little exists with regard to the impact of striatal cell loss on the main neurons that receive striatal input, pallidal neurons. Currently, there is a disagreement in the literature about the extent of pallidal neuron loss in the GP in HD. In 1976, Lange et al reported a quantitative loss of pallidal neurons in the GPe and GPi in HD (Lange et al., 1976). In contrast, a more recent study of the GPe and GPi in HD by Wakai et al reported no such loss of pallidal neurons (Wakai et al., 1993). However, the study by Lange et al was carried out in the 1970’s before design-based stereological techniques were used, utilised a small sample size with limited clinical information, did not use immunohistochemical markers to delineate the regions, and was conducted prior to the establishment of the striatal neuropathological grading criteria. Furthermore, the more recent study by Wakai et al also used non-stereological techniques, a small predetermined and biased selection of sections, and a small sample size. Thus, there is a disagreement in the literature about the extent of pallidal neuron loss in the GPe and GPi in Huntington’s disease.

Furthermore, there is a lack of stereological knowledge detailing the relationship between GPe and GPi neurodegeneration, with both striatal neuropathological grade and symptom heterogeneity. Most importantly, because the indirect enkephalinergic pathway from the striatum to the GPe and the direct substance-P pathway from the striatum to the GPi are differentially affected in the disease, this present study focuses on detailed stereological characterisation of the GPe and GPi nuclei in HD, to further understand the HD disease mechanism on the basal ganglia circuitry.

Despite the single gene aetiology of HD, there is considerable phenotypic variation in the pattern of symptoms exhibited by each individual. For example, some patients show major motor
dysfunction at clinical onset with minimal changes in mood or cognition, while others show major mood and cognitive related changes with minimal motor dysfunction until late stages of the disease (Myers et al., 1991, Claes et al., 1995, Zappacosta et al., 1996, Thompson et al., 2002, Tippett et al., 2007). What is interesting about the striatum in terms of HD pathology, is that the ventral (limbic) portion, which comprises the nucleus accumbens as its major component, appears to be relatively preserved in HD compared to the dorsal (motor) component (Vonsattel et al., 1985, Kassubek et al., 2004). However, no current evidence exists in the literature with regard to the fate of the main output structure of the ventral striatum in HD, the VP (Heimer and Wilson, 1975, Haber et al., 1990b). Although psychiatric symptoms in HD have been reported to affect 35-75% of HD individuals (van Duijin et al., 2007), nothing is known about the role of the VP in HD, despite being recognised as an integrator of sensory, emotional, and cognitive information with appropriate motoric responses largely garnered from studies in laboratory animals (Smith et al., 2009). These functional complexities are reflected in human neuroimaging and primate behaviours associated with pain experiences (Zubieta et al., 2002), reward-motivated function (Cilia et al., 2008), and social interaction and affiliation (Bales et al., 2007). Therefore, studies into the role of the VP in such a dynamic disease, such as HD; with the triad of motor, cognitive, and psychiatric impairments, might help to better understand the role of this structure in a disease with extensive heterogeneity in mood and motor symptoms.

The primary goal of this study is to investigate in HD, the overall volumetric changes, the extent of pallidal neuron loss, and neuronal soma volume changes in the GPe, GPi and VP. To better understand any links between pallidal neurodegeneration and other aspects of HD, the findings will be compared with: (1) striatal neuropathological grade, (2) various clinical data including CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, disease duration; and (3) symptom heterogeneity and clinical assessments. It is hoped that this study will provide a clear understanding of the extent of neurodegeneration for each pallidal structure in HD. In addition, it is hoped this research will aid in better understanding the anatomical-functional relationships in the human brain, through exploring the relationship between striatal degeneration and pallidal degeneration in HD. Furthermore, it is also hoped that studying three functionally different structures which interconnect with different parts of the basal ganglia circuitry will also aid in understanding symptom heterogeneity in HD. The literature review below outlines the main features of HD, with a focus on the role of basal ganglia pathways implicated in the disease, in addition to an outline of the neuroanatomical architecture of each pallidal segment with relevance to basal ganglia connectivity and possible roles in HD.
CHAPTER 2: LITERATURE REVIEW

2.1 Huntington’s disease

2.1.1 Introduction to Huntington’s disease

Huntington’s Disease (HD) is a debilitating autosomal dominant neurodegenerative disorder characterised by affective, cognitive, behavioural and motor dysfunctions (Wilson et al., 1987, Albin and Tagle, 1995, Nance, 1998, Margolis and Ross, 2003, Walker, 2007). HD aetiology was identified as a CAG (cytosine-adenosine-guanosine) trinucleotide repeat expansion in the \textit{IT15} gene (Interesting Transcript 15) on chromosome 4, which encodes a mutant cytoplasmic protein called \textit{huntingtin}, resulting in an abnormally long polyglutamine tract in the protein N-terminus (MacDonald et al., 1993). The disease affects approximately 7/100,000 individuals in New Zealand (Macleod, 1994). Worldwide, HD has a prevalence of 5-10 per 100,000 in South America, North America, Australia, and most European countries, but a lower prevalence of 0.5:100,000 in Asia, and even lower in Africa (Walker, 2007). Depending on the individual CAG expansion, HD onset usually ranges from 35-44 years of age. However, special cases have been reported for ages as young as 4 and as old as 80 years (Haslam et al., 1983, Harper, 1991). Typically, after the onset of symptoms, the lethal progression and death from the disease is 10 to 20 years (Reiner et al., 2011). In terms of neuropathology, characteristic features include pronounced degeneration in the neostriatum of the basal ganglia, which includes the caudate nucleus and putamen, in addition to pronounced cortical atrophy (Vonsattel et al., 1985, Vonsattel et al., 2008, Thu et al., 2010). The most prominent cell loss occurs in the neostriatum, affecting mainly the dominant cell type, the GABAergic medium sized spiny projection neurons (MSNs) (Seto-Ohshima et al., 1988, Goto et al., 1989, Ferrante et al., 1991).

However, despite the single-gene aetiology of HD there is remarkable variability in the types of motor, behavioural and cognitive symptoms present in different HD cases both at clinical onset and thereafter during the disease. As a result, there is considerable interest in whether there are any underlying pathological differences in HD brains which may account for symptom variability. Many studies have illustrated that there is a preferential pattern of MSN loss, which
leads to the preferential vulnerability and dysfunction of certain striatal pathways during the progression of HD, and attempted to relate this differential loss to HD symptomatology (Crossman, 1987, Albin et al., 1989, Hedreen and Folstein, 1995, Deng et al., 2004). However, in order to understand the functional roles which may underpin the characteristic phenotypic manifestation in HD, a clear understanding of brain regions which receive striatal input, such as the globus pallidus, will aid in further understanding the basal ganglia circuitry involvement in HD. While there is currently no cure, understanding the neuropathology in brain regions implicated in functions which are impaired in HD, will aid in understanding the heterogeneity of symptoms.

2.1.2 Genetic features

The gene for HD, known as IT15 (“Interesting Transcript” 15) is located on the tip of the short arm of chromosome 4, with a small segment of 4p16.3 identified as the site of mutation by the Huntington’s Disease Collaborative Research Group (Gusella et al., 1983, MacDonald et al., 1993). The genetic mutation is linked with the expansion of a CAG (cytosine-adenosine-guanosine) trinucleotide repeat, which codes for glutamine within the N-terminal coding region of the HD gene, encoding a mutant ~350 kDa protein termed huntingtin (htt) containing an expanded stretch of polyglutamine repeats, as is highlighted in Figure 2.1.

The inheritance pattern of HD proceeds via autosomal dominance, with unstable, polymorphic expression of CAG repeats that alter during meiosis (Myers et al., 1993). Normal individuals tend to have CAG repeats of 36 or less, and will not develop HD. However, incomplete penetrance with regard to the HD phenotype can occur at 36-39 repeats, whereas complete full penetrance of the HD phenotype will occur when the repeat length reaches more than 39 repeats. The majority of adult-onset HD patients have 40 to 55 CAG repeats, with expansions of greater than 70 being associated with juvenile HD (MacDonald et al., 1993, Rubinsztein et al., 1996, McNeil et al., 1997, Vonsattel and DiFiglia, 1998, Walker, 2007). An interesting phenomena of genetic anticipation can also occur, in which the age of onset of HD can become earlier in successive generations due to an expansion of an allele in the borderline normal range (28 to 35 repeats), usually on the paternal side (Trottier et al., 1994, Kremer et al., 1995, Walker, 2007).

One of the most striking features of HD is the robust inverse correlation between the age of disease onset and the number of CAG repeats. Studies also suggest that the length of the CAG
repeat is proportional to symptom severity, age of death, and striatal neuropathological grade. Although difficult to confirm, some data also suggest that the rate of disease progression might be faster with longer CAG repeats (Andrew et al., 1993, Snell et al., 1993, Claes et al., 1995, Penney et al., 1997, Foroud et al., 1999, Squitieri et al., 2002, Mahant et al., 2003, Andreson, 2007, Langbehn et al., 2010). It has also been shown that there is significant heterogeneity in disease onset amongst HD individuals, with the CAG repeat accounting for only 44-65% of the variance in age of onset, indicative of other considerations including modifier genes, and environmental influences which may play a role in disease progression and the age of onset (Andrew et al., 1993, Snell et al., 1993, Li et al., 2003, Djoussé et al., 2004, Wexler et al., 2004).

2.1.3 Huntingtin and pathogenesis of HD

Although many leads have been uncovered, the precise pathogenesis from the genetic mutation to neuronal dysfunction in HD is yet to be established. *Huntingtin* is a widely expressed predominantly cytoplasmic protein of unknown function found heterogeneously in neurons throughout the brain (Persichetti et al., 1994, DiFiglia et al., 1995, Gutekunst et al., 1999). In HD, both normal and mutant alleles are expressed, and both gain of function alterations, in which mutant *huntingtin* is toxic, and loss of function alterations, in which suppression of normal *huntingtin* functions might also be toxic, have been identified (Zuccato et al., 2001, Cattaneo et al., 2005).

Proteolysis of mutant *huntingtin*, whereby an abnormal and ultimately toxic N-terminus fragment of the protein is released, may contribute to causing HD (Goldberg et al., 1996, Wellington et al., 1998, Wellington et al., 2002). *Huntingtin* fragments have been shown in humans, in animals, and cell models to form protein aggregates in the nucleus, cytoplasm, and neuronal processes (Davies et al., 1997, Gutekunst et al., 1999). These aggregates induce chaperone expression and become ubiquitinated yet persist, indicating protein misfolding and failed proteolysis (Paulson, 1999). *Huntingtin* aggregates can also sequester other variable proteins, including chaperones (Jana et al., 2000), proteasomal proteins (Chai et al., 1999), normal *huntingtin* proteins (Kazantsev et al., 1999) and transcription factors (Steffán et al., 2000, Holbert et al., 2001, Nucifora Jr et al., 2001, Steffán et al., 2001), and thereby, disturb protein homeostasis. These protein aggregates can be detected in post-mortem brains of individuals at risk of developing HD who died prior to symptom presentation, and in brains of individuals who died throughout the course of HD (DiFiglia et al., 1997, Gutekunst et al., 1999, Gómez-Tortosa
et al., 2001). While the role of huntingtin aggregates continues to be debated (Perutz and Windle, 2001). Most literature points to a proximal toxicity residing in mutant huntingtin, or its proteolytic fragments, and its interactions with other proteins that have been shown to associate with huntingtin or huntingtin aggregates; these include a number of transcription factors that have been implicated in widespread transcriptional alterations that appear in HD.

Mutant huntingtin could also trigger direct negative effects on mitochondria through physical interaction with the huntingtin protein or its fragments. Mutant huntingtin has been localised to neuronal mitochondrial membranes, where incubation of normal mitochondria with mutant mitochondria leads to depolarisation of the mitochondrial membrane at lower calcium loads (Fernandes et al., 2007, Lim et al., 2008). Neuronal dysfunction could result secondary to disruption of the mitochondrial membrane potential, excitotoxic-induced calcium influx and diminished ATP production (Oliveira, 2010). Mitochondrial dysfunction and secondary energy (ATP) depletion could ultimately trigger uncontrollable free radical production and eventual cell death (Brouillet et al., 1995, Browne and Beal, 2006). There is wide evidence in human HD and in cellular and animal models suggesting that free radicals then cause cellular damage and upregulated antioxidant responses in the brain and periphery (Beal et al., 1990, Beal, 1992, 2000). Huntingtin also seems to be involved in axonal trafficking (Rong et al., 2006), which has been evidenced in in vitro assays, where mutant huntingtin impairs vesicle and mitochondrial trafficking in transgenic HD mice and in mammalian neurons (Trushina et al., 2004, Orr et al., 2008). These alterations are present prior to the onset of HD clinical symptoms, which could support the possible role of altered mitochondrial localisation in axons in HD. Thus, taken together, these studies suggest that cleavage of mutant huntingtin protein and aggregation may lead to neuronal cell death via several proposed mechanisms including transcriptional dysregulation, mitochondrial dysfunction, and impaired axonal transport, which is summarised in Figure 2.1.
The HD allele with more than 36 CAG repeats in the *IT15* gene produces a mutant ~350 kDa protein termed *huntingtin* (*htt*), which contains an expanded stretch of polyglutamine repeats. This mutant *htt* protein may lead to cell death via many mechanisms. Several studies suggest that cleavage of mutant *htt* protein and aggregation may lead to neuronal cell death via several proposed mechanisms including transcriptional dysregulation, mitochondrial dysfunction, and impaired axonal transport. Each process could directly influence mutant *htt* and contribute to neuronal dysfunction autonomously from other processes. Furthermore, each process could modulate other processes, enhancing neuronal dysfunction through many pathways.
2.1.4 Clinical features and symptom variability in HD

Huntington’s disease is characterised by a “triad” of symptoms which advance rapidly to affect motor control, mood changes, and cognitive deficits (Nance, 1998, Margolis and Ross, 2003, Walker, 2007). The HD symptoms were first described clinically by George Huntington in 1872 (Huntington, 1872, Neylan, 2003). The symptoms usually manifest during mid-life with the disease duration typically being from 10 to 20 years (Reiner et al., 2011). However, cases have been reported of symptoms emerging from early childhood to late in life (Nance, 1998, Walker, 2007).

Despite the aetiology of HD as a single-gene disorder, there is considerable variability in the types of motor, behavioural and cognitive symptoms both at clinical outset and thereafter during disease progression (Claes et al., 1995, Waldvogel et al., 2012). Observations in monozygotic twins who have inherited identical HD genes, and also have the same CAG repeat length, remarkably have an age of onset within several years of one another and exhibit differences in their symptoms (Georgiou et al., 1999, Friedman et al., 2005, Gomez-Esteban et al., 2006). Some HD cases present predominantly motor dysfunction at clinical outset, and no changes in mood for prolonged periods while, at the other extreme, others may present with predominantly mood and/or cognitive disturbances, with minimal involuntary movements until advanced stages of the disease (Andrew et al., 1993, Claes et al., 1995). However, others still present with the marked classical “triad” of symptoms simultaneously (Brandt and Butters, 1986, Folstein, 1989, Claes et al., 1995, Zappacosta et al., 1996, Thompson et al., 2002). The onset of clinical symptoms is generally correlated with CAG repeat number (Wexler et al., 2004), as is the severity of symptoms, but there is no clear relationship between CAG repeat number and the type of symptoms presented (MacMillan et al., 1993, Telenius et al., 1994, Claes et al., 1995, Zappacosta et al., 1996).

Motor Dysfunction

Typically, the onset of motor symptoms is used to define the clinical onset of HD (MacDonald et al., 1993, Waldvogel et al., 2012). The motor abnormalities associated with HD affect both involuntary and voluntary actions. Development of an involuntary “choreoathetotic” disorder, which involves rapid, irregular, involuntary choreiform (Latin for “dance-like”) movements, are one of the most distinct and well recognised symptoms of HD, which was documented in George Huntington’s original description of the disease in 1872 (Huntington, 1872). Chorea is characterised by sudden, quick, unintended movement of almost any part of the body, including
the face and limbs (Folstein, 1989). Although the presence of chorea is useful for the diagnosis of HD, it is a poor marker of disease severity. The majority of patients display hyperkinetic movements such as chorea, which tend to peak in frequency and amplitude about ten years after the age of onset, followed by a plateau or lessening in the extent of hyperkinetic movement (Kovach and Stearns, 1993). Analyses of HD populations have shown that approximately 50-70% of cases present with chorea at onset (Di Maio et al., 1993, Witjes-Ané et al., 2002). The hyperkinetic syndrome is progressively replaced by a more hypokinetic (akineti- rigid) syndrome which involves bradykinesia, rigidity and dystonia. Furthermore, the patient’s ability to speak and swallow is also affected, which can leave the patient susceptible to aspiration pneumonia secondary to dysphagia, and is the most common cause of death in HD (Young et al., 1986, Folstein, 1989, Berardelli et al., 1999, Mahant et al., 2003, Walker, 2007, Waldvogel et al., 2012).

**Cognitive impairment**

In terms of cognitive impairment, the symptoms begin early and become more severe as the disease progresses. Deficits in cognition tend to begin with the loss of mental flexibility and a gradual decline of intellectual processes that can lead to profound dementia. Early cognitive deficits included difficulties in concentration, dysfunction in short-term memory, and executive function impairment (Snowden et al., 2002, Ho et al., 2003, Montoya et al., 2006). As HD disease progression continues, these cognitive deficits can advance into widespread dementia, in addition to verbal language deterioration and difficulties in visuospatial function (Lawrence et al., 2000, Kirkwood et al., 2001, Montoya et al., 2006).

**Mood, behavioural and psychiatric changes**

Behavioural and psychiatric symptoms in HD are common, with 30-50% of symptom presentation at disease onset being behavioural and emotional problems such as irritability, aggression, anxiety, and obsessive behaviour, and most commonly, depression (Di Maio et al., 1993, Witjes-Ané et al., 2002). There is a vast range of mood and psychiatric symptoms including depression, dysphoria, agitation, irritability, labile mood, apathy and anxiety (Rosenblatt and Leroi, 2000, Paulsen et al., 2001). In addition, other symptoms can include obsessive-compulsive behaviours, sleep disturbances, personality changes, psychosis, and suicidal ideation, with suicide being estimated in patients with HD to be about 5 to 10 times that of the general population (about 5-10%) (Robins Wahlin et al., 2000, Anderson et al., 2001, Baliko et al., 2004, Beglinger et al., 2007, Walker, 2007).
2.2 The Basal Ganglia

2.2.1 Normal functional anatomy and organisation of the basal ganglia

The term “Basal Ganglia” refers to a group of large subcortical nuclei in the base of the forebrain involved in many processes including motor, associative, cognitive and mnemonic functions (Bolam et al., 2000). The nuclei of the basal ganglia were initially considered to be the principal components of the “extrapyramidal system” and by convention, the term basal ganglia was restricted mainly to the striatum, (comprising of the caudate nucleus and putamen), globus pallidus external and internal segments (also termed lateral and medial segments), the subthalamic nucleus, and the substantia nigra. The striatum is divided into the caudate nucleus (CN), which rostrally forms a head, more centrally a body, and posteriorly a tail region which extends dorsally, and, the putamen. The globus pallidus is divided into two parts; the external segment of the globus pallidus (GPe) and the internal segment of the globus pallidus (GPi). The subthalamic nucleus (STN) lays ventral to and intermediate between the GPe and GPi. The substantia nigra consists of two parts, the substantia nigra pars reticulata (SNr) which is located ventral in the midbrain, and the substantia nigra pars compacta (SNC), which in humans and primates is pigmented (Carpenter et al., 1976, Smith et al., 1998b, Bolam et al., 2000, Nieuwenhuys et al., 2008). The revolution of anatomical methods has redefined the basal ganglia from the “extrapyramidal” motor system, to a feed-forward part of a closed loop connecting all cortical areas sequentially through the striatum, pallidum, and thalamus back to the frontal cortex. This revised view involving the direct and indirect pathways of information flow through the basal ganglia will be covered in section 2.2.2.

The main cortical inputs into the striatum principally use the excitatory neurotransmitter, glutamate (Young et al., 1983, Graybiel and Penney, 1999). In contrast, the internal connectivity of the basal ganglia and the outputs predominantly use the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). GABA is the main inhibitory neurotransmitter used by the majority of neurons within the basal ganglia including the striatal, pallidal and SNr projection neurons, in addition to the projections from these structures out of the basal ganglia, including to the thalamus and brainstem (Parent, 1986, Chevalier and Deniau, 1990). Thus, classically speaking, in terms of principal neurotransmitters, the circuits within the basal ganglia are simple, as the input paths are mainly excitatory and the output paths are mainly inhibitory (Graybiel and Penney, 1999).
However there are exceptions, within the striatum there are a group of interneurons that use acetylcholine, neurons within the STN that use the excitatory neurotransmitter glutamate, and neurons of the SNc that use dopamine (Smith and Parent, 1988, Albin et al., 1989, Nieuwenhuys et al., 2008).

Finally, unlike the “motor-centric” view of the basal ganglia as simply an extrapyramidal motor system, current thinking emphasises the role of the basal ganglia in integrating the cognitive and limbic domains with the motor domain (Haber and Knutson, 2010). The concept of the basal ganglia has been widened by Heimer et al (Heimer and Wilson, 1975, Heimer et al., 1982, Heimer et al., 2007). The striatum, STN, GP and SN are now known to comprise the “dorsal division” of the basal ganglia, which plays a predominant role in associative and motor functions (Bolam et al., 2000, Nieuwenhuys et al., 2008). There is also a ventral division of the basal ganglia which is more associated with limbic and mood functions. The ventral division consists of the ventral striatum or nucleus accumbens, which closely resembles the caudate nucleus and putamen both cytoarchitectonically and histochemically; and the rostral portion of the substantia innominata, which represents the ventral extension of the globus pallidus, called the ventral pallidum (VP) (Bolam et al., 2000, Heimer et al., 2007, Nieuwenhuys et al., 2008, Haber and Knutson, 2010).

2.2.2 Basal ganglia pathways

The basal ganglia is integrated into connectional forebrain loops which form cerebro-cortical/basal ganglia/thalamo/cerebro-cortical circuits (Nauta and Domesick, 1984). The projections in the loops from several functionally segregated parallel and interconnected systems. The main functional loops include the motor “direct” and “indirect” circuits, and the limbic circuit (Alexander and Crutcher, 1990, Parent and Hazrati, 1995, Gerfen and Wilson, 1996, Smith et al., 1998a, Wilson, 2004, Nieuwenhuys et al., 2008). A summary of the cortico-basal ganglia-thalamo-cortico loops are shown in Figure 2.2.

*Indirect and direct motor circuits (Figure 2.2, Panel A and B)*

The cortex provides major excitatory glutamatergic extrinsic input into the striatum (caudate nucleus and putamen) (Carpenter et al., 1976). The main flow of cortical information through the motor circuits of the basal ganglia are termed the “direct” and “indirect” pathways (Albin et al., 1989, Alexander and Crutcher, 1990, DeLong, 1990, Parent and Hazrati, 1993, Smith et al., 1998a, Graybiel and Penney, 1999). Understanding these pathways is critical to understanding the
pathophysiology of HD. According to these pathway models, cortical information (originating from the premotor, supplementary motor and primary motor cortices), which is passed to the striatum, is processed and transmitted through the basal ganglia via two main routes. First, a **direct** GABAergic inhibitory input flows from the striatum to the output nuclei of the basal ganglia, which includes the GPi (**direct** pathway). Secondly, an **indirect** output from the striatum to the GPe which in turn, provides an inhibitory input to the STN, which projects an excitatory output to the GPi (**indirect** pathway). The **indirect** pathway can be viewed as a polysynaptic disinhibitory pathway through the GPe and glutamatergic STN. Therefore, the **direct** and **indirect** pathways converge on the GPi, which provides an inhibitory output projection to the ventral anterior and ventral lateral (VA/VL) nuclear regions of the thalamus. The VA/VL thalamic nucleus projects an excitatory input mainly to the frontal and premotor cortex, which subsequently influences the motor cortical output (Mehler, 1971, Faull and Mehler, 1978, Kayahara and Nakano, 1996).

The GPe is a component of the **indirect** circuit which relays striatal inputs towards the STN (Kita, 2007). In comparison, the GPi receives input from the striatum, GPe and STN in both the **indirect** and **direct** circuits, and projects the information outside the basal ganglia (Nambu, 2007). This highlights the fact that these nuclei have a differential involvement in the basal ganglia circuitry. In both of the **indirect** and **direct** pathways the excitatory cortico-striatal projection terminates onto striatal medium spiny projection neurons (MSNs), which are the principal neurons of the striatum. A dramatic set of findings made in the 1980’s found that the cells of origin leaving the striatum towards the **direct** and **indirect** pathways express different neuropeptides that coexist with GABA (Haber and Elde, 1981, Haber and Watson, 1985, Graybiel, 1986, Mai et al., 1986). The striatal efferents passing through the GPi arise from MSNs which contain GABA as the principal neurotransmitter and the neuropeptide substance-P (Beach and McGeer, 1984, Haber and Watson, 1985, Mai et al., 1986). In contrast, striatal efferents to the GPe arise from MSNs which contain GABA and the neuropeptide enkephalin (Haber and Elde, 1981, Haber and Watson, 1985, Maneuf et al., 1994). The two classes of MSNs can also be subdivided based on the kinds of dopamine receptors they express. The D1 dopamine receptor class is mainly expressed on substance-P containing MSNs, which form part of the **direct** circuitry, whereas the D2 class is specifically expressed on enkephalin-containing MSN’s, forming part of the **indirect** circuit (Surmeier et al., 1996). In essence, MSNs which express D1 receptors and substance-P project to the GPi, whereas those expressing D2 receptors and enkephalin project towards the GPe (Gerfen et al., 1990, Gerfen, 1992, Surmeier et al., 1996).
The *indirect* and *direct* pathways have apparently opposing effects upon the output nuclei and the thalamic target nuclei. In the *indirect* pathway, the excitatory glutamatergic cortico-striatal projection terminates onto striatal MSNs that contain GABA, enkephalin (ENK), and dopamine D2-class receptors, thereby reducing the output of GPe GABAergic activity. This leads to the decreased inhibition (or disinhibition) of STN neurons, triggering an increase in glutamatergic activation of the GPi and SNr output regions. The increase in GPi activation reduces thalamic activation of the cortex. Activation of the *indirect* circuit leads to a “negative feedback” as opposed to the “feed forward” effect of *direct* circuit activation (Figure 2.2, panel A). In the *direct* pathway, the excitatory glutamatergic fibres of the cortico-striatal projection terminate onto striatal MSNs that contain GABA, substance-P, and dopamine D1-class receptors. This increased activity of GABAergic MSNs exerts an inhibitory influence on GPi and SNr neurons. Because the neurons of the GPi and SNr are GABAergic (inhibitory), their inhibition leads to an increase in activity of the glutamatergic thalamo-cortical neurons onto which they synapse. Thus, activation of the *direct* pathway is opposite to that of the *indirect* pathway, supporting thalamo-cortical interactions via a “positive feedback” mechanism. This emphasises that excitation of *direct* pathway MSNs by cortical input leads to *reinforcement*, rather than reduction in activity of the motor cortex (Figure 2.2, panel B) (DeLong and Georgopoulos, 1981, Penney and Young, 1986, DeLong, 1990, Gerfen, 1992, Flaherty and Graybiel, 1994, Gerfen and Wilson, 1996, Smith et al., 1998b, Wilson, 2004). This completes the cerebro-cortical/basal ganglia/thalamus/cerebro-cortical motor circuit.

**Limbic circuit (Figure 2.2, Panel C)**

In comparison to the dorsal components of the basal ganglia circuitry and their involvement in the direct/indirect motor circuits, the ventral striatum (also known as the nucleus accumbens), combined with the ventral pallidum (VP), form part of a loop system involved in the regulation of mood and emotion (Heimer, 1978, Heimer et al., 1982, Alexander and Crutcher, 1990, Haber and Knutson, 2010, Petrasch-Parwez et al., 2012). The ventral striatum receives excitatory glutamatergic input from the orbital and medial prefrontal (OMPFC) cortical areas (Yeterian and Pandya, 1991, Carmichael and Price, 1996, Chiba et al., 2001, Heimer et al., 2007). The GABAergic ventral striatal regions that receive fibres from the OMPFC, in turn, project to the GABAergic VP (Heimer, 1978, Haber et al., 1990a). This information from the VP then projects towards the mediodorsal (MD) thalamic nucleus, and subsequently back to the OMPFC (Figure 2.2, Panel C) (Haber et al., 1993, Parent et al., 1999, Öngür and Price, 2000).
Figure 2.2 Schematic diagrams of motor "indirect", "direct" and limbic cortico-basal ganglia-thalamo-cortical loops

The projections in the cortico-basal ganglia-thalamo-cortical loop form several functionally segregated parallel and interconnected systems. The main functional loops include the motor "indirect" (A) and "direct" (B) circuits, and the limbic (C) circuit. In the indirect pathway (A), the excitatory cortico-striatal projection terminates onto striatal medium spiny neurons that contain GABA/ENK. The striatal output first passes to the inhibitory GPe and then to the GPI via the excitatory STN, whereby disinhibition of the STN neurons reduces thalamic activation of the cortex. In the direct pathway (B), the cortical excitatory fibres terminate on striatal projection neurons that contain GABA/SP which project to the GPI and SNr. These result in inhibition of the GPI and SNr, and disinhibition of the VA/VL thalamic output to the cerebral cortex. Thus, the result of cortical activation in the direct pathway is opposite to that of the indirect circuit: reinforcement rather than reduction of cortical activity to generate movement. In the limbic circuit (C), the ventral (limbic) striatum, combined with the VP, form part of a loop system involved in the regulation of mood and emotion. The GABAergic ventral striatum receives excitatory glutamatergic input from orbital and medial PFC. The ventral striatum in turn projects to the GABAergic VP. This information from the VP then projects towards the MD thalamic nucleus, and subsequently back to the orbital and medial PFC. The continuous and dotted lines indicate excitatory and inhibitory pathways, respectively. ENK, enkephalin; SP, substance-P; GPe, globus pallidus external segment; GPI, globus pallidus internal segment; VP, ventral pallidum; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral thalamic nuclei; MD, medial dorsal thalamic nucleus; Orbital and medial PFC, orbital and medial prefrontal cortex.
2.3 The Globus Pallidus

2.3.1 Introduction to the globus pallidus

The globus pallidus or pallidum (GP) is the principle target of striatal outflow and is a core structure of the basal ganglia. It is a triangular mass of cells which lies along the medial aspect of the putamen. The GP is separated from the putamen by a fibre sheet, known as the lateral medullary lamina. Furthermore, the GP is divided by a medial medullary lamina into the globus pallidus external (GPe) and internal (GPi) segments, where the GPi can be further divided into inner and outer parts. The ventral pallidum (VP) has been distinguished from the dorsal GPe and GPi, based on its close connections with limbic structures, with the anterior commissure serving as an anatomical border. The term “globus pallidus” comes from the pale appearance of the GP, which is due to the low density of neurons surrounded and encapsulated by large numbers of myelinated axons (white matter) (Difiglia and Rafols, 1988, Nieuwenhuys et al., 2008, Goldberg and Bergman, 2011). The GPe and GPi receive mainly sensorimotor and associative cortical information via the indirect and direct motor circuits involving the dorsal striatum. As components of the dorsal striatopallidal system, the GPe and G Pi both play a predominant role in initiating motor activities. In contrast, the VP largely receives limbic cortical information via the ventral striatum, and as part of the ventral striatopallidal system, the VP has a role in regulating emotion and initiating movements in response to emotional or motivation stimuli (Heimer et al., 1982, Alexander et al., 1986, Alexander and Crutcher, 1990, Joel and Weiner, 1997, Kita, 2007, Nambu, 2007, Haber and Knutson, 2010).

The neuronal population in the GP comprises mainly of GABAergic pallidal projection neurons, which suggests that they have an inhibitory effect on their target neurons (Penney and Young, 1981, Oertel et al., 1983, Oertel and Mugnaini, 1984, Albin et al., 1989). Structurally, the GP differs from the striatal nuclei, being predominantly composed of large, widely spaced fusiform cells, containing triangular or polygonal cell bodies, with up to four thick dendrites extending to 700 µm in length (Fox et al., 1974, Difiglia et al., 1982, Francois et al., 1984, Percheron et al., 1984, Yelnik et al., 1984). Pallidal neurons are sparse in distribution compared to striatal neurons, and are 100 times less numerous than spiny striatal projection neurons, meaning that a single striatal neuron innervates ~100 pallidal neurons (Yelnik, 2002, Goldberg and Bergman,
The axon of a single pallidal neuron can travel for a very long distance, innervating a diverse range of neuronal types (Bevan et al., 1998, Smith et al., 1998a, Kita et al., 1999).

Pallidal neurons have long, thick, smooth, sparsely spiny, poorly branching dendrites (François et al., 1984). The long dendrites of pallidal projection neurons sometimes create dendritic radii of more than one millimetre in their main plane. In the central areas of the primate GPe and GPi, these dendrites appear as a large, disc-like territory (Yelnik et al., 1984). The flat plane of the discoidal dendritic field parallels the border between the GPe and the putamen, and is orientated perpendicular to incoming striatal fibres (Percheron et al., 1984, Yelnik et al., 1984, Kita and Kitai, 1994). This topographic organisation of dendrites and fibres enables convergence of incoming information in the GPe and GPi. However, unlike GPe pallidal neurons, GPi neurons do not have extensive local axon collaterals and if present, have poor arborisation and therefore few intrinsic connections (Parent et al., 1999). Pallidal dendrites are densely innervated by synapses which cover the entire dendrite (Fox et al., 1974, Difiglia et al., 1982, Difiglia and Rafols, 1988). The majority of these synapses represent GABAergic input, provided by the GABAergic striato-pallidal pathway, which terminates on cell dendrites and soma of pallidal efferent neurons (Öertel and Mugnaini, 1984, Shink and Smith, 1995, Bevan et al., 1998).

Myelinated striatal axons cross perpendicular to pallidal discoidal dendritic fields and provide unmyelinated collaterals (described as “woolly fibres”) parallel to pallidal dendrites with which they repeatedly synapse (Haber and Nauta, 1983, Difiglia and Rafols, 1988). These woolly fibres can be stained using immunohistochemistry specific for peptides, such as enkephalin and substance-P (Haber and Watson, 1985). The majority of glutamatergic and excitatory input to both the GPe and GPi is derived from the STN (Nauta and Cole, 1978, Smith and Parent, 1988, Smith et al., 1990, Hazrati and Parent, 1992). Even though only a small percentage of synapses impinge onto pallidal neurons from the STN, this is the main excitatory input to the GP (Chan et al., 2005). There are far more GABAergic synapses from the striatum, than glutamatergic STN synapses on the GPe and GPi pallidal neurons. Pallidal dendrites are covered in synaptic boutons, 90% of which come from the striatum, and 10% of which come from other sources, including the subthalamic nucleus (STN) and pedunculopontine nucleus within the brainstem (Difiglia et al., 1982). In addition to afferent input from the striatum and STN, GPe neurons receive collateral innervation from other GPe neurons, and the GPi also receives input from the GPe. These pallido-pallidal connections terminate close to, or on the soma (Hazrati et al., 1990, Shink and Smith, 1995, Smith et al., 1998a, Parent et al., 2000, Sadek et al., 2007).
The cellular morphology of GPi neurons is similar to that of the GPe. Three types of pallidal projection neurons have been identified based on their neurochemistry and morphology properties: Type 1 and 2 are the largest in size, have been described in multiple mammalian species, and make up 80-90% of the total population of pallidal neurons in the GP (Fox et al., 1974, Difiglia et al., 1982, François et al., 1984, Waldvogel et al., 1999). When labelled with Nissl stains, the majority of these neuronal cell soma in the GP are large (35-70 µm), contain varying amounts of Nissl granules, and appear elongated with a triangular or spindle-shaped cell soma (Difiglia and Rafols, 1988). Type 1 pallidal neurons contain GABA (gamma-aminobutyric acid) (Smith et al., 1987) and the calcium-binding protein parvalbumin (Kita, 1994), but are immunonegative for any other immunohistochemical markers and make up about 10% of the large pallidal neuron population (Waldvogel et al., 1999). Type 2 cells are identical in cell morphology to type 1 cells, but are subdivided into “type 2” based on their double calcium-binding protein immunoreactivity (Fortin and Parent, 1994). These large pallidal neurons co-label for both parvalbumin and calretinin (another calcium-binding protein), on the same cells, and make up 90% of the large human pallidal neuron population (Fortin and Parent, 1994, Waldvogel et al., 1999). However, in the GPi, most of the type 2 pallidal neurons are weakly immunolabelled with parvalbumin compared to the GPe, and are surrounded by large parvalbumin-immunoreactive boutons. Medium-sized type 3 neurons in the globus pallidus have also been described in previous studies (Difiglia et al., 1982), and in primates they are intensely immunoreactive for calretinin only (Fortin and Parent, 1994), are less than 25 µm in cell soma size, and in the human make up approximately 10-20% of the total number of neurons in the human globus pallidus (Waldvogel et al., 1999). It is possible that the smaller neurons with spiny dendrites are considered to be local circuit neurons/interneurons (Difiglia et al., 1982, François et al., 1984). The main subdivisions of the globus pallidus, the external segment (GPe), internal segment (GPi) and ventral pallidum (VP) are highlighted in Figure 2.3 and will be discussed in detail.
Figure 2.3 Schematic diagram of a lateral view of the human brain depicting the location of the globus pallidus externus (GPe), internus (GPi) and ventral pallidum (VP) in the basal ganglia.

Diagram showing a (A) lateral view of the brain with the location of the basal ganglia, and a (B), 3-dimensional schematic lateral view of the GPe, GPi and VP. Note the putamen (Put) is located most laterally, and the globus pallidus internal segment (GPI) is located most medially (signified with dotted lines). The GPe (filled in red) is located most caudomedial to the striatum, separated by the lml. The GPi (filled in yellow) is located medially to the GPe and is separated by the mml. The VP (filled in blue) is located ventrally to the GPe, and is separated by the anterior commissure. Abbreviations: Put, putamen; CN, caudate nucleus; ac, anterior commissure; Thal, thalamus; GPe, globus pallidus external segment; GPI, globus pallidus internal segment; VP, ventral pallidum; lml, lateral medullary lamina; mml, medial medullary lamina.
2.3.2 The globus pallidus external segment (GPe)

Located caudomedial to the striatum (Figure 2.3), the external segment of the globus pallidus (GPe), is a relatively large nucleus, which receives major inputs from two major basal ganglia nuclei, the striatum and the subthalamic nucleus (Kita, 2007). The GPe receives massive GABAergic afferent fibres from the striatum and glutamatergic afferent fibres from the subthalamic nucleus. The GPe also receives sparse afferents from the cerebral cortex, thalamus, GPi, SNc, raphe nuclei and pedunculopontine tegmentum (Hazrati et al., 1990, Fink-Jensen and Mikkelsen, 1991, Kita, 1994, Parent and Hazrati, 1995, Deschênes et al., 1996, Yasukawa et al., 2004). In both rodents and primates, the majority, if not all of the striatal projection neurons project to the GPe, with approximately half of the neurons projecting to the GPe only, and the other half emitting collaterals to the GPe en route to the GPi and SNr (Kawaguchi et al., 1990, Parent et al., 1995, Wu et al., 2000). Electron microscopy has shown that large areas of the soma and dendrites of GPe pallidal projection neurons were covered with synaptic boutons, of which 80% formed symmetrical synapses, with 80% of those synapses belonging to striatal axons, and the remaining are local collateral axons. The boutons which formed asymmetrical synapses (i.e. less than 20% of the total synapses in the GPe) belong mainly to the fibres of the STN, while a minor proportion are from the cortex and thalamus (Difiglia et al., 1982, Falls et al., 1983, Kita, 1994, Shink and Smith, 1995).

The output of the GPe is GABAergic, and inhibitory on its targets (Jessel et al., 1978). Most neurons of the GPe are projection neurons, which contain glutamate decarboxylase and are thereby GABAergic in nature (Penney and Young, 1981, Oertel and Mugnaini, 1984, Smith et al., 1987, Kita, 1994, Kita and Kitai, 1994). GPe neurons project to most of the basal ganglia nuclei, including the subthalamic nucleus (Nauta and Mehler, 1966, Carpenter et al., 1968, Carpenter et al., 1981), striatum (Beckstead, 1983), the internal globus pallidus (GPi) (Hazrati et al., 1990, Smith et al., 1994) the substantia nigra (Parent and De Bellefeuille, 1983), and itself (Kita, 2007, Jaeger and Kita, 2011). A single-axon tracing study of neurons in the GPe of primates demonstrated that based on their axonal targets, ~84.2% of the total number of GPe axons studied projected to the STN. However, none of these axons projected only to the STN. In fact, 52.6% branched to both the STN and SNr, 18% branched to both the STN and GPi, and 13.2% branched to the STN, SNr, and GPi (Sato et al., 2000). **Because GPe output connects to virtually every other basal ganglia nucleus, this places the GPe as a crucial component of the indirect pathway of the basal ganglia motor circuit, which is critical in relaying striatal**
inputs to the subthalamic nucleus (STN) (Gerfen and Wilson, 1996, Smith et al., 1998a). Furthermore, this literature suggests that the GPe is an important integrative locus in the basal ganglia. This organisation allows single GPe pallidal neurons to exert a multifarious effect not only on the STN, which is the main GPe target, but also on two major output structures of the basal ganglia, the SNr and GPi, which is considered to be its primary mode of action.

In terms of neurochemical architecture, the GPe can be distinguished laterally from the striatum, and medially from the GPi in the human brain based on the high expression of the neuropeptide enkephalin, which originates from striatal fibres that project directly into the region. The anterior commissure separates the GPe dorsally from the subcommissural VP (Haber and Elde, 1981, Haber and Watson, 1985).

2.3.3 The globus pallidus internal segment (GPi)

Located medially to the external segment (GPe), the GPi is separated from its larger counterpart by the medial medullary lamina (Figure 2.3). The GPi is a final output station of the basal ganglia, through which body movements are controlled (DeLong, 1971, Nambu, 2007). Although the GPe and GPi share a common neurotransmitter (GABA), and similar cellular morphology, they are distinct in terms of function and place in the basal ganglia circuitry. GPi neurons have relatively large 20-60 µm triagonal or polygonal soma with thick, sparsely-spined, poorly branching dendrites (Yelnik et al., 1984). The dendrites occupy a disk-like territory orientated perpendicular to incoming striatal fibres (Percheron et al., 1984). This topographic organisation of dendrites and fibres enables convergence of incoming information towards the GPi, similarly to the GPe. However, unlike GPe pallidal neurons, GPi neurons do not have extensive local axon collaterals (Parent et al., 1999).

The striatum sends rich GABAergic afferents to the GPi, with about 70% of the total number of synaptic terminals in contact with GPi neurons originating from striatal spiny neurons (Shink and Smith, 1995). The striatum projects to the GPi via two major projection systems, the direct and indirect pathways (Alexander and Crutcher, 1990). The direct pathway arises from GABAergic striatal neurons which contain substance-P and projects monosynaptically to the GPi. The indirect pathway arises from GABAergic striatal neurons containing enkephalin, and projects
polysynaptically to the GPi by a sequence of connections involving the GPe and STN (Nambu, 2007). Two other major inputs to the GPi are glutamatergic afferents from the STN (about 10%), and GABAergic afferents from the GPe (about 15%) (Smith et al., 1994, Shink and Smith, 1995, Nambu, 2007). Other inputs into the GPi include glutamatergic inputs from the intralaminar nuclei of the thalamus, serotonergic inputs from the dorsal raphe nucleus, glutamatergic and cholinergic inputs from the pedunculopontine nucleus, and dopaminergic inputs from the substantia nigra (Lavoie et al., 1989, Lavoie and Parent, 1990, 1994).

The neuronal population in the GPi comprises of GABAergic pallidal neurons, which suggests that they have an inhibitory effect onto their target neurons (Albin et al., 1989). The GPi projects to the ventral anterior/ventral lateral (VA/VL) nucleus of the thalamus in primates, where GPi pallidal fibres entering the thalamus give off several collaterals and terminate primarily onto thalamic projection neurons (Kuo and Carpenter, 1973, Kim et al., 1976). The thalamic neurons which receive GPi input project to the striatum as well as the primary motor cortex, supplementary motor area, and premotor cortex (Nambu, 2007). Therefore, the GPi has a major role in gathering movement-related activity from the striatum, GPe and STN, integrating this information, and finally, conveying this processed information outside the basal ganglia towards the thalamus, and motor cortices. In addition, GPi pallidal neurons also project to the lateral habenular nucleus (involved in the limbic reward pathway) and the motor pedunculopontine nucleus, which suggests an integrative role of the GPi in other circuits in the brain, including the reward pathway (Parent et al., 1981, Parent and De Bellefeuille, 1983).

In terms of neurochemical architecture, the GPi can be distinguished medially from the GPe in the human brain, based on the high expression of the neuropeptide substance-P, which originates from striatal fibres that project directly into the region. This difference in neuropeptide expression in the GPi compared to the GPe reflects that the striatal afferents to the GPi originate from a different population of striatal neurons, containing either enkephalin (for the GPe) or substance-P (for the GPi) (Haber and Elde, 1981, Haber and Watson, 1985, Mai et al., 1986).
2.3.4 The ventral pallidum (VP)

The ventral pallidum (VP) was first identified in 1975 as a primary GABAergic output for the ventral striatum, located immediately ventral to the anterior commissure (Figure 2.3) (Heimer and Wilson, 1975, Haber et al., 1990b). This area has been included as part of the pallidum based on sharing a similar histological criteria, including the presence of “pallidal-like” projection neurons, and the fact that it receives its main source of input from the ventral striatum (Heimer, 1978). It has also been shown that the VP contains heterogeneous cell types, including cholinergic and GABAergic projection neurons (Smith et al., 2009). Notions of the VP as a striatal output for movement, comparable to the GP, contributed originally from the view that it functioned as a motor expression region (Heimer et al., 1982). For example, based on a series of behavioural studies, Mogenson et al proposed that the nucleus accumbens projections to the VP translated limbic motivation signals into motor output (Mogenson and Yang, 1991). This account attributed the “limbic-motor integration” to the accumbens-pallidal system, and specifically identified ventral pallidal projections to the brainstem (i.e. pedunculopontine tegmentum) as a primary motor output for limbic motivation signals. The VP also receives glutamatergic input from the subthalamic nucleus (Kita and Kitai, 1987, Turner et al., 2001). Likewise, dopaminergic projections from both the substantia nigra and ventral tegmental area (midbrain regions associated with the basal ganglia and limbic systems, respectively) terminate in the VP (Napier et al., 1991, Klitenick et al., 1992).

The VP projects back to nearly all of its input sources, including the nucleus accumbens for reciprocal information exchange (Spooren et al., 1996). The VP also projects to the subthalamic nucleus and hypothalamus, as well as midbrain structures including the substantia nigra and ventral tegmental area (Haber et al., 1990b, Haber et al., 1993). Fibres also innervate the pedunculopontine nucleus, a key structure involved in the reward circuit (Kobayashi and Okada, 2007). Furthermore, outputs from the VP re-enter corticolimbic loops via direct projections to the medial prefrontal cortex, and dense projections to the mediodorsal nucleus of the thalamus (Zahm et al., 1987, Haber et al., 1993, Pirot et al., 1994, Parent et al., 1999). The VP also projects back to both the GPe and GPi, which is a unique projection, because the dorsal structures (the GPe and GPi) do not project to the VP (Haber and Knutson, 2010). Parts of the VP also project to the lateral habenular nucleus, a structure now considered to be part of the reward circuit (Matsumoto and Hikosaka, 2007, Morissette and Boye, 2008).
Currently, the VP is an area of focus in the study of addictive behaviours (Mitrovic and Napier, 2002, Tindell et al., 2006, Smith and Berridge, 2007). Evidence supporting the idea that the VP may be needed for normal motivation and hedonics has been shown in both animal studies and humans. A recent clinical report describes a drug-addicted human patient with partial lesions to the VP, who, after the lesions, reported the disappearance of all drug cravings and remained abstinent from all recreational drugs, reporting a loss in “pleasure.” The patient also endorsed a depressed mood, with noted “anhedonia” or an inability to feel pleasure (Miller et al., 2006). Furthermore, a patient with bilateral damage to the globus pallidus, which extended to the VP, reported an “inability to feel emotions” and a “profound lack of motivation” (Vijayaraghavan et al., 2008). Reward cues also activate the VP, as shown by cue-triggered “wanting” of drugs. Childress et al. presented cocaine addicted subjects with pictures of drug-associated cues (i.e. images of taking drugs). These images triggered VP activation as shown through functional MRI (Childress et al., 2008). Furthermore, it has also been shown that the VP plays a role in sex and social affiliation. VP activity through functional MRI is shown to increase during male sexual arousal, and in response to subliminally presented images of happy human faces or sexual images (Whalen et al., 1998, Rauch et al., 1999, Childress et al., 2008).

Thus, the key role of the VP in reward, mood, and pleasure processing as evidenced by these studies, indicates that an exploration into the role of the VP in mood disorders, or disorders with a mood component, is warranted. Such limbic-related anatomical connectivity sets the stage for the VP to mediate reward and motivation functions at many levels of the brain, beyond merely aiding translation to movement (Smith et al., 2009). The close relation between the ventral striatum and VP within the limbic loop of the basal ganglia coined the term “ventral striatopallidum” (Petrasch-Parwez et al., 2012). The ventral striatopallidum is known as an integrator of emotional, cognitive and sensory information, and in linking motivation to behaviour (Waraczynski, 2006, Heimer et al., 2007).

The human VP, like the GPe and GPi can be divided chemoarchitectonically into areas based on the extent of substance-P and enkephalin innervation, which originates from the ventral striatum (Haber and Watson, 1985). Staining for these peptides has been useful in defining the boundaries of the VP (Fox et al., 1974, Difiglia et al., 1982, Haber and Watson, 1985, Mai et al., 1986, Reiner et al., 1999). The rostral pole of the VP can be ventrally separated from the GPe by the anterior commissure (Heimer et al., 1999).
2.4. Neuropathology of the basal ganglia in HD

2.4.1 Neuropathology of the striatum in HD

Gross examination of the post-mortem HD human brain, as well as more recent in vivo neuroimaging of brains of HD patients demonstrate striking characteristic bilateral atrophy of the striatum (caudate nucleus and putamen) (de la Monte et al., 1988, Aylward et al., 1997, Vonsattel and DiFiglia, 1998, Vonsattel et al., 2008). The pattern of degeneration tends to follow an ordered topographical distribution. The pattern of degeneration in the caudate nucleus and putamen usually progresses from the tail of the caudate nucleus to the head and body in a caudo-rostral, and, simultaneously dorsal-ventral and medial-lateral directions (Roos, 1986, Vonsattel and DiFiglia, 1998, Vonsattel et al., 2008). With HD progression, the caudate nucleus becomes more atrophic, becoming thinner and ultimately more concave in shape, with subsequent enlargement of the lateral ventricles occurring in parallel. The major atrophy of the striatum is due to the loss of GABAergic medium-sized spiny projection neurons (MSNs) which make up ~90-95% of the striatal neuronal population, combined with their dendritic arbors and heavily myelinated axonal projections. In addition to the heavy extent of neurodegeneration in the striatum, there is also marked gliosis by astrocytes and oligodendrocytes. The extent of striatal degeneration provides a basis of the Vonsattel grading system which as detailed below, assigns a grading nomenclature to the extent of striatal degeneration in each post-mortem HD case (Vonsattel and DiFiglia, 1998, Vonsattel et al., 2008).

2.4.2 Striatal neuropathological grading

A neuropathological grading system, the framework of which is based on the distinctive, temporospatial pattern of degeneration in the HD striatum was developed by Vonsattel et al (Vonsattel et al., 1985, Vonsattel et al., 2008). The assignment of the grade of neuropathological severity is based on gross and microscopic findings on post-mortem HD tissue. The system has five grades (0-4) of severity of striatal involvement. This grading system applies to brains from individuals diagnosed clinically as having HD, with or without genetic testing confirmation.

Grade 0 comprises less than 1% of all HD brains (N=1250 brains). For grade 0 cases, there are no are gross changes seen in the human brain. However, the cases have a strong clinical or
familial history of HD. Further evaluations including cell counts indicate a 30-40% loss of neurons in the head of the caudate nucleus, and no visible reactive astrocytosis.

**Grade 1** comprises 4% of all HD brains. The tail of the caudate nucleus (CN) is smaller than normal. Neuronal loss and astrogliosis involve the tail and body of the CN, as well as the dorsal portion of both the head and nearly dorsal putamen. Cell counts show a 50% or greater loss of neurons in the head of the CN.

**Grade 2** comprises 16% of all brains. In grade 2, macroscopic atrophy of the CN and putamen are both present, with atrophy of the head of the CN being the most pronounced at this grade. The medial outline of the head of the CN is only slightly convex but still bulges into the lateral ventricle. The lateral ventricle is also slightly enlarged. The GP does not show gross macroscopic changes at this level. Microscopically, the dorsal striatum exhibits neuronal loss and concomitant astrocytosis at the medial half, lateral half and head of the CN, and dorsal portion of the putamen. The ventral striatum (including nucleus accumbens) shows no change.

**Grade 3** comprises 52% of all brains. In grade 3, the CN is dramatically atrophic. The medial outline of the head of the CN forms a straight line, or is slightly concave medially. Both the putamen and globus pallidus now exhibit moderate reductions in size. However, the nucleus accumbens remains unchanged. Microscopically, there is severe neuronal loss and astrocytosis in the CN. However, grade 3 is characterized by gliosis and neuronal loss in the putamen, which becomes more striking in this grade. The GPe shows slight to moderate fibrillary astrocytosis, whereas the GPi does not exhibit the same change.

**Grade 4** comprises 28% of all HD brains. The neostriatum neuronal loss is 90% or more. In the most severe grade, gross macroscopic examination reveals an extremely atrophic CN, which presents in a concave appearance accompanied with widened lateral ventricles. The medial contour of the head of the CN is concave, as is the CN at the anterior limb of the internal capsule. The putamen decreases markedly in size, with widened perivascular spaces in its ventral portion. At this grade, the nucleus accumbens shows some shrinkage, but is relatively preserved in comparison to the dorsal striatum. In at least 50% of grade 4 brains, the underlying nucleus accumbens (ventral striatum) remains relatively preserved, but is not normal. The external medullary lamina of the GP is unseen; with the GP showing marked atrophy (approximately half the size in comparison to age matched controls). Microscopically, neuronal loss and gliosis is
evident throughout the entire CN and putamen. The nucleus accumbens exhibits slight to moderate astrocytosis, mainly in the dorsal region. The GP exhibits astrocytosis, especially in the GPe, and the neurons are seen to be more closely packed together than in the age matched controls. There is an overall dorsal to ventral, anterior to posterior, and medial to lateral progression of neuronal death observed; with the dorsal medial striatum affected the earliest in comparison to the ventral striatum.

2.4.3 Cellular and receptor changes in the basal ganglia in HD – relation to the indirect and direct motor circuits

In HD, it has been postulated that the degeneration of the projection neurons within the striatum leads to aberrant activation of the basal ganglia output nuclei, resulting in uncoordinated movement, cognition, and emotional control. Various autoradiographic, in situ hybridisation and immunohistochemical studies have reported the loss of neurochemicals and neurotransmitters associated with receptors in the striatum. As mentioned above, the most affected neuronal populations in the striatum are the medium-sized spiny GABAergic projection neurons (MSNs) that constitute ~90-95% of the striatal neuronal population. There are two major GABAergic populations of MSNs: (1) those projecting mainly to the external segment of the globus pallidus (GPe), which are typically rich in the neuropeptide enkephalin and devoid of the neuropeptide substance-P; and (2) those projecting to the internal segment of the globus pallidus (GPi), which are rich in the neuropeptide substance-P, and poor in enkephalin. MSNs are also associated with a large number of ion channel and metabotropic receptors on the surface membranes including cannabinoid (CB1) (Glass et al., 2000), GABA$_A$ receptors (Waldvogel et al., 1999) and dopamine receptors (D1 and D2) (Joyce et al., 1988, Khan et al., 1998).

As mentioned in section 2.2.2, MSNs of the indirect motor circuit contain GABA and enkephalin, whereas MSNs of the direct motor circuit contain GABA and substance-P. In HD, the MSNs degenerate with advancing HD grade (Vonsattel et al., 1985, Vonsattel and DiFiglia, 1998). Both enkephalin and substance-P MSNs are lost (Emson et al., 1980, Marshall et al., 1983). However, MSNs projecting to the GPe (indirect pathway) that express enkephalin and dopamine D2 receptors have been shown to be most vulnerable to the disease process (Reiner et al., 1988, Albin et al., 1992, Augood et al., 1996). The enkephalinergic MSNs are shown to degenerate in advance of MSNs that express substance-P and dopamine D1 receptors that project to the GPi and SNr (direct pathway) (Gerfen et al., 1990, Deng et al., 2004). In addition, a
reduction in glutamate NMDA receptor binding, GABA$_\alpha$ receptor binding and cannabinoid receptor binding are all evident in the HD striatum (Whitehouse et al., 1985, Young et al., 1988, Glass et al., 2000), which could be indicative of dysfunction or downregulation of these receptors in addition to the loss of MSNs.

The projections of striatal MSNs to the pallidal output nuclei which contain enkephalin, substance-P and cannabinoid receptors are progressively lost with advancing HD grade as shown in post-mortem tissue, mirroring the loss of MSNs in the striatum. Staining for the neuropeptide enkephalin is dramatically lost in a grade dependent manner in the GPe. This reflects the loss of the GABAergic enkephalin-positive pathway to the GPe (indirect pathway), based on the presymptomatic and early HD grade (grade 0-1) loss of enkephalin immunoreactivity from striatal efferent terminals in the GPe (Reiner et al., 1988, Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009). There is also a later loss of substance-P in the GPi, reflecting the loss of the GABAergic substance-P positive pathway to the GPi (direct pathway) (Reiner et al., 1988, Albin et al., 1990). Quantification of substance-P immunolabelled terminals within the GPi, in a sample of HD cases ranging from 0-4, highlighted that the loss of striatal projections to the GPi proceeded far more gradually than the loss of enkephalin immunolabelled striatal terminals to the GPe (Deng et al., 2004). These results have been confirmed by a previous study from our group (Allen et al., 2009), that this substance-P immunoreactivity from striato-pallidal terminals is progressively reduced in later stages of HD (Glass et al., 2000, Deng et al., 2004). There is also a loss of cannabinoid receptors on the presynaptic terminals of both the direct and indirect pathways (Glass et al., 2000). Furthermore, associated with the loss of neurotransmitter GABA from the striato-pallidal indirect and direct pathways, there is a major increase in post-synaptic GABA$_\alpha$ and GABA$_\beta$ receptors, which is proposed to be a compensatory upregulatory response of GABA$_\alpha$ receptors on the pallidal output neurons (Penney and Young, 1982, Faull et al., 1993, Allen et al., 2009). A summary of the main cellular and receptor changes in Huntington’s disease is shown in Figure 2.4.
In the striatum, the GABAergic medium spiny projection neurons (in red) are divided into 2 groups, (1) those that stain for enkephalin (Enk) and (2) those staining for substance P (Sub P): These project to the large GABAergic pallidal neurons in the GPe and GPi (in blue). The small boxes near the axon terminals in the GPe and GPi represent the presynaptic D1 or D2 dopamine receptors and CB1 cannabinoid receptors. The boxes on the pallidal cells represent post-synaptic GABA_A receptors (GA) and GABA_B receptors (GB). In Huntington’s disease the medium spiny neurons that degenerate are indicated by dotted lines. Arrows indicate up or down regulation of receptors. (1)The GABA/Enk striato-GPe neurons of the *indirect pathway* and their associated receptors are affected in the early stages of the disease (2) while the GABA/Sub P (striato-GPi) neurons of the *direct pathway* and receptors are affected in HD cases with advanced pathology. Abbreviations: GPe, globus pallidus external segment; GPi, globus pallidus internal segment; GABA, gamma amino butyric acid.
The disruptions of these striatal pathways in HD have led to hypotheses as to how they affect the basal ganglia circuitry in HD. Clear hypotheses have been raised to relate the early loss of enkephalinergic indirect pathway MSNs to the symptomatology of HD (Albin et al., 1989, Deng et al., 2004). The loss of striatal neurons that give rise to the indirect pathway could result in increasing the inhibitory action of the GPe upon the STN. The STN then becomes hypofunctional and causes a reduction of the inhibitory action of the GPi upon the thalamus. This subsequent disinhibition of the thalamus is postulated to result in the appearance of uncontrolled, chorea movements, a characteristic feature of HD (Crossman, 1987, Crossman et al., 1988, Albin et al., 1989, Hedreen and Folstein, 1995, Deng et al., 2004). In contrast, the loss of the substance-P direct pathway MSNs that project from the striatum to the GPi may contribute to dystonia in advanced (grade 3) HD. The near complete loss of this projection system by grade 4 may be associated with the akinesia in terminal HD (Albin et al., 1989, Berardelli et al., 1999, Deng et al., 2004). This could be due to an increase in the inhibitory action of the GPi upon the thalamus, reducing the thalamocortical neuronal excitatory input to the cortex, and thereby contributing to a symptom shift from hyperkinesia to hypokinesia (Albin et al., 1989, DeLong, 1990, Berardelli et al., 1999, Deng et al., 2004).

2.4.4 Involvement of the GPe, GPi and VP in HD

Neuroimaging studies have reported severe atrophy of the globus pallidus in HD patients (Aylward et al., 1997, Rosas et al., 2003, Fennema-Notestine et al., 2004, Douaud et al., 2006). However, in vivo imaging studies have not specifically isolated the GPe, GPi and VP and separately evaluated the extent of volumetric decline in HD. Post-mortem pathology studies have also reported that significant progressive atrophy of the GPe occurs, with greater atrophy and gliosis observed compared to the GPi based on qualitative or morphometric analysis (Lange et al., 1976, Vonsattel et al., 1985, Roos, 1986). However, only one stereological approach has reported a decline in GPe volume (42%), and GPi volume (21%) specifically in HD relative to controls (Halliday et al., 1998). No data has related the extent of GP volume loss to striatal neuropathological grade or clinical symptom information.

Currently, there has been no stereological study which has evaluated the degree of pallidal neuron loss in Huntington’s disease. However, two studies using non-stereological methods have reported conflicting conclusions in regards to GPe and GPi pallidal neuron involvement in HD. In one quantitative study by Lange et al in 1976, which compared 6 HD cases of unreported
grades with 15 control cases, the absolute number of pallidal neurons was shown to decrease by up to 40% in the GPe and 43% in the GPi, which was accompanied by a 50% reduction in pallidal volume (Lange et al., 1976). However, the neuronal density was up to 42% higher within the GPe, and 27% higher in the GPi, and no changes were found in relation to the soma volume of individual pallidal neurons in HD. This morphometric study suggested that pallidal neuronal loss was due to primary degeneration (cell-autonomous processes), rather than solely the consequence of striatal degeneration (non-cell-autonomous processes), thereby suggesting that pallidal neuron loss also contributes to GP atrophy.

However, more recent studies have reached a conflicting conclusion, suggesting that the GP atrophy is mainly due to neuropil loss, resulting from striatal fibres and terminals, and to a lesser extent the loss of neurons (Reiner et al., 1988, Albin et al., 1990, Storey and Beal, 1993). Wakai et al histometrically examined 6 HD cases (of advanced striatal neuropathological grading), in comparison to 10 control cases. Examination of pallidal neurons in 5 selected coronal sections taken along the rostral-caudal axis, using a non-stereological cell-counting method, detected no pallidal neuron loss in HD (Wakai et al., 1993). This study supported the hypothesis that no pallidal neuronal depletion was recognised in HD, despite marked atrophy of tissue volume, thereby implicating overall GP atrophy to striato-pallidal fibre loss and non-cell autonomous processes.

Currently, neuropathological analyses of limbic associated regions in human HD brains are relatively sparse, with no reported studies in HD *post-mortem* tissue which documents cellular changes within the VP. The ventral (limbic) striatum, which comprises of the nucleus accumbens as its major component, appears to be relatively preserved in HD compared to the dorsal (motor) striatum (Vonsattel et al., 1985, Kassubek et al., 2004). In one such study of Parkinson’s disease patients with pathological gambling, single-photon emission computed tomography showed enhanced resting state activity (regional cerebral blood flow) in the VP of these individuals, compared with non-gambling PD patients (Cilia et al., 2008). Therefore, it could be possible that in HD, sharing commonalities with Parkinson’s disease as a neurodegenerative disorder involving both the limbic system and basal ganglia, could also have an association with changes in VP activity.

Despite the extensive literature examining the implications of HD on the striatum, very little exists with regard to the impact of striatal loss on the main neurons that receive striatal input,
pallidal neurons. There is also a lack of detailed knowledge involving the relationship between pallidal neurodegeneration with striatal neuropathological grade and symptom heterogeneity. Furthermore, as the GPe and GPi are critical structures involved in the indirect and direct basal ganglia motor circuits, a better understanding of the extent of their involvement in HD is crucial in order to gain a better understanding of basal ganglia dysfunction. Also, as there is currently no literature which implicates the VP in HD, despite being a key structure of the limbic pathway, a detailed examination of the VP in HD post-mortem human tissue is a very novel endeavour, and would therefore provide interesting results in being the first examination at the involvement of the VP in HD.
2.5 Aims of this study

Huntington’s disease (HD) is characterised by pronounced pathology of the basal ganglia, with numerous studies documenting the pattern of striatal neurodegeneration. However, there are no studies currently to date which have used detailed stereology-based research to characterise neurodegeneration of a principle target of striatal outflow, the globus pallidus (GP). The external segment (GPe) is a major output of the dorsal striatum, connecting widely to other basal ganglia nuclei via the indirect motor pathway. The internal segment (GPi) is a final output station of both the direct and indirect motor pathways of the basal ganglia. The ventral pallidum (VP), in contrast, is a primary output of the limbic ventral striatum. Currently, there is a lack of consensus in the limited literature with regard to GPe and GPi neurodegeneration in HD, with a disagreement between pallidal neurons being preserved (Wakai et al., 1993), and pallidal neurons being lost (Lange et al., 1976). In addition, no current evidence exists in the literature with regard to the fate of the VP in HD, despite being a key structure involved in reward and motivation. Understanding the involvement of these structures in HD will help to determine their involvement in basal ganglia pathway dysfunction in HD. Furthermore, a clear understanding of the impact of striatal projection loss on the main neurons that receive striatal input, pallidal neurons, will aid in the understanding of disease pathogenesis. Furthermore, a clearer picture of pallidal involvement in HD may contribute to providing a morphological basis to the considerable variability in the types of motor, behavioural and cognitive symptoms in HD.

This study is directed towards determining the extent of pallidal involvement in the pathogenesis of HD. The specific aims of this study are to:

- Quantify using design-based stereology, the overall regional volume, total pallidal neuron number, and pallidal soma volume in three functionally diverse regions of the basal ganglia, the globus pallidus externus (GPe), internus (GPi), and ventral pallidum (VP) of the human brain in Huntington’s disease and neurologically normal control cases.

- In addition, to compare any changes in regional volume, pallidal neuron number, and pallidal soma volume with: (1) striatal neuropathological grade; (2) CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, disease duration; and (3) symptom heterogeneity and clinical assessments.
CHAPTER 3: MATERIALS AND METHODS

3.1 General Introduction

In this study, post-mortem human brain tissue was used to investigate, in Huntington’s disease (HD), compared to neurologically normal control brains, the overall changes in three functionally diverse regions within the basal ganglia: the globus pallidus externus (GPe), the globus pallidus internus (GPi) and the ventral pallidum (VP). First, the boundaries of each region were delineated using immunohistochemistry for two neuropeptides in the basal ganglia, enkephalin (to delineate the GPe and VP) and substance-P (to delineate the GPi). Secondly, a histochemical stain using cresyl violet (Nissl) was applied to label the cellular population within each region. Pallidal neurons were easily identifiable using Nissl stain due to their specific morphology. For cell morphology measurements, accurate delineation was carried out by immunohistochemically staining large pallidal neurons for the calcium-binding protein parvalbumin. Light microscopy was used to qualitatively compare each region in cross-section at low magnification, and examine the distribution of pallidal neurons at high microscopic magnifications. Finally, unbiased design-based stereological techniques were used to compare total regional volumes (Cavalieri Estimator), total number of pallidal neurons (Optical Fractionator), and pallidal neuron soma volume (Isotropic Nucleator) in both HD and control cases. Figure 3.1 highlights the general overview of the methodology used in this study.
Figure 3.1. Overview of the methodology

Human brain tissue
- Normal control cases
- Huntington’s disease cases
  - CAG repeat length assessment
  - Striatal pathological grading
  - Clinical symptom scores

Human brain tissue processing
- Basal ganglia VP-GP-anterior thalamus block
  - Globus pallidus externus (GPe)
  - Globus pallidus internus (GPi)
  - Ventral pallidum (VP)
  - Perfusion fixed
  - Sectioned at 70 μm

Immunohistochemistry and Histology
- Single immunoperoxidase labelling
  - Enkephalin (Enk)
  - Substance-P (Sub-P)
  - Parvalbumin (PV)
  - Nissl staining
  - Cresyl violet - Counterstain Enk/Sub-P sections to delineate pallidal neurons

Required for:
- Cavalieri Estimator
- Optical Fractionator

Design-based Stereology
- Obtain total volume of GPe, GPi, VP

Required for: Isotropic Nucleator

Optical Fractionator
- Obtain total number of pallidal neurons in GPe, GPi

Cavalieri Estimator
- Obtain average volume of pallidal neuronal soma in GPe, GPi

Statistical analyses
- Coefficient of error (CE) estimators for stereology precision
- Mann-Whitney, Kruskal-Wallis and Dunn’s post test
- Spearman’s correlation
3.2 Human brain tissue processing

3.2.1 Brain tissue

The human brain tissue was received from the Neurological Foundation of New Zealand Human Brain Bank in the Centre for Brain Research, The University of Auckland. Informed consent was obtained from all families and the research protocols were approved by the University of Auckland Human Participants Ethics Committee. The Huntington’s disease (HD) brains were obtained through a donor program where fully informed consent was granted by the patient and the family in advance of the patient’s death. The control brains used for this study showed no history of neurological disorder and there were no underlying pathological conditions were identified by the independent neuropathologist. In all cases, the diagnosis of HD was confirmed through genetic testing (number of CAG repeats in both alleles of the HD gene), and all cases were thoroughly examined histologically by a neuropathologist with specialized expertise in the neuropathology of HD (Dr B. Synek at Auckland City Hospital, Auckland, NZ). Each HD case was graded based on striatal pathology according to the internationally recognized standard Vonsattel 0-4 grading criteria (Vonsattel et al., 1985, Vonsattel et al., 2008).

Brain tissue from 8 Huntington’s disease (HD) cases (6 males and 2 females, mean age 56 ± 17 years, mean post-mortem delay 15 ± 6 hours) of neuropathological grades 1-3, and 8 control brains (5 males and 3 females, mean age 68 ± 7 years, mean post-mortem delay 19 ± 15 hours) were used for this study (see Table 3.1 and 3.2 for case details), with a post-mortem (PM) interval prior to perfusion extending from 5-49 hours. The HD and control cases were matched as far as possible for age, sex and PM delay.
Table 3.1 Control cases used in this study

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Post-mortem delay (hours)</th>
<th>Cause of death</th>
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<tbody>
<tr>
<td>H153R</td>
<td>76</td>
<td>M</td>
<td>8</td>
<td>ischaemic heart disease</td>
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<td>ischaemic heart disease</td>
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<td>H186R</td>
<td>68</td>
<td>M</td>
<td>21</td>
<td>ischaemic heart disease</td>
</tr>
<tr>
<td>H204R</td>
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<td>M</td>
<td>9</td>
<td>ischaemic heart disease</td>
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<tr>
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<td>73</td>
<td>F</td>
<td>49</td>
<td>mesothelioma</td>
</tr>
<tr>
<td>H227R</td>
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<td>F</td>
<td>4</td>
<td>cerebellar cerebrovascular accident (cortex and basal ganglia unaffected)</td>
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<tr>
<td>H230R</td>
<td>57</td>
<td>F</td>
<td>32</td>
<td>carcinomatosis (renal sarcoma) (brain not affected)</td>
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<tr>
<td>H231R</td>
<td>65</td>
<td>M</td>
<td>8</td>
<td>ischaemic heart disease</td>
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</table>

Table 3.2 Huntington’s disease cases used in this study

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<thead>
<tr>
<th>Case</th>
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<th>Sex</th>
<th>Post-mortem delay (hours)</th>
<th>CAG repeats</th>
<th>Vonsattel HD grade</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC101L</td>
<td>35</td>
<td>M</td>
<td>24</td>
<td>17/44</td>
<td>1</td>
<td>asphyxia</td>
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<td>51</td>
<td>M</td>
<td>15.5</td>
<td>17/48</td>
<td>3</td>
<td>dehydration</td>
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<tr>
<td>HC120R</td>
<td>51</td>
<td>M</td>
<td>15</td>
<td>10/46</td>
<td>2</td>
<td>pneumonia</td>
</tr>
<tr>
<td>HC125L</td>
<td>67</td>
<td>F</td>
<td>13</td>
<td>17/43</td>
<td>3</td>
<td>pneumonia</td>
</tr>
<tr>
<td>HC132L</td>
<td>32</td>
<td>M</td>
<td>14</td>
<td>17/47</td>
<td>1</td>
<td>submandibular squamous carcinoma</td>
</tr>
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<td>83</td>
<td>M</td>
<td>13</td>
<td>17/41</td>
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<td>broncho pneumonia</td>
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<tr>
<td>HC139R</td>
<td>67</td>
<td>F</td>
<td>5</td>
<td>14/41</td>
<td>3</td>
<td>bilateral subdural haematoma (basal ganglia unaffected)</td>
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<tr>
<td>HC140R</td>
<td>62</td>
<td>M</td>
<td>22</td>
<td>17/40</td>
<td>3</td>
<td>chronic renal failure</td>
</tr>
</tbody>
</table>

Abbreviations: H, Neurologically normal control cases; HC, HD cases; R, Right cerebral hemisphere; L, Left cerebral hemisphere; M, Male; F, Female.
3.2.2 Fixation of tissue

All brains used for this study were fixed via perfusion through the basilar and internal carotid arteries. Perfusion was first carried out with phosphate-buffered saline (PBS) with 1% sodium nitrite, followed by 15% formalin in 0.1M phosphate buffer, pH 7.4. After perfusion, the brain was post-fixed for 6-12 hours in the same fixative before being dissected into blocks. Blocks containing the basal ganglia were dissected out and placed in fresh formalin fixative for 24 hours. This was followed by immersion in 20% sucrose in 0.1M phosphate buffer with 0.1% sodium azide for 1 week. The blocks were then transferred into 30% sucrose in 0.1M phosphate buffer with 0.1% sodium azide for further cryoprotection and left for ~1 month or until the blocks had been completely infiltrated. Blocks were then rapidly snap frozen using powdered dry ice, double wrapped in tin foil, and stored at -80°C until required (Waldvogel et al., 2006). Basal ganglia blocks were obtained from either the left or right hemisphere, due to tissue availability. However, previous studies have shown that basal ganglia atrophy is symmetrical and affects both sides of the brain equally (Vonsattel et al., 2008).

For this stereological study, the basal ganglia were collected as three consecutive blocks as detailed below:

1: The CN block: containing the head of the caudate nucleus.

2: The VP-GP-anterior THAL block: containing the body of the caudate nucleus, the putamen, the anterior thalamus, and the entire globus pallidus which includes the GPe, GPi and VP.

3: The posterior THAL block: containing the posterior putamen, tail of caudate nucleus, and posterior thalamus.

This method of tissue dissection ensured that the entire globus pallidus was obtained in a single block.

The VP-GP-anterior THAL block was separated rostrally from the CN block by cutting through the basal ganglia just rostral to the anterior commissure level, and separated caudally from the posterior THAL block by cutting through the basal ganglia just caudal to the globus pallidus. This block was roughly four centimetres in rostral-caudal extent for a control brain, and three centimetres for an HD brain. Figure 3.2 shows the location of the basal ganglia blocks taken.
3.2.3 Tissue sampling

For each case, an entire fixed-frozen block containing the ventral pallidum and globus pallidus (VP-GP-anterior THAL block) was cut in the coronal plane into 70 µm serial sections using a Leica freezing microtome. All sections were collected, and available for sampling, and no bias was introduced for stereological purposes. The cut sections were stored in strict serial order at 4°C in PBS with sodium azide. Nissl staining and immunohistochemistry were performed on a separate series of sections for every case by staining every \( n^{th} \) section, with a randomly determined starting point (1 to \( n \)) for each case and marker. These sampling intervals were selected to ensure that sufficient sections were selected to represent the entire area of interest for each case (West, 1999) (see Table 3.3).

<table>
<thead>
<tr>
<th>Region</th>
<th>Sampling interval (every nth section)</th>
<th>Immunohistochemical/Histology Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globus pallidus external segment</td>
<td>n=20</td>
<td>Enkephalin + Cresyl Violet (Nissl)</td>
</tr>
<tr>
<td>Globus pallidus internal segment</td>
<td>n=20</td>
<td>Substance-P + Cresyl Violet (Nissl)</td>
</tr>
<tr>
<td>Ventral pallidum</td>
<td>n=5</td>
<td>Enkephalin + Cresyl Violet (Nissl)</td>
</tr>
</tbody>
</table>

Table 3.3 Sampling schemes for stereological probes used in this study
Figure 3.2 Location of the basal ganglia block dissected and analysed for this study

Diagram showing (A) lateral view of the brain with the location of the basal ganglia, and a (B), 3-dimensional schematic lateral view of the basal ganglia, with the location of core structures for this study identified. Note the putamen (Put) is located most laterally, and the globus pallidus internal segment (GPI) is located most medially (signified with dotted lines). The red vertical lines depict the positions where the dissections were made to isolate the VP-GP-anterior THAL block. Abbreviations: Put, putamen; CN, caudate nucleus; ac, anterior commissure; Thal, thalamus; GPe, globus pallidus external segment; GPI, globus pallidus internal segment; VP, ventral pallidum.
3.2.4 Immunohistochemistry

Immunohistochemistry using standard single peroxidise labelling techniques was performed on one complete series for each marker in the GPe, GPi and VP (Table 3.3). Immunohistochemistry was carried out on free-floating sections in a six well plate for three different primary antibodies: enkephalin, substance-P and parvalbumin (Table 3.4).

Enkephalin and Substance-P are both neuropeptides which are found to coexist with the classic inhibitory neurotransmitter GABA in striatal projection neurons (Haber and Elde, 1981, Haber and Watson, 1985, Mai et al., 1986). These immunohistochemically detected peptides are differentially distributed in the globus pallidus, which allows accurate visualisation of the subdivisions (Beach and McGeer, 1984, Haber and Watson, 1985, Mai et al., 1986). Enkephalin specifically stains the GPe and VP, and substance-P specifically stains the GPi.

Enkephalin is a peptide which specifically labels GABAergic neurons projecting from the striatum to the GPe and the VP (Haber and Watson, 1985, Reiner et al., 1999). Substance-P labels GABAergic neurons which project from the striatum to the GPi (Haber and Elde, 1981, Haber and Watson, 1985, Mai et al. 1986). Both neuropeptides are localised in punctuate terminals and fibres that outline the dense network of long branching dendrites of pallidal cells (Allen et al., 2009) and are seen as “woolly fibres” (Haber and Elde, 1981). Parvalbumin is a calcium-binding protein expressed in 80-90% of pallidal neurons in the human GP (Waldvogel et al., 1999), labelling all of the large pallidal neurons (types 1 and 2) in both the external and internal segments.
Table 3.4 List of primary antibodies used for this study

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Remarks</th>
<th>Purpose for this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enkephalin</td>
<td>Mouse monoclonal</td>
<td>1:1,000,000</td>
<td>Seralab Ltd, United Kingdom</td>
<td>Peptide co-label of GABAergic neurons that project from the striatum to the globus pallidus external segment. The enkephalin immunoreactive terminal field of neostriatal projection neurons defines the full extent of the external segment. (Haber and Elde, 1981, Haber and Watson, 1985, Waldvogel et al., 2006)</td>
<td>Delineating the extent of the external segment of the globus pallidus and ventral pallidum for stereological cell quantification and regional volume analysis - Optical Fractionator and Cavalieri Estimator</td>
</tr>
<tr>
<td>Substance-P</td>
<td>Rabbit polyclonal</td>
<td>1:50,000</td>
<td>Watpa Enterprises, New Zealand</td>
<td>A peptide which labels GABAergic neurons that project from the striatum to the globus pallidus internal segment. The Substance-P immunoreactive terminal field of neostriatal projection neurons defines the full extent of the internal segment. (Haber and Elde, 1981, Haber and Watson, 1985, Waldvogel et al., 2006)</td>
<td>Delineating the extent of the internal segment of the globus pallidus for stereological cell quantification and regional volume analysis - Optical Fractionator and Cavalieri Estimator</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Mouse monoclonal</td>
<td>1:5000</td>
<td>SWant, Switzerland</td>
<td>GPe: Labels a majority of large pallidal neurons (types 1 and 2) (entire cell body and some proximal processes). GPI: Outlines the soma of large pallidal neurons with punctuate terminal labelling in the internal segment of the globus pallidus. (Waldvogel and Faull, 1993, Hardman and Halliday, 1999, Waldvogel et al., 1999, Waldvogel et al., 2006)</td>
<td>Delineates the pallidal cell soma for size analysis - Isotropic Nucleator</td>
</tr>
</tbody>
</table>
Sections used for immunohistochemistry were initially incubated overnight in PBS-triton at 4°C. All of the sections were then washed once in PBS-triton, and incubated for 20 minutes in 50% methanol, 1% \( \text{H}_2\text{O}_2 \) solution at room temperature. The sections were washed in PBS-triton, 3x 10 minutes before primary antibody incubation, and after every subsequent incubation. Sections were then incubated on a rocker for 2 days in the primary antibody diluted in 1% normal goat serum at 4°C. An adjacent series of sections for each case was used as a negative control, incubated in 1% normal goat serum without the addition of the primary antibody. Following the 3x 10 minute PBS-triton washes, the primary antibody sections were incubated overnight at room temperature in secondary antibody (anti-mouse, dilution 1:1000, Sigma, USA; or anti-rabbit, dilution 1:2500, Sigma, USA) in 1% normal goat serum. Following the PBS-triton washes, the sections were incubated for 4 hours at room temperature in tertiary antibody (HRP-avidin complex) consisting of streptavidin peroxidase (ExtrAvidin, dilution 1:1000, Sigma, USA). The tertiary antibody was washed off in PBS-triton, and the peroxidase was reacted by incubation of the sections for 5 to 20 minutes in 3,3-diaminobenzidine tetrahydrochloride (DAB) solution (0.05M DAB in 0.1M Phosphate, pH 7.4 and 0.01% \( \text{H}_2\text{O}_2 \)) to produce a brown reaction product. For sections stained with enkephalin or substance-P antibodies, the DAB incubation time was 5 and 15 minutes respectively. This was to ensure that the DAB intensity does not interfere with the subsequent Nissl counterstain. Once the desired DAB intensity was achieved, the reaction was deactivated with three PBS-triton washes.

In the final step, the sections were mounted in gelatine, and either dehydrated according to a standard protocol (for parvalbumin stained sections), or Nissl stained with Cresyl violet and subsequently dehydrated afterwards (enkephalin and substance-P sections). The standard dehydration protocol involved immersion in a series starting with water (5 minutes), then 70%, 85%, 95% alcohol (5 minutes each), then 3 changes of 100% alcohol (for 10 minutes each), followed by 3 changes of xylene (20 minutes each). The sections were then coverslipped using DPX mounting medium (Waldvogel et al., 2006).
**Antibody specificity and controls**

The ability to control antibody specificity and tissue binding patterns for each antibody used in this study ensures the overall quality of the stain. Following the internationally accepted criteria of the *Journal of Comparative Neurology*, suitable controls for immunohistochemistry include (in order of preference) (Saper and Sawchenko, 2003, Saper, 2005):

- Showing that the antibody does not stain anything in sections from a transgenic animal where the protein of interest has been “knocked out”;
- If the above is not possible, then showing in Western blots that the antibody identifies bands of correct specific molecular weight.

Because these studies are human-based, the specificity of antibodies used is reported based on human-tissue western blotting references. Parvalbumin was the only antibody used in this study which was involved in quantification methods, and therefore western-blotting evidence has been provided for this antibody. In the case of parvalbumin (SWant), western-blots analysis has shown that the signals found using immunohistochemistry with this antibody were specifically due to the presence of human parvalbumin. In protein extracts from human parathyroid glands, the parvalbumin (SWant) antibodies against parvalbumin detected a protein band with a molecular weight of Mr 12,000, as expected for human parvalbumin, indicating that the antibodies indeed detected human parvalbumin for immunohistochemistry (Pauls et al., 2000). This antibody has also met the criteria for immunohistochemical labelling as specified by the *Journal of Comparative Neurology* (Saper and Sawchenko, 2003, Saper, 2005, J.C.N., 2012).

Controls for staining caused by non-specific binding of the secondary antibodies were also used for this study. The primary antibody was replaced with immunobuffer (a no-first primary control) for each case used.
3.2.5 Histochemistry - Nissl staining

The Nissl stain is a classic basophilic staining method for nucleic acids developed by Franz Nissl in the 19th century. Several different basophilic chemicals are suitable for Nissl stains; cresyl violet is the most commonly used. Nissl stains label granules within a cell known as “Nissl bodies,” which comprise ribosomal mRNA in the rough endoplasmic reticulum located in the cell soma and proximal dendritic processes. The Nissl stain protocol was optimised to ensure that pallidal neurons take up and retain the stain. The term “globus pallidus” (Latin for "pale globe") derives from the pale appearance of the GP due to the low density of large pallidal neurons (Nieuwenhuys et al., 2008, Goldberg and Bergman, 2011).

The mounted sections immunolabelled with enkephalin and substance-P were washed in water and dehydrated in an ascending series of 70%, 85%, 95% alcohol (5 minutes each), then 3 changes of 100% alcohol, followed by 20 minutes in xylene. The sections were subsequently rehydrated in reverse (with the same incubation times) before being placed into a 0.1% cresyl violet solution for 45 minutes. Finally, the stained sections were differentiated starting with water (30 seconds), then 70%, 85%, 95% alcohol (1 minute wash), then 2 baths of 100% alcohol for 5 minutes each, followed by 3 changes of xylene (20 minutes each) and covered using DPX mounting medium. Care was taken with the differentiation step to minimise the amount of stain lost by pallidal neurons, leaving many surrounding basal ganglia nuclei over-stained as a result.
3.3 Analysis

After immunohistochemical and histological staining procedures were completed, the sections were viewed and photographed using a Nikon Eclipse E800 bright field microscope coupled to a high-resolution digital camera (DXM1200F, Nikon) (for high power imaging), or a dissecting microscope (Leica MZ6) coupled to a Nikon Digital Sight DSU1 CCD camera (for low power imaging). Macroview images at 3 coronal levels (rostral, middle and caudal) were taken of control and HD cases at the regional level (low power) based on substance-P (GPe) and enkephalin (GPe and VP) staining using the dissecting microscope, allowing qualitative observations at the regional level. Furthermore, qualitative comparisons were also made at the cellular level (high power) based on examination of Nissl and parvalbumin-positive pallidal neurons in all regions. High power photomicrographs (20x) were taken at 3 coronal levels (rostral, middle and caudal) to compare the distribution of Nissl-positive pallidal neurons between control and HD cases for the GPe, GPi and VP. The morphological changes of parvalbumin-positive pallidal neurons in each region were also compared using 60x oil immersion high-power imaging.

Quantitative analysis of the GPe, GPi and VP using design-based stereology was performed using a Nikon E800 microscope equipped with a motorised stage and connected to a computer with Stereoinvestigator software installed (Version 10, MBF Bioscience, MicroBrightField Ltd, USA). Using the software, total volume changes were assessed for control and HD cases for each region using the Cavalieri Estimator probe. Stereoinvestigator was also used to quantify the total number of Nissl-positive pallidal neurons for all HD and control cases for each region using the Optical Fractionator probe. Finally, Stereoinvestigator was also used to analyse parvalbumin-positive pallidal cell soma size for HD and control cases for the GPe and GPi using the Isotropic Nucleator probe.

3.3.1 Design-Based stereology

An unbiased stereological cell quantification method, combined with systematic random sampling techniques was used to quantify the changes in Nissl-positive pallidal neurons and regional volumetric changes in the GPe, GPi and VP in HD and control cases. The Optical Fractionator probe was used to obtain an estimate in the total number of pallidal neurons in each
region, and the *Cavalieri Estimator* was used to quantify regional volume changes. The differences in cell number and regional volume between HD and control cases were compared for the GPe, GPi and VP (West and Gundersen, 1990, Oorschot, 1996).

### 3.3.1.1 Definition of the regions of interest

To perform stereological analysis for the GPe, GPi and VP, the exact boundaries for each region were defined so that the entire region of interest was sampled for each case. The anatomical boundaries were delineated based on enkephalin (GPe and VP) or substance-P (GPi) immunoreactivity. An outline was traced around the immunolabelled boundary under a lower power dissection microscope (DXM1200F, Nikon) using a fine waterproof marker. A diagram summarising the anatomical boundaries and regions of interest are shown in Figure 3.3.

**Globus pallidus external segment**

The external segment of the GP was distinguished laterally from the striatum, and medially from the internal segment by its extremely dense enkephalinergic terminals, which was visualised using enkephalin immunolabelling. The enkephalinergic immunoreactivity appeared as “woolly fibres” at low magnification. At high magnification, this pattern can be seen to be composed of many individual beaded axons wrapping pallidal dendrites. The enkephalin positive woolly fibres which make up the external segment are quite dense in distribution, which was the rationale behind using a low antibody concentration and short peroxidase reaction time, as the dense fibre distribution can mask the Nissl stained pallidal neurons. The rostral pole of the external segment was determined by the posterior limit of the temporal limb of the anterior commissure, which marks the boundary between the external segment and subcommissural ventral pallidum (Haber and Elde, 1981, Haber and Watson, 1985).

**Globus pallidus internal segment**

The internal segment was distinguished laterally from the external segment by dense substance-P immunoreactive terminals, which was visualised using substance-P immunolabelling (appearing as peptidergic tubular profiles, similar to the enkephalinergic woolly fibres in the GPe). The full
extent of the GPi was defined by the intense substance-P immunoreactivity which was readily visible to the unaided eye (Haber and Watson, 1985, Mai et al., 1986).

**Ventral pallidum**

The border between the ventral pallidum and GPe is defined by the ventral region of the pallidum which receives input from the ventral striatum (nucleus accumbens) lying immediately ventral to the anterior commissure (Haber et al., 1990b). Unlike the clear separation between the GPe and GPi based on differential neurochemical markers, the delineation of the ventral pallidum borders both rostrally and caudally in coronal sections were more difficult. This is because both substance-P and enkephalinergic-positive profiles are found within the subcommissural region, where these peptidergic tubular profiles intermingle. The ventral pallidum was distinguished ventrally from the GPe in this study by a dense wedge of enkephalin-positive terminals extending directly under the temporal limb of the anterior commissure. Enkephalin immunoreactivity was selected because of the continuity seen between the GPi and VP based on substance-P immunoreactivity, thereby using substance-P to delineate between these regions caudally could not be consistently carried out across cases (Mai et al., 1986). The rostral pole of the VP was defined by the first appearance of enkephalin immunoreactivity clearly separated from the GPe by the anterior commissure, whereas the caudal pole was marked by caudal limit of the temporal limb of the anterior commissure, which coincides with the loss of the enkephalin positive ventral wedge (Heimer et al., 1999).

3.3.1.2 Blinding of cases

After immunohistochemistry and Nissl staining was complete, sections from each case were blinded by an independent researcher who had no involvement in specimen selection, delineation, or measurement. Case numbers were coded based on the region analysed, with a random number between 1 and 16 allocated (16 cases in total per region). For example, a control case such as H170 used for a stereological study of the globus pallidus external segment would be blinded to GPe14, and a Huntington’s case used in the same study, such as HC125 would be GPe6. Therefore, the cases were blinded to avoid knowledge of the case number, and knowledge of the disease state.
Figure 3.3 Schematic drawings of the human basal forebrain in a series of coronal sections

This figure of schematic drawings of the human basal forebrain shows the regions of interest in a series of coronal sections starting rostrally at the level of the ventral pallidum and ventral striatum (A), and ending at the level of the caudal amygdala and thalamus (C). Panel (A) highlights the globus pallidus external segment (GPe) as defined in red, which is separated from the ventral pallidum (VP) as defined in blue, by the anterior commissure (ac). Panel (B) highlights the globus pallidus internal segment (GPI), as defined in yellow, which is located medially from the GPe. Panel (C) highlights the location of both the GPe and GPI more caudally in the basal ganglia. The GPe and VP are delineated from neighbouring structures based on enkephalin immunoreactivity, with the location of the ac being the boundary between these structures (A). The GPI is delineated based on substance-P immunoreactivity, allowing separate visualisation from the GPe (B), (C). Abbreviations: LV, lateral ventricle; Cl, claustrum, VCl, ventral claustrum; B, basal nucleus of Meynert; VS, ventral striatum; Put, putamen, CN, caudate nucleus; ac, anterior commissure; GPe, globus pallidus external segment, GPI, globus pallidus internal segment; VP, ventral pallidium; Th, thalamus; BL, basolateral nucleus of amygdala; BM, basomedial nucleus of amygdala; La, lateral nucleus of amygdala; Ce, central nucleus of amygdala; Me, medial nucleus of amygdala.
In order to investigate the total number of objects in a well delineated area of interest, the entire basal ganglia was sectioned at a thickness of 70 µm and a systematic random sampling (SRS) protocol was employed to select a serial section, for example, with a fixed known periodicity from a random starting point. Every \( n^{th} \) section series, characterised by a series of sections at equal intervals sampled in a systematic random manner, were collected and stained according to the sampling scheme (see 3.1.3 tissue sampling, and Table 3.3). The \( n^{th} \) section series is determined based upon the size of the region. For statistical feasibility, 10-15 sections per region from each case is sufficient for a stereological counting study (Gundersen and Jensen, 1987, West, 1999, Slomianka and West, 2005). Using the globus pallidus external segment as an example, 300 coronal serial sections made up the total number of sections in the GPe for an average case. Every 20\( ^{th} \) section (i.e. 1/20 series) was sampled systematically, collected, and stained with enkephalin and Nissl, resulting in 15 sections (300 divided by 20, or total section number divided by sampling interval). For this study, an average of 15 sections per case were used to study the GPe, 10 sections per case were used to study the GPi, and 9 sections per case were used to study the VP. As the VP is a substantially smaller region compared to the GPe and GPi, care was taken when selecting the sampling interval to ensure that the region was adequately sampled, without compromising the other regions.

Optimal stereological estimates are based on a sampling protocol that yields sufficient data to achieve the required level of precision, while making the work efficient by performing as few measurements as possible to achieve this level of precision. Once the section interval was selected for each region (section 3.2.3, Table 3.3), pilot studies were run to determine the optimal stereological parameters required to run all stereological probes. A sufficient amount of oversampling and resampling of each region was carried out with a grade 3 Huntington’s case, and a representative control case, to determine the optimal number of sampling sites required (based on altering the grid size), the optimal dissector height and guard zone thickness (based on measuring the total tissue thickness and several sampling sites), and the optimal counting frame dimensions (based on the maximal number of pallidal neurons which could be captured in one screenshot) (Slomianka and West, 2005). The coefficient of error (CE) was used to evaluate the
precision of stereological estimates (see section 3.3.3). Therefore, the optimal parameters which produced the lowest CE were used for the rest of the studies.

3.3.1.5 Optical Fractionator Workflow

The *Optical Fractionator* is a 3-dimensional systematic random sampling method that enables the objects of interest (cells, organelles, objects, or particles) to be sampled with an equal probability that is independent of their size, shape and orientation in the tissue (West and Gundersen, 1990, West, 1993, Pakkenberg and Gundersen, 1997). This method uses thick sections, and estimates the total number of objects sampled with a systematically randomly sampled set of unbiased virtual counting spaces covering the entire region of interest, with uniform distance between the counting spaces in x, y and z directions. This probe required a tissue thickness that can be broken up optically by the focal point of a high power numerical aperture (NA) objective. Typically, tissue between 15-30 microns thick post-processing generates several optical Z-planes when using a 1.4 NA objective, which is the reason why the sections for this study were cut at 70 µm, as tissue which has been sectioned at ~60 µm normally generates usable results (Dorph-Petersen et al., 2001). The *Optical Fractionator* probe was performed using Stereoinvestigator software (Version 10, MBF Bioscience, MicroBrightField Ltd, USA), to estimate the total number of Nissl-positive pallidal neurons in the external, internal, and ventral pallidum regions.

*Optical Fractionator* based sampling was performed on live images of each section on a Nikon E800 microscope equipped with a digital camera (MBF Bioscience, MicroBrightField Ltd, USA) and automated mechanical stage (Ludl Electronic Products Ltd, USA). The microscope was calibrated with various measures to ensure that movements of the stage in the x, y and z directions could be measured and displayed. Rotational alignment of the camera was constantly checked and recalibrated to ensure correct physical alignment of the camera with the microscope. The objective lenses were also calibrated with a graticule slide to ensure accurate alignment in the x and y directions. Parcentral and parfocal calibration was also performed to account for parfocal (focal plan) deviations and parcentric (imperfect collimation) differences among different objectives.

To achieve unbiased sampling, each pallidum region was sampled in a systematically random method (see section 3.3.1.3). Using the *Optical Fractionator* workflow, several steps are carried
out for each case to ensure all procedures were kept constant. The first step involved entering general case information for each region; including the number of sections to be analysed, the section cut thickness (70 µm), section evaluation interval (every 20th section for the external and internal segment, and every 5th section for the ventral pallidum), the starting section number (one), and the z-value for the first section (which is always 0.00 since the starting section is one). The second and third step involved setting the microscope to a low power objective for contour drawing around each pallidum region. Each section was traced using a 2x air objective (Nikon plan UW 2x/0.06 NA), and a grid of known dimensions was placed at random on to the trace. To ensure that an adequate number of sampling sites were selected, pilot studies were performed to determine an optimal grid size for each region (West and Gundersen, 1990, Slomianka and West, 2005).

Steps 4 and 5 involved setting the microscope to a high power objective which gives a good depth of view to resolve cells in close proximity in the z axis, and measuring the mounted tissue thickness post-processing. Each section was sampled at the top left hand corner of each square in the grid using a 60x oil immersion objective for cell counting (Nikon Plan Apo 1.40 NA). The thickness of the section was found by focusing through the tissue; and it was measured as the distance, in micrometres, between the first point in which the tissue comes into focus at the top of the section, and the last point to come into focus at the bottom of the section when the stage was moved in the z direction. The mounted thickness was measured at every sampling site for accuracy.

Step 6 involved defining the counting frame size. A 3-dimensional disector counting frame was superimposed on to the image. The counting frame is composed of “inclusion” (green top and right adjacent surfaces) and “exclusion” (red bottom and left adjacent surfaces) lines (Figure 3.4). The counting frame was 95µm by 95µm, a square frame which allows approximate 1-5 pallidal neurons to fit within the frame. Within each frame, pallidal neurons which were found inside the counting frame, did not cross the exclusion lines, crossed the inclusion lines, and fell in the disector height were counted (Figure 3.3). Neurons which were outside the counting frame, crossed the exclusion lines, or fell out of the disector height were not counted. If a pallidal neuron was found to cross both an inclusion and exclusion line, it was not counted. To maintain an unbiased quantification, each cell must have an equal opportunity to be counted once (without the chance of repetition) (West, 1993). Each cell is represented by a “unique point,” which is a feature that can only occur in a cell once (for example, a cell top) (König et al., 1991). In this
study, the nucleolus of a pallidal neuron was a unique feature which was easily identified and therefore was chosen as the main identifier. Pallidal neurons were morphologically identified on Nissl-stained sections as large, ellipsoid-shaped, nucleolated cells. The visibility of the nucleolus was a main identifier for a pallidal neuron to be counted. For any pallidal neuron which was a valid candidate based on the exclusion and inclusion criteria, it was only counted if the nucleolus came into focus within the disector height. Therefore, for this study, the pallidal neuron was only counted if its “unique point” (König et al., 1991) was found within the optical disector or virtual counting space (West, 1993, Schmitz, 1998). A flow chart summarising the inclusion and exclusion criteria is outlined in Figure 3.5.

Pilot studies were conducted to determine all stereological parameters including counting frame dimensions, grid size, disector height and guard zones to produce the desired number of pallidal neuron observations in each pallidal region. Step 7 involved determining the number of sampling frames required based on such pilot work, and step 8 involved defining the disector height and guard zones based on the measured mounted thickness. Guard zones are placed to ensure that over or under-estimation of total pallidal neurons does not occur. Due to tissue cutting procedures, section deformation and section thickness changes; cells are often damaged, cut in half, or plucked from tissue, particularly near the top and bottoms of sections, creating a “lost cap” effect (Andersen and Gundersen, 1999). A calibration study was carried out to observe bulk pallidal neuron distribution (without guard zones, the full thickness of the section) and it was found that the 70 µm sections reduced in thickness to ~16-25 µm post-processing. Thus, the disector height was set to 10 µm with guard zones of 2 µm on the top and bottom. The stereological parameters for the Optical Fractionator are summarised in Table 3.5. Steps 9 and 10 involved the saving of parameters and the running of the fractionator.

<table>
<thead>
<tr>
<th>Region (marker)</th>
<th>Cell (marker)</th>
<th>Grid size (µm)</th>
<th>Counting frame (µm)</th>
<th>Disector height (µm)</th>
<th>Guard zones (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globus pallidus external segment (enkephalin)</td>
<td>Pallidal neurons (Nissl)</td>
<td>500 x 500</td>
<td>95 x 95</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Globus pallidus internal segment (substance-P)</td>
<td>Pallidal neurons (Nissl)</td>
<td>400 x 400</td>
<td>95 x 95</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Ventral pallidum (enkephalin)</td>
<td>Pallidal neurons (Nissl)</td>
<td>300 x 300</td>
<td>95 x 95</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>
This figure illustrates a screenshot of a Nissl cell count in a control case using a 60x oil immersion objective. Nissl-positive pallidal neurons have been illustrated, with a “counted cell” (yellow arrow) and “excluded cell” (white arrow) highlighted as shown. A 3-dimensional disector counting frame (95 µm by 95 µm) was superimposed on the image. The counting frame is composed of “inclusion” (green top and right adjacent surfaces) and “exclusion” (red bottom and left adjacent surfaces) lines. The optical disector height was 10 µm. Within each frame, pallidal neuronal soma which were found inside the counting frame, did not cross the exclusion lines, and came into focus within the disector height, were counted (as shown by the “included cell” above). Whereas, pallidal neuronal soma which were outside the counting frame, crossed the exclusion lines, or fell out of the disector height, were not counted. If a pallidal neuron was found to cross both an inclusion and exclusion line, it was not counted (as shown by the “excluded cell” above). Pallidal neurons were morphologically identified on Nissl-stained sections as large, ellipsoid-shaped, nucleolated cells. The visibility of the nucleolus was a main identifier for a pallidal neuron to be counted (highlighted with a green star).
This figure summarises the stereological counting criteria for the quantification of Nissl-positive pallidal neurons in the globus pallidus externus, globus pallidus internus, and the ventral pallidum. Pallidal neurons were counted based on their position within the counting frame, visibility of the nucleolus, crossing position with regard to inclusion (green) and exclusion (red) lines, and focal position within the optical disector.
A total of 100-300 cells were counted for a set of approximately ~10 sections to produce a coefficient of error (CE) of ≤0.1 per case. Data collected from each case for number of neurons counted, number of sampling frames, averaged measured section thickness, disector height, total area counted, area of counting frame, sampling interval and total section number were entered into Equation 3.1 below by StereoInvestigator to obtain an unbiased estimate of total neuron number (West and Gundersen, 1990). The raw data files were used for the basis of the analysis instead of the precalculated results given by the software package. However, the manually calculated results were compared with reference to the calculated results by StereoInvestigator (estimated population using mean section thickness).

**Equation 3.1 – The Optical Fractionator**

\[
N = \sum Q^- \cdot \frac{t}{h} \cdot \frac{1}{asf} \cdot \frac{1}{ssf}
\]

Where:

- \( N \) = total number of neurons
- \( \sum Q^- \) = number of neurons in a disector frame
- \( asf \) = area sampling fraction – The ratio of the counting frame’s area to the area formed by the Optical Fractionator sampling grid. Calculated by: area of disector frame / (sampling grid area x \( \sum F \))
- \( t \) = average mounted thickness of the section (measured defined mounted thickness)
- \( h \) = disector height
- \( ssf \) = section sampling fraction – The known interval of sections sampled through an object of interest. Calculated by: number of sections sampled / total section number
- \( \sum Q^- = \sum Q / \sum F \)
- \( \sum Q \) = total number of neurons for the set of profiles
- \( \sum F \) = total number of sampling frames

**3.3.1.6 The Cavalieri Estimator**

The overall regional volumes of the external and internal segments, and the ventral pallidum, were estimated using the Cavalieri principle in the same series of sections used for neuronal counting (Cavalieri, 1653, Gundersen and Jensen, 1987). The **Cavalieri Estimator** is a design-based stereological probe for determining the total volume of all regions using a point-counting
method, and was run using the StereoInvestigator software. The point counting-method consists of overlaying each selected section with a regular grid of test points, which are randomly positioned. After each superimposition, the number of test points which are within the contour are counted, and the volume is determined by multiplying the section thickness, total number of points, and the representing area per point in the grid. A regularly spaced grid was superimposed at 2x/0.06 NA objective lens on each slide at random grid rotation, and the points which fell within the boundary of the external segment, internal segment and ventral pallidum respectively were counted separately for each case (Figure 3.6). The number of points to be counted (or the grid spacing to be used) was the same as the grid size selected using the Optical Fractionator (i.e. 500 x 500 for the external segment, Table 3.5). Equation 3.2 was used to determine the volume using the point counting method (Gundersen and Jensen, 1987, García-Fiñana et al., 2003). For each region, >200 points (ΣP) per case were counted to ensure that the coefficient of error (CE) was within acceptable limits (West, 1999, Slomianka and West, 2005).

The area of each section was determined from the number of points and the area associated with each point (Ap). The mean section interval (m) was either 20 for the GPe and GPi (every 20th section) or 5 for the VP (every 5th section). The mean section cut thickness (t), or block advance, was 70 microns. These values were substituted into Equation 3.2, together with the number of points falling on each sampled section (ΣP) to calculate the total volume of the globus pallidus external and internal segments, and the ventral pallidum.

**Equation 3.2: Cavalieri Estimator equation**

\[
V = A_p \cdot m \cdot \bar{t} \cdot \left( \sum_{i=1}^{n} P_i \right), \quad A_p = g^2
\]

Where:

- \( V \) = estimated volume
- \( A_p \) = area associated with a point
- \( m \) = mean section evaluation interval
- \( \bar{t} \) = mean section cut thickness
- \( \sum_{i=1}^{n} P_i \) = sum of points falling on the region of interest for each section
- \( g \) = grid size
This figure illustrates a screenshot of the *Cavalieri Estimator* probe, showing the delineation of a 70 µm coronal section from the globus pallidus external segment using a 2x objective. The *Cavalieri Estimator* consists of overlaying each selected section with a regular grid of test points, which are randomly positioned. After each superimposition, the number of test points which are within the contour are counted (counted test points marked with red stars).
3.3.2 Morphometric analysis

Morphometric analysis was carried out on HD and control tissue immunohistochemically stained for parvalbumin in the globus pallidus external and internal segments in order to quantify changes in pallidal neuron cell soma size. The use of a design-based estimator, called the *Isotropic Nucleator*, was implemented with an *Optical Fractionator* sampling scheme and systematic random sampling to estimate the cross-sectional area and volume of pallidal cell soma.

### 3.3.2.1 Isotropic Nucleator

The *Isotropic Nucleator* is a design-based estimator available in the StereoInvestigator software package that uses the intersection of rays and the cell surface for the estimation of the volume and cross-sectional area of cells or small objects. In many studies, it has been shown that the estimation of mean neuronal volume is a very sensitive parameter to detect pathological changes in neurons, often preceding changes in neuronal number (Tandrup and Braendgaard, 1994, Tandrup and Jakobsen, 2002, Iacono et al., 2009). As illustrated in Figure 3.7, the somal volume of every parvalbumin-positive pallidal neuron was estimated using the nucleator method (Gundersen, 1988). This method requires uniformly random sampling of neurons in either isotropic uniformly random sections (IUR) (Nyengaard and Gundersen, 1992) or vertical uniformly random sections (VUR) (Baddeley et al., 1986); that is, sections cut either with full randomness in orientation (IUR), or randomly rotated in relation to the vertical axis (VUR). However, this requirement was not met, as the sections were cut as coronal sections (therefore without randomness in orientation). The magnitude of the resulting bias in somal volume measurements depends on the degree of anisotropy in shape and orientation of neurons in the globus pallidus. Therefore, statistical analysis was also carried out using the cross-sectional area estimates generated, which is not subject to bias.
Table 3.6 Stereological counting parameters – *Isotropic Nucleator*

<table>
<thead>
<tr>
<th>Region (marker)</th>
<th>Cell (marker)</th>
<th>Grid size (µm)</th>
<th>Counting frame (µm)</th>
<th>Disector height (µm)</th>
<th>Guard zones (µm)</th>
<th>Number of Rays</th>
<th>Number of intersections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globus pallidus external segment (enkephalin)</td>
<td>Pallidal neurons (parvalbumin)</td>
<td>400 x 400</td>
<td>95 x 95</td>
<td>10</td>
<td>2</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Globus pallidus internal segment (substance-P)</td>
<td>Pallidal neurons (parvalbumin)</td>
<td>400 x 400</td>
<td>95 x 95</td>
<td>10</td>
<td>2</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

Serial coronal sections (70 µm thick), containing the globus pallidus internal and external segments, were stained immunohistochemically in a 1 in 40 series with the calcium-binding protein parvalbumin. The majority of large pallidal neurons in the globus pallidus are immunoreactive for parvalbumin, showing clear delineation of the entire cell body in the external segment, and punctate terminal labelling outlining the cell soma in the internal segment (see Table 3.4) (Waldvogel and Faull, 1993, Hardman and Halliday, 1999, Waldvogel et al., 1999, Waldvogel et al., 2006). This was the rationale for selecting parvalbumin to delineate pallidal neuron soma morphology for the Nucleator, as opposed to using the Nissl stain, which due to its pale nature, renders it difficult to accurately delineate the pallidal neuron soma. The *Isotropic Nucleator* probe samples cells based on the same systematic sampling principle as the *Optical Fractionator*; therefore it is launched in conjunction with the fractionator. The *Optical Fractionator* workflow (section 3.3.1.5) was launched and steps 1-8 were carried out to set up sampling parameters, with some minor adjustments included. Separate contours were traced around both the internal and external segments under low power on the same section, and counting frame dimensions were analogous to those used for Nissl-positive pallidal neuron quantification (Table 3.5). The systematic random sampling grid dimensions and all parameters were altered based on pilot studies (Table 3.5). Once all parameters were set up (i.e. upon completion of steps 1-8), the *Isotropic Nucleator* probe was launched (Moller et al., 1990). Upon clicking the nucleolus of pallidal neurons to mark the central feature of the cell, 16 rays were
placed within the focal plane, resulting in 16 systematically randomly rotated radii intersected with the cell membrane (see Figure 3.7). Based on these measurements, the software calculates the volume of the cell body from the lengths of the line segments using the following equations (Equation 3.3):

**Equation 3.3: Isotropic Nucleator equation**

Cross sectional area estimation:

$$a = \pi l^2$$

Volume estimation:

$$v_N = \frac{4\pi}{3} \cdot l_n^3$$

Where:

- $v_N$ = number weighted volume
- $l$ = length of the intercept
- $a$ = area
- $n$ = number of nucleator estimates

If a ray extended into a dendrite, the ray was cut at the base of the dendrite (Figure 3.6). A total of 8 to 377 pallidal neurons were measured in the external and internal segments for each case to achieve a coefficient of error of ≤0.1. Following the same inclusion and exclusion criteria depicted in Figure 3.4, pallidal neurons were measured if their cell bodies were inside the counting frame, or if the nucleolus was crossing the inclusion (green) line. Neurons were excluded if the nucleolus crossed the exclusion (red) line, or if they were outside the counting frame. Thus, the neurons measured were selected with optical dissectors so that the selection was number-weighted, not size-weighted. All stereological assessments were performed blinded (section 3.3.1.2) to the clinical and neuropathological classifications.
This figure illustrates a screenshot of the *Isotropic Nucleator* probe, showing a parvalbumin-positive pallidal neuron in a control case using a 60x oil immersion objective. Upon clicking the nucleolus of pallidal neurons (marked with yellow arrow), 16 rays were placed within the focal plane, resulting in 16 systematically randomly rotated radii intersected with the cell membrane (marked with red arrow). If a ray extended into a dendrite, the ray was cut at the base of the dendrite (marked with green arrow). Following the same inclusion and exclusion criteria (Figure 3.4), the neurons measured were selected with optical disectors so that the selection was number-weighted, not size-weighted.
3.3.3 Evaluation of the precision of estimates (coefficient of error)

In practical applications of design-based stereology, the amount of sampling error (the difference between an estimate and the true value) is unknown. Thus, many methods have been used to predict the accuracy of a stereological estimate without the need to repeat the estimate multiple times. The results of these methods of prediction are presented in the form of a coefficient of error or sampling error (CE), which represents the precision or reproducibility of estimates in stereology (West and Gundersen, 1990, Slomianka and West, 2005). The CE is expressed as a ratio of the standard error of the mean (SEM) to the mean of the repeated estimates (CE = SEM / mean) (West and Gundersen, 1990). It is important to have a low CE value in order to ensure that any variation seen between or within cases is due to real biological differences, rather than experimental procedures.

The precision and accuracy of the values obtained by the Optical Fractionator to estimate the total number of pallidal neurons in each pallidal region can be derived using a revised Matheron’s quadratic approximation formula (Matheron, 1971, Gundersen and Jensen, 1987, Gundersen et al., 1999). Regional volume estimation using the Cavalieri probe was also evaluated using a revised Matheron’s quadratic approximation formula (Gundersen and Jensen, 1987, García-Fiñana et al., 2003). The precision and accuracy of the values obtained using the Isotropic Nucleator were also evaluated using a CE, and is based on the assumption that individual neuronal volume estimates are independent (Gundersen, 1988).

At the beginning of this study, a CE value of less than or equal to 10% was decided as an ideal precision for all stereological data to be considered valid. For quantification of pallidal neurons in the internal, external and ventral pallidum, the CE should be less than half of the total observed variation (coefficient of variation, CV), or CE² / CV² < 0.5 (Gundersen and Jensen, 1987, Pakkenberg and Gundersen, 1997, Slomianka and West, 2005). This was applied to stereological counts in all regions in the pallidum, and was especially applicable for those regions were the CE was slightly over 10%. The CV is calculated as the standard deviation of the population divided by the mean number of cells. For this, the equation CV² = BV² + CE² was used. Previous stereological studies have shown that the CV of the human cerebral cortex was ~0.2, thus, assuming from previous studies that the biological variation (BV) of a human brain was approximately 0.15 or 15% (Pakkenberg and Gundersen, 1997), a CE of ≤0.1 was considered to be within the acceptable range (0.2² – 0.15² = 0.1²). For the vast majority of studies
based on stereological principles to date, a sampling scheme of 100-200 counts in ~10 sections, provides a corresponding CE of 0.07-0.1, which is enough precision that the variance of the estimator itself makes a smaller contribution to the observed variance of group estimates than the biological variance (Gundersen and Jensen, 1987, Gundersen et al., 1999, West, 1999). The CE is automatically calculated by the StereoInvestigator software for the *Optical Fractionator*, *Cavalieri Estimator* and *Isotropic Nucleator* separately or manually calculated using equations 3.4, 3.5 and 3.6.

3.3.3.1 Coefficient of Error for the *Optical Fractionator*

The precision, CE, of the estimate for the total number of pallidal neurons is expressed as a ratio of the standard error of the mean (SEM) to the mean of the repeated estimates (CE = SEM / mean) (West and Gundersen, 1990). For estimates of total numbers obtained with the *Optical Fractionator*, various methods to predict the CE have been described. The StereoInvestigator software automatically calculates the CE based on the following formula (Gundersen et al., 1999, Slomianka and West, 2005):

**Equation 3.4: Coefficient of error for the *Optical Fractionator***

$$CE = \sqrt{\frac{\text{Total Variance}}{\text{Nugget Variance}}}$$

$$\text{Total Variance} = \text{VAR}_{\text{SRS}} + \text{Nugget Variance}$$

$$\text{Nugget Variance} = \sum_{i=1}^{n} Q^{-}$$

$$\text{VAR}_{\text{SRS}} = \frac{3(A - \text{Nugget Variance}) - 4B + C}{240}, \quad m = 1$$

$$A = \sum_{i=1}^{n} (Q_i^{-})^2, \quad B = \sum_{i=1}^{n-1} Q_i^{-} Q_{i+1}^{-}, \quad C = \sum_{i=1}^{n-2} Q_i^{-} Q_{i+2}^{-}$$

$$\therefore CE = \sqrt{\frac{3(A - \text{Nugget Variance}) - 4B + C}{\text{VAR}_{\text{SRS}}} + \text{Nugget Variance}}$$
Where:

A = the sum of the squares of counts obtained from all individual sections of the sample, i.e. \( A = \sum_{i=1}^{n} (Q_i)^2 \)

B = the sum of the products of the count in the section \( i \) with the count in the subsequent section \( (i+1) \) for all sections of the sample, i.e. \( B = \sum_{i=1}^{n-1} Q_i Q_{i+1} \)

C = the sum of the products of the count in the section \( i \) with the count in section \( i+2 \) for all sections in the sample, i.e. \( C = \sum_{i=1}^{n-2} Q_i Q_{i+2} \)

Nugget variance = is the variance introduced by local errors for disector sampling. It is also known as \( S^2 \) and is equal to \( \sum_{i=1}^{n} Q^- \) (where \( Q^- \) is the sum of the cell counts obtained in all sections of the sample).

Smoothness factor \( m=1 \) = the decided smoothness factor was \( m=1 \), therefore the value given for a smoothness factor of \( m=1 \) is 1/240.

Extensive computer simulations have shown that the CE of estimated total numbers of cells can be precisely predicted with the Optical Fractionator depending on the spatial distribution of cells (Glaser and Wilson, 1998, Glaser and Glaser, 2000). Because the spatial distribution of cells within the pallidal regions resemble homogeneity or spatial randomness (pallidal neurons are not clustered), the Gunderson-Jenson Estimator was used to estimate the CE with a smoothness factor of \( m=1 \) as most biological tissues are best described by the \( m=1 \) class (Gundersen et al., 1999, Slomianka and West, 2005). This estimator examines how well a sequence of counts approximates the smooth outline of the true distribution of cells, and therefore, how close the estimate is to the true number.

The CE using the Gunderson-Jenson estimator is influenced by two independent sources of variance. One is the variability of the estimates made within each of the individual sections which is influenced by the amount of sampling performed at the individual section level (i.e. the number and or size of the optical disectors), which is referred to as the Nugget variance, or variance due to local errors, \( S^2 \). This is also known as the intra-section contribution to the coefficient of error, or (VAR_{section noise}). Local errors can be estimated under the assumption that the cells counted in disector samples are selected from a Poisson distribution. The sum of Poisson distributions is itself a Poisson distribution, and the variance of a Poisson distribution is equal to its mean. Because the CE estimation is based on one sample, which means there is only one sample from which the mean is determined, the sum of all cells counted estimates both its mean and its variance, as well as the variance due to local errors, \( S^2 \), introduced by disector sampling. Therefore, the sum of all disectors is equal to the mean of the Poisson distribution, thereby equal to the variance of the Poisson distribution. Thus \( S^2 = \sum Q^- \) (where \( Q^- \) is the sum of
the cell counts obtained in all sections of the sample). The second source of variance is referred
to as VAR$_{SRS}$ which is related to the inter-section contribution to the coefficient of error. This
variance is related to the variability between sections attributable to the systematic random
sampling scheme (differences in number from section to section) and can be calculated with the
quadratic approximation formula (Slomianka and West, 2005).

3.3.3.2 Coefficient of error for the *Cavalieri Estimator*

The precision, CE, of the estimate for the overall regional volumes is expressed as a ratio of the
standard error of the mean (SEM) to the mean of the repeated estimates ($CE = \frac{SEM}{\text{mean}}$)
(West and Gundersen, 1990). For estimates of total regional volume for each pallidal region
obtained with the *Cavalieri Estimator*, various methods to predict the CE have been described.
The StereoInvestigator software automatically calculates the CE based on the following formula
(Gundersen and Jensen, 1987):

**Equation 3.5: Coefficient of error for the *Cavalieri Estimator***

\[
CE = \frac{\sqrt{\text{Total Variance}}}{\text{Nugget Variance}}
\]

*Total Variance = Nugget Variance + VAR$_{SRS}$*

*Nugget Variance of point counting*  \( \sum P = 0.0724 \cdot \left( \frac{b}{a} \right) \cdot \sqrt{n \cdot \sum P_i} \)

*VAR$_{SRS}$ of*  \( \sum Q^- = \frac{3(A - \text{Nugget Variance}) - 4B + C}{240} \), \( m = 1 \)

\[
A = \sum_{i=1}^{n} (Q^-_i)^2 , \quad B = \sum_{i=1}^{n-1} Q^-_i Q^-_{i+1} , \quad C = \sum_{i=1}^{n-2} Q^-_i Q^-_{i+2}
\]

\[
\therefore CE = \frac{\sqrt{3(A - \text{Nugget Variance}) - 4B + C} \div 240 + \text{Nugget Variance}}{\sum_{i=1}^{n} Q^-}
\]
Where:

\[ A = \sum_{i=1}^{n} (Q_{i})^2 \]

\[ B = \text{the sum of the product of the number of points in the section } i \text{ with the number of points in the subsequent section in the series } (i+1) \text{ for all sections in the sample} \]

\[ C = \text{the sum of the products of the number of points in section } i \text{ with the number of points counted on the second next section in the series } i+2 \text{ for all the sections in the sample} \]

Nugget variance = is the variance introduced by local errors for point counting and is also known as the variance due to noise or the intra-section contribution to the coefficient of error. It is also known as \( S^2 \) and is equal to \( 0.0724 \cdot \left( \frac{L}{\sqrt{a}} \right) \cdot \sqrt{n} \cdot \sum_{i=1}^{n} P_i \).

Smoothness factor \( m=1 \) = the decided smoothness factor was \( m=1 \), therefore the value given for a smoothness factor of \( m=1 \) is 1/240.

Estimates of the CE were calculated with the Gunderson-Jensen estimator based on Matherson’s quadratic equation using the \( m=1 \) smoothness constant, as most biological tissues are modelled using this constant (Gundersen and Jensen, 1987, Gundersen et al., 1999). This estimator examines how well a sequence of counts approximates the smooth outline of the true distribution of a volume, and therefore, how close the estimate is to the true number.

The CE using the Gunderson-Jensen estimator is influenced by two independent sources of variance. One is the variability of the area estimates made within each of the individual sections which is influenced by the amount of sampling performed at the individual section level (i.e. the variability in the number of points counted when the point counting grid is placed repeatedly over the same section in a random manner), which is referred to as the Nugget variance, or variance due to local errors, \( S^2 \). This is also known as the intra-section contribution to the error or \( \text{VAR}_{\text{section noise}} \). The shape factor in the Nugget variance equation can either be read from a nomogram (Gundersen and Jensen, 1987), or calculated as the average length of the boundaries \( b \) of the structure divided by the square root of its average area \( \sqrt{a} \) of the sections, where \( n \) is the number of sections and \( Q \) is the sum of points counted in all sections of the sample (Gundersen and Jensen, 1987, Slomianka and West, 2005). The second source of variance is referred to as \( \text{VAR}_{\text{SRS}} \). This variance is related to the variability between sections attributable to the systematic random sampling scheme (differences in number from section to section) and can be calculated with the quadratic approximation formula (Slomianka and West, 2005). It is also known as the inter-section contribution to the coefficient of error.
3.3.3.3 Coefficient of error for the Isotropic Nucleator

The CE equation for the Isotropic Nucleator is based on the assumption that individual pallidal neuronal volume estimates are independent. The estimated coefficient of error for \( n \) independent observations for either cross-sectional area or volume estimates \( \text{CE}_n(R) \) is determined using the following formula: (Gundersen, 1988, Dorph-Petersen et al., 2001, Dorph-Petersen et al., 2004).

**Equation 3.6: Coefficient of error for the Isotropic Nucleator**

\[
\text{CE}_n(R) = \frac{CV(R)}{\sqrt{n}}
\]

\[
est CV(R) = \sqrt{\frac{1}{n-1} \cdot \sum_{i=1}^{n} (R_i - \bar{R})^2}, \text{ where } \bar{R} = \frac{1}{n} \cdot \sum_{i=1}^{n} R_i
\]

**Where:**

\( R = \) either area or volume estimates

\( n = \) number of nucleator estimates
3.4 Statistical analysis

3.4.1 Nonparametric statistical analysis for comparing mean ranks between groups

The mean, standard deviation (SD) and standard error of the mean (SEM) for total regional volume, total pallidal neuron number and average pallidal neuron body volume were calculated for all HD and control cases, as well as HD cases sub grouped according to striatal grade. All statistical analyses were conducted using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). Average changes in total regional volume, total pallidal neuron number, and average pallidal neuron soma volume in HD compared to control were assessed using a two-sided Mann-Whitney test, whereas HD multiple subgroup comparisons with control cases were carried out using a Kruskal-Wallis test, with the Dunn’s post-test. P-values < 0.05 were considered statistically significant (statistical significance expressed as *P < 0.05, **P < 0.01, ***P < 0.001.

Non-parametric methods were selected for statistical analysis based on considerable screening of the data for departures from assumptions required for parametric tests (Sokal and Rohlf, 1995). These assumptions include normality and homogeneity of variance (homoscedasticity). Normality was graphically assessed using a dot plot of the data, and if 95% of data points fell within two SD of the mean, the data was considered to be from a normally distributed population (Drummond and Tom, 2011). Normality tests were also used to assess normality using GraphPad Prism. The D-Agostino-Pearson omnibus normality test was used to quantify how far from normal the distribution from the samples were in terms of asymmetry and shape (D’Agostino, 1986). Data was considered normally distributed according to this test if \( P = 0.05 \). However, it is well documented that normality tests are unreliable on samples with low \( n \) numbers (due to potentially giving false negatives and possibly identifying data to be normally distributed even if it is not) (Quinn and Keough, 2002, Field, 2009). Also, for HD subgroup comparisons, normality tests could not be conducted as a minimum sample size of 6 is required. Therefore, graphical assessments of normality were the main deciding factor to determine whether the data came from a Gaussian population. Homoscedasticity was tested using the Brown-Forsythe test using GraphPad Prism, where \( P < 0.05 \) was considered to be heteroscedastic (the groups have different variances). Although the majority of stereological data sets collected from the control cases were homoscedastic and were normally distributed, many data sets from the HD cohort did
not satisfy all the above assumptions for parametric tests. Therefore, based on screening, it was decided to use non parametric methods.

**3.4.2 Correlation of Stereological data with case and symptom information**

A nonparametric Spearman’s correlation analysis was used to analyse any covariation or correlation between the stereological data obtained for each region and all case information (CAG repeat length, *post-mortem* delay, brain weight, age of death, age of disease onset, disease duration). Spearman’s correlations were also made between stereological data clinical assessment scales (see section 3.5 Symptom profile information). A two-tailed *P-value* of < 0.05 was considered a statistically significant correlation. An *R-value* of +1.0 or -1.0 is considered a perfect correlation, or inverse correlation, respectively.

**3.5 Symptom profile**

Clinical information relating to mood and motor symptoms was obtained from 5 of the 8 HD cases studied. This served as a basis for a preliminary investigation to determine whether there was any correlation between symptom profile and the stereological data (regional volume, cell loss, cell soma volume) obtained from the GPe, GPi and VP. Clinical measures of motor impairment and chorea were gathered from a neurologist who administered the Quantitated Neurological Examination (QNE) (Folstein et al., 1983) and The Unified Huntington’s Disease Rating Scale (UHDRS) (Kremer, 1996). Measures of Total Functional Capacity (TFC) were also obtained as part of the Unified Huntington’s Disease Rating Scale (Shoulson et al., 1989). A clinical psychologist assessed formal measures of mood using the Hospital Anxiety and Depression Scale (Zigmond and Snaith, 1983) and the Irritability, Depression and Anxiety Scale (Snaith et al., 1978). The Mini-Mental State Examination score (MMSE) was included as a cognitive measure (Folstein et al., 1975). The interval between the times for which the symptoms were examined and the death of the Huntington’s disease patients ranged from 3-10 years for mood scores (mean ± SD, 7 ± 2 years), and 3-12 years for motor scores (mean ± SD, 8 ± 3 years).
CHAPTER 4: RESULTS

The Globus Pallidus External Segment

4.1 Introduction

Huntington’s disease (HD), a neurodegenerative disorder, involves generalised loss of brain tissue, but the most prominent pathology is in the basal ganglia. The most pronounced neuropathology in HD occurs within the striatal part of the basal ganglia, in which there is gross atrophy. This is principally due to the loss of medium spiny GABAergic projection neurons in the striatum (Vonsattel et al., 1985, Vonsattel et al., 2008). Located caudomedial to the striatum, the external segment of the globus pallidus (GPe), is a relatively large nucleus which receives major input from the striatum (Kita, 2007). GPe neurons project to most of the basal ganglia nuclei, including the subthalamic nucleus, striatum, the internal globus pallidus (GPi), and the substantia nigra (Kita, 2007).

Of the three major output pathways of the striatum, the enkephalin-containing indirect pathway projecting to the GPe is involved earlier and more predominantly in the disease, than the substance-P containing direct pathway to the GPi, based on the presymptomatic and early HD grade loss of enkephalin immunoreactivity from striatal efferent terminals in the GPe (Reiner et al., 1988, Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009). Clear hypotheses have been raised to relate the early loss of enkephalinergic indirect pathway neurons to the symptomatology of HD (Albin et al., 1989, Deng et al., 2004). Loss of the indirect pathway to the GPe with sparing of the direct pathway could result in imbalance of these pallidal circuits favouring decreased GABA in the motor thalamus of the direct pathway, with loss of surround inhibition generated by the indirect pathway. This is postulated to result in the appearance of uncontrolled, chorea movements, a characteristic feature of HD (Albin et al., 1989, Hedreen and Folstein, 1995, Deng et al., 2004).

Neuroimaging studies have reported severe atrophy of the globus pallidus in HD patients (Aylward et al., 1997, Rosas et al., 2003, Fennema-Notestine et al., 2004, Douaud et al., 2006). However, in vivo imaging studies have not specifically isolated the external segment and
separately evaluated the extent of volumetric decline in HD. Post-mortem pathology studies have also reported that significant progressive atrophy of the globus pallidus external segment occurs, with greater atrophy and gliosis observed compared to the internal segment, based on qualitative or morphometric analysis (Lange et al., 1976, Vonsattel et al., 1985, Roos, 1986). However, only one stereological approach has reported a decline in GPe volume specifically in HD, with Halliday et al reporting a decline of 42% relative to controls (Halliday et al., 1998). However, no data relating the extent of GPe volume loss to striatal neuropathological grade or clinical symptom information was presented.

The neuronal population in the GPe comprises of GABAergic pallidal neurons, which suggests that they have an inhibitory effect on their target neurons (Albin et al., 1989). Pallidal neurons are sparse in distribution, compared to striatal neurons, and are 100 times less numerous than spiny striatal projection neurons (Yelnik, 2002). Three types of pallidal neurons have been identified based on their neurochemistry and morphology properties: Type 1 and 2 are the largest in size and have been described in multiple mammalian species and make up 80-90% of the total population of pallidal neurons in the globus pallidus (Fox et al., 1974, Difiglia et al., 1982, Francois et al., 1984, Waldvogel et al., 1999). When stained with Nissl, the majority of these neuronal cell soma in the GPe are large (35-70 µm), contain varying amounts of Nissl granules, and appear elongated with a triangular or spindle shaped cell soma (Difiglia and Rafols, 1988). Type 1 pallidal neurons contain GABA (Smith et al., 1987) and the calcium binding protein parvalbumin (Kita, 1994) but are immunonegative for any other immunohistochemical markers and make up about 10% of the large pallidal neuron population (Waldvogel et al., 1999). Type 2 cells are identical in cell morphology to type 1 cells, but are subdivided into “type 2” based on their double calcium binding protein immunoreactivity. These large pallidal neurons co-label for both parvalbumin and calretinin (another calcium-binding protein), on the same cells, and make up ninety percent of the large human pallidal neuron population (Fortin and Parent, 1994, Waldvogel et al., 1999). Medium-sized type 3 neurons in the globus pallidus have also been described in previous studies (Difiglia et al., 1982), and in primates they are intensely immunoreactive for calretinin only (Fortin and Parent, 1994), are less than 25 µm in cell soma size, and in the human make up approximately 10-20% of the total number of neurons in the human globus pallidus (Waldvogel et al., 1999).

Currently, there has been no stereological study which has evaluated the degree of pallidal neuron loss in Huntington’s disease. However, two studies using non-stereological methods have
reported conflicting conclusions in regards to GPe pallidal neuron involvement in HD. In one quantitative study by Lange et al in 1976, which compared 6 HD cases of unreported grades with 15 control cases, the absolute number of pallidal neurons was shown to decrease by up to 40% in the GPe, which was accompanied by a 50% reduction in pallidal volume (Lange et al., 1976). However, the neuronal density was up to 42% higher within the GPe, and no changes were found in relation to the soma volume of individual pallidal neurons in HD. This morphometric study suggested that pallidal neuronal loss was due to primary degeneration, rather than solely the consequence of striatal degeneration, thereby suggesting that pallidal neuron loss also contributes to GPe atrophy.

However, more recent studies have reached a conflicting conclusion, suggesting that the GP atrophy is mainly due to neuropil loss, resulting from striatal fibres and terminals, and to a lesser extent the loss of neurons (Reiner et al., 1988, Albin et al., 1990, Storey and Beal, 1993). Waikai et al histometrically examined 6 HD cases (of severe striatal neuropathological grading) in comparison to 10 control cases. Examination of pallidal neurons in 5 selected regions of coronal sections taken along the rostral-caudal axis, and using a non-stereological cell-counting method of large pallidal neurons, detected no cell loss in HD GP (Wakai et al., 1993). This study supported the hypothesis that no pallidal neuronal depletion was recognised in HD despite marked atrophy of tissue volume, thereby implicating overall GP atrophy to striato-pallidal fibre loss.

Thus, there is a disagreement in the literature about the extent of pallidal neuron loss in the GPe in Huntington’s disease. Furthermore, there is a lack of detailed stereological knowledge detailing the relationship between GPe atrophy, with both striatal neuropathological grade and symptom heterogeneity. There is also a current lack of understanding of pallidal neuron morphology changes in HD. Most importantly, since the GPe is a key nucleus involved in the indirect enkephalinergic pathway from the striatum in HD, the major aims of this section of the thesis are to investigate:

(1) The overall volumetric changes of the GPe in HD with regard to striatal pathological grade and symptom heterogeneity;
(2) the extent of pallidal neuron loss in HD with regard to striatal pathological grade and symptom heterogeneity; and
(3) pallidal neuron soma volume changes in HD with regard to striatal pathological grade and symptom heterogeneity.
These aspects will be investigated using design-based stereological techniques, including an unbiased method of quantifying GPe regional volume (Cavalieri Estimator), total pallidal neuron number (Optical Fractionator), and pallidal neuron volume (Isotropic Nucleator) using the whole region of interest. The research performed in this study is novel, as very few laboratories have access to tissue which is extensively characterised in terms of symptom and clinical information.

For each stereological technique used, the data was obtained using the StereoInvestigator software on 8 Huntington’s disease (HD) (HC101L, HC119R, HC120R, HC125L, HC132L, HC137R, HC139R, HC140R) and 8 control (H153R, H170R, H186R, H204R, H226R, H227R, H230R and H231R) cases (see Chapter 3 on detailed methodology). The stereological estimates for overall volume (in mm$^3$), total pallidal neuron number (total number of Nissl-positive pallidal neurons) and average pallidal cell soma volume (µm$^3$) for the GPe within this chapter are presented for each HD case ($N=8$) and each control case ($N=8$) in the form of a scatter plot with the standard error of the mean expressed as mean ± SEM. An estimation of cell density was also derived based on the quotient of the Optical Fractionator data and Cavalieri Estimator data (cells/mm$^3$). The standard deviation (SD) was also reported to assess variation amongst the samples. The mean changes in total regional volume, total pallidal neuron number, average pallidal cell soma volume, and pallidal neuron density in HD compared to control were assessed using a two-sided Mann-Whitney test, where P-values <0.05 were considered statistically significant (statistical significance expressed as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

In order to investigate if stereological estimates for overall volume, total pallidal neuron number and average pallidal cell soma volume for the GPe within this chapter is related to striatal pathology, the 8 HD cases were also independently examined by a neuropathologist (Dr B Synek) and each case was designated a grade based on striatal neuropathology criteria according to the Vonsattel grading system (Vonsattel et al., 1985, Vonsattel et al., 2008). The grades of the 8 HD cases ranged from 1 to 3. There were three grade 0-1 cases (HC101L, HC132L and HC137R) one grade 2 case (HC120R) and four grade 3 cases (HC119R, HC125L, HC139R, HC140R) (case details are listed in Table 3.2, Chapter 3). For the purpose of this investigation, the only grade 2 case was pooled with the grade 3 cases, with analyses conducted with and without this case to ensure it did not skew the data.
Grade-wise comparisons were conducted for the stereological estimates for overall volume (in mm³), total pallidal neuron number (total number of Nissl-positive pallidal neurons) and pallidal cell soma volume (µm³) for the GPe within this chapter. The mean changes were presented for each HD grade 0-1 case (N=3), each HD grade 2-3 case (N=5) and each control case (N=8) in the form of a scatter plot with the standard error of the mean expressed as mean ± SEM. The mean changes were grouped according to striatal neuropathological grade and compared to control using a Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted \(P\)-value. \(P\)-values <0.05 were considered statistically significant (statistical significance expressed as \(*P < 0.05, **P < 0.01, ***P < 0.001\).
4.2 The pattern of volumetric changes in the globus pallidus external segment in Huntington’s disease using the *Cavalieri Estimator*

In order to study volumetric changes of the globus pallidus external segment in detail, immunohistochemistry was performed using standard single peroxidise labelling techniques on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire GPe (1 in every 20th section) using antibodies to the neuropeptide enkephalin, which allows accurate delineation of the GPe from other structures in the basal ganglia. Secondly, an unbiased design-based stereological technique to quantify the volume of the GPe was performed using StereoInvestigator. The *Cavalieri Estimator* is a design-based stereological probe for determining the total volume of the entire globus pallidus external segment using a point-counting method, and was run using the StereoInvestigator software (see Chapter 3 on detailed methodology).

4.2.1 The pattern of volumetric changes in the globus pallidus external segment in all HD and control cases (Table 4.1, Figure 4.1)

The results of stereological volume quantification using the *Cavalieri Estimator*, shows that there is a striking 54% reduction in the mean volume of the GPe in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 4.1). The mean GPe volume ± SEM of 8 controls was 836.0 ± 68.1 mm$^3$, compared to the mean GPe volume ± SEM of all 8 HD cases which was 381.1 ± 32.2 mm$^3$ (Table 4.1). The mean changes in total regional volume in HD compared to control were significant according to the two-sided Mann-Whitney test, with the $P$-value being <0.05 (***$P = 0.0002$).

It was also interesting to note that there was considerable variation in the total regional volume of the GPe within the control and HD cohorts as shown by the differential standard deviations (SD). The mean for all control cases has a larger standard deviation (SD = 194.6 mm$^3$) compared to the HD cohort where the cases show a smaller standard deviation from the mean (SD = 91.13 mm$^3$) (Table 4.1). This is also reinforced by the larger range within the control cohort as shown by Figure 4.1, with the difference between case H231R (largest GPe volume 1172.2 mm$^3$) and case H204R (smallest GPe volume 550.9 mm$^3$) being 621.3 mm$^3$. In
comparison, the HD cohort has a smaller range, with the difference between case HC132L (largest GPe volume 512.4 mm³) and case HC119R (smallest GPe volume of 251.7 mm³) being 260.7 mm³.

For the stereological analysis of control and HD groups carried out using the *Cavalieri Estimator*, the average coefficient of error (CE) for the total volume of the GPe was always less than 0.10. The average CE for mean estimates of volume for the control and HD cases was 0.006 and 0.007 respectively, which are ≤ 0.10 and therefore a generally reliable estimation of volume (Table 4.1) (Gundersen and Jensen, 1987, Slomianka and West, 2005). This reinforces that any variability observed is due to a true difference between cases in the total volume rather than a lack of precision in terms of the stereological technique used (see Chapter 3, Materials and Methods) (Slomianka and West, 2005).

Table 4.1 Variation in the mean overall volume of the globus pallidus external segment (GPe) between control cases and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean total volume of the globus pallidus external segment (mm³)</td>
<td>836.0</td>
<td>381.1</td>
<td>461.1</td>
<td>333.1</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>194.6</td>
<td>91.13</td>
<td>63.11</td>
<td>69.78</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>68.81</td>
<td>32.22</td>
<td>36.44</td>
<td>31.21</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage reduction (%) as compared to control</td>
<td>-</td>
<td>***54% reduced</td>
<td>45% reduced</td>
<td>**60% reduced</td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.006</td>
<td>0.007</td>
<td>0.006</td>
<td>0.008</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
Figure 4.1 Overall volume of the globus pallidus external segment (GPe) in all HD cases compared to control cases in the human brain

The graph shows the variation of the total volume of the globus pallidus external segment (GPe) (defined by the Enkephalin immunoreactivity) in 8 HD and 8 control brains, determined using design-based stereological methods involving the Cavalieri Estimator protocol. Each blue diamond indicates the total volume of the GPe (in mm$^3$) for each control case, and each red diamond indicates the total volume of the GPe (in mm$^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 54% reduction in the mean total volume of the GPe in Huntington’s disease, which is statistically significant based on a 2-tailed $P$-value from the Mann-Whitney test (***(P = 0.0002). See Table 4.1 for detailed results.
4.2.2 The pattern of volumetric changes in the globus pallidus external segment in HD correlated with striatal neuropathological grade (Table 4.1, Figure 4.2, 4.3)

To investigate if the volumetric reduction of the pallidus external segment (GPe) is globus related to striatal pathology, the HD results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological volume quantification using the Cavalieri Estimator, shows that there is a **45% reduction in the mean volume of the GPe in grade 0-1 HD**, when compared with 8 controls (Figure 4.2). The mean GPe volume ± SEM of 8 controls was 836.0 ± 68.1 mm³, compared to the mean GPe volume ± SEM of the grade 0-1 cases which was 461.1 ± 36.4 mm³ (Table 4.1). However the mean changes in total GPe volume in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of >0.05 ($P = 0.14$). **Analysis of the grade 2-3 group showed a 60% reduction in mean GPe volume** compared to 8 controls (Figure 4.2), highlighted by the mean ± SEM of 333.1 ± 31.21 mm³ (Table 4.1). This striking reduction in mean GPe volume in HD grade 2-3 compared to control was significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of <0.05 (**$P = 0.001$**). Qualitative descriptions showcase the cross-sectional reduction of the GPe with advancing striatal neuropathological grade as highlighted in Figure 4.3.

In summary, it was observed that the overall mean volume of the GPe decreased with advancing HD striatal neuropathological grade. However with a small cohort of HD cases in the grade 0-1 group, the reduction observed was not statistically significant. This reduction was even greater beyond grade 2, with the mean GPe volume for the grade 2-3 group reduced by 60%, reinforcing that atrophy of the GPe coincides with striatal pathology.
Figure 4.2 Overall volume of the globus pallidus external segment (GPe) in HD cases grouped according to striatal neuropathological grade compared to control cases

The graph shows the variation of the total volume of the globus pallidus external segment (GPe) defined by the Enkephalin immunoreactivity in 8 control brains compared with 3 HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the *Cavalieri Estimator* protocol. Each blue diamond indicates the total volume of the GPe (in mm³) for each control case, each red diamond indicates the total volume of the GPe (in mm³) for each grade 0-1 HD case, and each green diamond indicates the total volume of the GPe (in mm³) of each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a **45% reduction in the mean total volume of the GPe in HD grade 0-1**, compared to control, though this was not statistically significant (*P* = 0.14). By HD grade 2-3, the mean GPe volume reduced by **60% compared to control**, and is statistically significant (**P** = 0.001) based on a multiplicity adjusted *P*-value from a Kruskal-Wallis test combined with Dunn’s multiple comparisons post-test. See Table 4.1 for detailed results.
Figure 4.3 Macroscopic cross-sectional examination of the globus pallidus external segment (GPe) of control and HD cases of striatal neuropathological grade 1 and 3

This figure shows representative macroscopic images at three coronal levels (rostral, middle, caudal) through the globus pallidus external segment (GPe) defined by enkephalin immunoreactivity and heavily counter-stained with Nissl. Images are taken of each 70 µm coronal section at low power from the rostral, middle and caudal areas of the GPe from a control (H227R), HD grade 1 (HC101L), and grade 3 (HC125L) case respectively. Note the progressive reduction of the delineated GPe cross-section with progressive HD grade.

A-C: Three representative coronal sections immunohistochemically stained with enkephalin (cross-section delineated) at rostral (A), middle (B) and caudal (C) levels of the GPe of control case H227R.

D-F: Three representative coronal sections immunohistochemically stained with enkephalin (cross-section delineated) at rostral (D), middle (E) and caudal (F) levels of the GPe of HD grade 1 case HC101L. Note the reduction in the outlined GPe cross-section observed at all levels in comparison to the control (H227R) equivalent levels.

G-I: Three representative coronal sections immunohistochemically stained with enkephalin (cross-section delineated) at rostral (D), middle (E) and caudal (F) levels of the GPe of HD grade 3 case HC125L. Note the reduction in the outlined GPe cross-section observed at all levels in comparison to case HC101L, and H227R.

Abbreviations: Put, putamen; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; CN, caudate nucleus; ac, anterior commissure; ant. Thal, anterior thalamus; mid. Thal, middle thalamus.

Scale bar: A-I = 5000 µm
Figure 4.3 Macroscopic cross-sectional examination of the globus pallidus external segment (GPe) of control and HD cases of striatal neuropathological grade 1 and 3

(A) H227R Rostral GPe

(B) H227R Middle GPe

(C) H227R Caudal GPe

(D) HC101L Rostral GPe (Grade 1)

(E) HC101L Middle GPe (Grade 1)

(F) HC101L Caudal GPe (Grade 1)

(G) HC125L Rostral GPe (Grade 3)

(H) HC125L Middle GPe (Grade 3)

(I) HC125L Caudal GPe (Grade 3)
4.2.3 The pattern of volumetric changes in the globus pallidus external segment compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration (Table 4.2, Figure 4.4)

The total volume of the globus pallidus external segment (GPe) was next correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both HD and control cases for which all this information was available (Figure 4.4). In general terms, the overall GPe volume decreased with brain weight for HD cases only (panel C), which is statistically significant according to a two-tailed Spearman’s correlation (*P = 0.03). No significant correlation was found between GPe total volume and repeat length, post-mortem delay, age of death, age of disease onset, disease duration for all HD and control cases.

4.2.4 The pattern of volumetric changes in the globus pallidus external segment compared with pallidal neuron number, average pallidal soma volume, and pallidal neuron density (Table 4.2, Figure 4.5)

It was next investigated whether the total volume of the GPe was correlated with total pallidal neuron number, average pallidal soma volume, and pallidal neuron density in both HD and control cases as determined using design-based stereology (Figure 4.5). In general terms, the overall GPe volume decreased with pallidal neuron number for control cases only (panel A), which is statistically significant according to a two-tailed Spearman’s correlation (*P = 0.04). No significant correlation was found between GPe total volume and average pallidal soma volume, and pallidal neuron density for all HD and control cases.
This figure shows the correlation of the total volume of globus pallidus external segment (GPe) with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration for all HD and control cases which have the information available. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total volume of the GPe and the CAG repeat number in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.22$, $P = 0.59$).

B: There was no significant correlation between the total volume of the GPe and the post-mortem delay in control ($r = -0.47$, $P = 0.23$) and HD ($r = 0.23$, $P = 0.59$) cases, according to a two-tailed Spearman’s correlation analysis.

C: There was no significant correlation between the total volume of the GPe and the post-mortem brain weight in control cases according to a two-tailed Spearman’s correlation analysis ($r = -0.26$, $P = 0.66$). However, there was a strong positive correlation between the total volume of the GPe and the post-mortem brain weight in HD cases, which was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = 0.82$, $P = *0.03$).

D: There was no significant correlation between the total volume of the GPe and the age of death for control ($r = 0.29$, $P = 0.50$) and HD ($r = 0.34$, $P = 0.39$) cases, according to a two-tailed Spearman’s correlation analysis.

E: There was no significant correlation between the total volume of the GPe and the age of disease onset in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.36$, $P = 0.36$).

F: There was no significant correlation between the total volume of the GPe and the duration of disease from age of onset to death in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.35$, $P = 0.38$).
Figure 4.4 Comparison between the total volume of the globus pallidus external segment with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration, in both HD and control cases.
Figure 4.5 Comparison between the total volume of the globus pallidus external segment with total pallidal neuron number, average pallidal soma volume, and pallidal neuron density, in both HD and control cases

This figure shows the correlation of the total volume of globus pallidus external segment (GPe) with total pallidal neuron number, average pallidal soma volume, and pallidal neuron density for all HD and control cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was a strong positive correlation between the total volume of the GPe and the total number of Nissl-positive pallidal neurons in control cases, which was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = 0.76$, $P = 0.04$). However, there was no significant correlation between the total volume of the GPe and the total number of Nissl-positive pallidal neurons in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50$, $P = 0.22$).

B: There was no significant correlation between the total volume of the GPe and the average pallidal soma volume in control ($r = -0.12$, $P = 0.79$) and HD ($r = 0.75$, $P = 0.27$) cases, according to a two-tailed Spearman’s correlation analysis.

C: There was no significant correlation between the total volume of the GPe and the density of pallidal neurons in control ($r = 0.62$, $P = 0.11$) and HD ($r = 0.33$, $P = 0.43$) cases, according to a two-tailed Spearman’s correlation analysis.
4.2.5 The pattern of volumetric changes in the HD globus pallidus external segment compared with symptomatology and clinical assessments (Table 4.2, Figure 4.6, 4.7)

It was next investigated whether the total volume of the GPe was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 4.6 highlights the correlation between the total volume of the GPe with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between GPe total volume and all variables examined. However, there is a general trend as shown in panel B, which shows that the overall GPe volume decreased with Mini Mental State Examination score, this was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), although the highly positive regression value of $r = 0.9$ highlights a very strong trend.

Figure 4.7 shows the correlation between the total volume of globus pallidus external segment (GPe) with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between GPe total volume and all variables examined. However, there is a general trend as shown in panel A and C, which shows that the overall GPe volume decreased with increased QNE and UHDRS motor impairment scores, this was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), although the highly negative inverse regression value of $r = -0.9$ highlights a very strong trend.
Figure 4.6 Comparison between the total volume of the HD globus pallidus external segment with measures of Total Functional Capacity, cognition and mood

This figure shows the correlation between the total volume of globus pallidus external segment (GPe) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases.

A: There was no significant correlation between the total volume of the GPe and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.40, P = 0.52$).

B: There is a clear trend that the overall GPe volume decreased with Mini-Mental State Examination score, although this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$). However, the highly positive regression value of $r = 0.9$ highlights a very strong trend.

C: There was no significant correlation between the total volume of the GPe and the Hospital Anxiety scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50, P = 0.45$).

D: There was no significant correlation between the total volume of the GPe and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.67, P = 0.27$).

E: There was no significant correlation between the total volume of the GPe and the Outward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50, P = 0.45$).

F: There was no significant correlation between the total volume of the GPe and the Inward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.79, P = 0.20$).
Figure 4.6 Comparison between the total volume of the HD globus pallidus external segment with measures of Total Functional Capacity, cognition and mood

A

**Overall GPp Volume (x10^2 mm^3)**

- **HD (n=5)**
- \( r = 0.40 \)
- \( P = 0.52 \)

Total Functional Capacity

B

**Mini-Mental State Examination Score**

- **HD (n=5)**
- \( r = 0.90 \)
- \( P = 0.08 \)

C

**Overall GPp Volume (x10^2 mm^3)**

- **HD (n=5)**
- \( r = 0.50 \)
- \( P = 0.45 \)

Mood Hospital Anxiety Scale

D

**Mood Hospital Depression Scale**

- **HD (n=5)**
- \( r = 0.67 \)
- \( P = 0.27 \)

E

**Overall GPp Volume (x10^2 mm^3)**

- **HD (n=5)**
- \( r = 0.50 \)
- \( P = 0.45 \)

SNAITH Outward Irritability Score

F

**SNAITH Inward Irritability Score**

- **HD (n=5)**
- \( r = 0.79 \)
- \( P = 0.20 \)
This figure shows the correlation between the total volume of globus pallidus external segment (GPe) with the Quantitative Neurological Exam (QNE) motor impairment and chorea scores, in addition to the Unified Huntington's Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case. In general terms, no significant correlation was found between GPe total volume and all variables examined.

A: There is a clear trend that the overall GPe volume decreased with increased QNE motor impairment score, although this was not statistically significant according to a two-tailed Spearman's correlation analysis ($P = 0.08$). However, the large negative inverse regression value of $r = -0.9$ highlights a very strong trend.

B: There was no significant correlation between the total volume of the GPe and the QNE chorea score in HD cases according to a two-tailed Spearman's correlation analysis ($r = -0.30$, $P = 0.68$).

C: There is a clear trend that the overall GPe volume decreased with increased UHDRS motor impairment score, although this was not statistically significant according to a two-tailed Spearman's correlation analysis ($P = 0.08$). However, the large negative inverse regression value of $r = -0.9$ highlights a very strong trend.

D: There was no significant correlation between the total volume of the GPe and the UHDRS chorea score in HD cases according to a two-tailed Spearman's correlation analysis ($r = -0.36$, $P = 0.50$).
4.2.6 Overall summary of volumetric changes in the globus pallidus external segment compared with all variables (Table 4.2)

A summary of the comparisons between the total volume of the GPe for all cases and all variables discussed in sections 4.2.3, 4.2.4 and 4.2.5 is highlighted in Table 4.2. Key findings include:

(1) The overall GPe volume decreased with brain weight for HD cases only
(2) The overall GPe volume decreased with pallidal neuron number for control cases only
(3) The overall GPe volume decreased with Mini-Mental State Examination score for HD cases only
(4) The overall GPe volume decreased with increased QNE and UHDRS motor impairment scores
Table 4.2 Summary of comparisons between globus pallidus external segment volume and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size (n)</th>
<th>Relationship</th>
<th>( r ) value (GPe volume)</th>
<th>Two-tailed ( P ) value (GPe volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD CAG repeat length</td>
<td>-</td>
<td>Inverse</td>
<td>-0.22</td>
<td>0.59</td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>8</td>
<td>Inverse Direct</td>
<td>-0.47</td>
<td>0.23 0.23 0.59</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>6</td>
<td>Inverse Direct</td>
<td>-0.26 0.82</td>
<td>0.66 *0.03</td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8</td>
<td>Direct Inverse</td>
<td>0.29 -0.34</td>
<td>0.50 0.39</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>-</td>
<td>Inverse</td>
<td>-0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>-</td>
<td>Inverse</td>
<td>-0.35</td>
<td>0.38</td>
</tr>
<tr>
<td>Total number of Nissl-positive pallidal neurons</td>
<td>8</td>
<td>Direct Direct</td>
<td>0.76 0.50 *0.04</td>
<td>0.22</td>
</tr>
<tr>
<td>Average parvalbumin-positive pallidal soma volume (µm³)</td>
<td>8</td>
<td>Inverse Direct</td>
<td>-0.12 0.45 0.79 0.27</td>
<td></td>
</tr>
<tr>
<td>Density of Nissl-positive pallidal neurons</td>
<td>8</td>
<td>Direct Direct</td>
<td>0.62 0.33 0.11 0.43</td>
<td></td>
</tr>
<tr>
<td>Total Functional Capacity</td>
<td>-</td>
<td>Direct</td>
<td>0.40</td>
<td>0.52</td>
</tr>
<tr>
<td>Mini-Mental State Examination score</td>
<td>-</td>
<td>Direct</td>
<td>0.90</td>
<td>0.08</td>
</tr>
<tr>
<td>Mood Hospital Anxiety Scale</td>
<td>-</td>
<td>Direct</td>
<td>0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>Mood Hospital Depression Scale</td>
<td>-</td>
<td>Direct</td>
<td>0.67</td>
<td>0.27</td>
</tr>
<tr>
<td>SNAITH Outward Irritability Score</td>
<td>-</td>
<td>Direct</td>
<td>0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>SNAITH Inward Irritability Score</td>
<td>-</td>
<td>Direct</td>
<td>0.79</td>
<td>0.20</td>
</tr>
<tr>
<td>QNE Motor Impairment score</td>
<td>-</td>
<td>Inverse</td>
<td>-0.90</td>
<td>0.08</td>
</tr>
<tr>
<td>QNE Chorea Score</td>
<td>-</td>
<td>Inverse</td>
<td>-0.30</td>
<td>0.68</td>
</tr>
<tr>
<td>UHDRS Motor Assessment Score</td>
<td>-</td>
<td>Inverse</td>
<td>-0.90</td>
<td>0.08</td>
</tr>
<tr>
<td>UHDRS Chorea Assessment Score</td>
<td>-</td>
<td>Inverse</td>
<td>-0.36</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \). Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
4.3 The pattern of pallidal neuron loss in the globus pallidus external segment in Huntington’s disease using the Optical Fractionator

In order to study pallidal neuron loss in the globus pallidus external segment in detail, first, immunohistochemistry using standard single peroxidise labelling techniques was performed on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire GPe (1 in every 20th section) using antibodies to the neuropeptide enkephalin, allowing accurate delineation of the GPe from other structures in the basal ganglia. Secondly, a Nissl stain with cresyl violet was used to stain all cells within the globus pallidus. Pallidal neurons were morphologically identified on Nissl-stained sections as large, ellipsoid-shaped, nucleolated cells. Thirdly, an unbiased design-based stereological technique to quantify pallidal neurons in the GPe was performed using StereoInvestigator. The Optical Fractionator is a 3-dimensional systematic random sampling method used to estimate the total number of Nissl-positive pallidal neurons in the GPe and was run using the StereoInvestigator software on 8 Huntington’s disease (HD) and 8 control cases (see Chapter 3 on detailed methodology).

4.3.1 The pattern of pallidal neuron loss in the globus pallidus external segment in all HD and control cases (Table 4.3, Figure 4.8)

The results of stereological pallidal neuron quantification using the Optical Fractionator, shows that there is a striking 59% reduction in the mean total pallidal neuron number in the GPe in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 4.8). The mean cell number ± SEM of 8 controls was 439,564 ± 77,197 pallidal neurons compared to the mean cell number ± SEM of all 8 HD cases which was 181,049 ± 46,920 pallidal neurons (Table 4.3). The mean changes in total pallidal neuron number in HD compared to control were significant according to the two-sided Mann-Whitney test, with the P-value being <0.05 (**P = 0.007).

It was also interesting to note that there was considerable variation in the total pallidal neuron number in the GPe within the control and HD cohorts as shown by the differential standard deviations (SD). Control cases had a larger standard deviation (SD = 218,347 cells) compared to the HD cohort where the cases show a smaller standard deviation from the mean (SD = 132,709
cells) (Table 4.3). This is also reinforced by the larger range within the control cohort as shown by Figure 4.8, with the difference between case H186R (highest count of 785,197 cells) and case H230R (lowest count of 172,150 cells) being 613,047 cells. In comparison, the HD cohort has a smaller range, with the difference between case HC101L (highest count of 412,022 cells) and case HC137R (lowest count of 52,576 cells) being 359,446 cells. It can also be noted that HC101L and HC132L produced cell counts which were well above the HD group mean (Figure 4.8), and with these cases both being classified with low striatal neuropathological grades of 0-1, it is not surprising that these cases produced counts higher than the HD cluster of cases with more severe pathology (i.e., >HD grade 2).

For the stereological analysis of HD and control groups carried out using the *Optical Fractionator*, the average coefficient of error (CE) for the total number of pallidal neurons in the GPe was always less than 0.10. The average CE for mean estimates of total cell number for the control and HD cases was 0.05 and 0.08 respectively, which are ≤ 0.10 and therefore a generally reliable estimation of pallidal neuron number, (Table 4.3) (Gundersen and Jensen, 1987, Slomianka and West, 2005). This reinforces that any variability observed is due to a true difference between cases in total pallidal neuron number rather than a lack of precision in terms of the stereological technique used (see Chapter 3, Materials and Methods) (Slomianka and West, 2005).

Table 4.3 Variation in the mean total pallidal neuron number in the globus pallidus external segment between control and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of Nissl-positive pallidal neurons</td>
<td>439,564</td>
<td>181,049</td>
<td>274,855</td>
<td>124,766</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>218,347</td>
<td>132,709</td>
<td>194,251</td>
<td>37,342</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>77,197</td>
<td>46,920</td>
<td>112,151</td>
<td>16,700</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage loss (%) as compared to normal</td>
<td>-</td>
<td><strong>59% loss</strong></td>
<td>37% loss</td>
<td><strong>72% loss</strong></td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.05</td>
<td>0.08</td>
<td>0.08</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with normal: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
The graph shows the variation in the total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) in 8 HD and 8 control brains, determined using design-based stereological methods involving the Optical Fractionator. Each blue diamond indicates the total number of Nissl-positive neurons in the GPe for each control case, and each red diamond indicates the total number of Nissl-positive neurons in the GPe for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a striking 59% reduction in the mean total number of Nissl-positive pallidal neurons in the GPe in Huntington's disease, which is statistically significant based on a 2-tailed P-value from the Mann-Whitney test (**P = 0.007). See Table 4.3 for detailed results.
4.3.2 The pattern of pallidal neuron loss in the globus pallidus external segment compared with striatal neuropathological grade (Table 4.3, Figure 4.9, 4.10)

To investigate if the loss of pallidal neurons in the globus pallidus external segment (GPe) is related to striatal pathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological pallidal neuron quantification using the Optical Fractionator, shows that there is a **37% reduction in the mean total number of pallidal neurons in the GPe in grade 0-1 HD**, when compared with 8 controls (Figure 4.9). The mean total cell number ± SEM of 8 control cases was 439,564 ± 77,197 cells, compared to the mean total cell number ± SEM of the grade 0-1 cases which was 274,855 ± 112,151 cells (Table 4.3). However, the mean changes in total cell number in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $>0.05$ ($P = 0.44$).

It is important to note that there is substantial variability amongst cases in the grade 0-1 group, as highlighted by the very large standard deviation of 194,251 cells, which is almost as large as the mean of the group itself (Table 4.2). **Analysis of the grade 2-3 group showed a 72% reduction in the mean total number of pallidal neurons** compared to 8 controls (Figure 4.9), highlighted by the mean ± SEM of 124766 ± 16700 cells (Table 4.3). This striking loss of pallidal neurons in HD grade 2-3 compared to control was significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $<0.05$ (**$P = 0.001$**). Figure 4.10 highlights these findings qualitatively through microscopic examination of pallidal neuron distribution in a representative control, HD grade 1, and HD grade 3 case.

In summary, it was observed that the **total number of pallidal neurons in the GPe decreased with advancing HD striatal neuropathological grade**. However, with a small cohort of HD cases in the grade 0-1 group, the reduction observed was not statistically significant. This reduction was even greater beyond grade 2, with the mean total number of pallidal neurons for the **grade 2-3 group reduced by a statistically significant 72%**, reinforcing that pallidal neuron loss coincides with striatal pathology.
Figure 4.9 Total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) in HD cases grouped according to striatal neuropathological grade compared to control cases.

The graph shows the variation in the total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) in 8 control brains compared with 3 HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the Optical Fractionator. Each blue diamond indicates total number of Nissl-positive neurons in the GPe for each control case, each red diamond indicates the total number of Nissl-positive neurons in the GPe for each grade 0-1 HD case, and each green diamond indicates the total number of Nissl-positive neurons in the GPe for each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 37% reduction in the mean total number of Nissl-positive pallidal neurons in the GPe in HD grade 0-1, compared to control, though this was not statistically significant ($P = 0.44$). By HD grade 2-3, a striking 72% reduction in the mean total number of Nissl-positive pallidal neurons was found compared to control, and is statistically significant (**) $P = 0.001$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn’s multiple comparisons post-test. See Table 4.3 for detailed results.
Figure 4.10 Representative photomicrographs showing the distribution of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) from control and HD cases of striatal neuropathological grades 1 and 3

This figure shows high power photomicrographs at 3 coronal levels (rostral, middle, caudal) through the globus pallidus external segment (GPe), highlighting the overall distribution of Nissl-positive pallidal neurons from a control (H170R), HD grade 1 (HC137R) and grade 3 (HC125L) case respectively (red arrows). Note the major reduction of pallidal neurons observed with progressive HD grade. It is also interesting to note the increase in small punctate Nissl-positive glial cells with progressive HD grade, these are likely to be a collection of astrocytes, oligodendrocytes and microglia.

A-C: Three representative coronal sections of a control case, H170R, stained with Nissl at rostral (A), middle (B) and caudal (C) levels of the GPe, showing the distribution of pallidal neurons. Red arrows denote representative pallidal neurons.

D-F: Three representative coronal sections stained with Nissl at rostral (D), middle (E) and caudal (F) levels of the GPe, showing the distribution of pallidal neurons within the HD grade 1 case HC137R. Red arrows denote representative pallidal neurons. Note the slight reduction in the number of Nissl-positive pallidal neurons observed at all levels in comparison to the control (H170R) equivalent regional levels. This is accompanied by an increase in the number of small punctate glial cells compared to H170R.

G-I: Three representative coronal sections immunohistochemically stained with Nissl at rostral (D), middle (E) and caudal (F) levels of the GPe of HD grade 3 case HC125L. Red arrows denote representative pallidal neurons. Note the major reduction in the number of Nissl-positive pallidal neurons observed at all levels in comparison to the control case (H170R), and the grade 1 HD case HC137R. This is accompanied by an increase in the number of small punctate glial cells compared to HC137R.

Scale bar: A-I = 100 µm
Figure 4.10 Representative photomicrographs showing the distribution of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) from control and HD cases of striatal neuropathological grades 1 and 3.
4.3.3 The pattern of pallidal neuron loss in the globus pallidus external segment compared with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration (Table 4.4, Figure 4.11)

It was next investigated whether the pattern of pallidal neuron loss in the GPe, was correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both HD and control cases for which all this information was available (Figure 4.11). In general terms, the total number of Nissl-positive pallidal neurons decreased with increasing disease duration for HD cases (panel F), but was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.07$). No significant correlation was found between the total number of Nissl-positive pallidal neurons and CAG repeat length, post-mortem delay, brain weight, age of death and age of disease onset for all control and HD cases.

4.3.4 The pattern of pallidal neuron loss in the globus pallidus external segment compared with pallidal neuron number, average pallidal soma volume, and pallidal neuron density (Table 4.4, Figure 4.12)

It was also explored whether the total number of Nissl-positive pallidal neurons was correlated with overall GPe volume, average pallidal soma volume, and pallidal neuron density in both control and HD cases, which were variables measured using design-based stereology (Figure 4.12). In general terms, the loss of pallidal neurons was greater with reducing GPe volumes for control cases only (panel A), which is statistically significant according to a two-tailed Spearman’s correlation ($*P = 0.03$). In addition to this, the loss of Nissl-positive pallidal neurons coincides with a reduction in overall pallidal neuron density, as shown by strong positive correlations for both control ($**P = 0.001$) and HD ($**P = 0.001$) cases. No significant correlation was found between the total number of Nissl-positive pallidal neurons and average pallidal soma volume.
Figure 4.11 Comparison between the total number of Nissl-positive pallidal neurons in the globus pallidus external segment with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration, in both HD and control cases

This figure shows the correlation of total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration for all control and HD cases which have the information available. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the CAG repeat number in HD cases according to a two-tailed Spearman's correlation analysis ($r = 0.30$, $P = 0.47$).

B: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the post-mortem delay in control ($r = -0.20$, $P = 0.61$) and HD ($r = 0.23$, $P = 0.59$) cases, according to a two-tailed Spearman's correlation analysis.

C: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the post-mortem brain weight in control ($r = 0.09$, $P = 0.92$) and HD ($r = 0.64$, $P = 0.14$) cases according to a two-tailed Spearman's correlation analysis.

D: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the age of death for control ($r = 0.10$, $P = 0.84$) and HD ($r = 0.64$, $P = 0.09$) cases, according to a two-tailed Spearman's correlation analysis.

E: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the age of disease onset in HD cases according to a two-tailed Spearman's correlation analysis ($r = -0.37$, $P = 0.34$).

F: There is a clear trend that the total number of Nissl-positive pallidal neurons decreased with a longer duration of disease from age of onset to death in HD cases, although this was not statistically significant according to a two-tailed Spearman's correlation analysis ($r = -0.67$, $P = 0.07$).
Figure 4.11 Comparison between the total number of Nissl-positive pallidal neurons in the globus pallidus external segment with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration, in HD and control cases

**A**

- **HD (n=8)**
  - \( r = 0.30 \)
  - \( P = 0.47 \)

**B**

- **Control (n=8)**
  - \( r = 0.23 \)
  - \( P = 0.59 \)

**C**

- **HD (n=7)**
  - \( r = 0.64 \)
  - \( P = 0.14 \)

**D**

- **Control (n=6)**
  - \( r = 0.09 \)
  - \( P = 0.92 \)

**E**

- **HD (n=8)**
  - \( r = -0.37 \)
  - \( P = 0.34 \)

**F**

- **Control (n=8)**
  - \( r = -0.67 \)
  - \( P = 0.07 \)
Figure 4.12 Comparison between the total number of pallidal neurons in the globus pallidus external segment with overall GPe volume, average pallidal soma volume, and pallidal neuron density, in HD and control cases

This figure shows the correlation of total number of Nissl-positive pallidal neurons with Overall GPe volume, average pallidal soma volume, and pallidal neuron density for all HD and control cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was a strong positive correlation between the total number of Nissl-positive pallidal neurons and the overall GPe volume in control cases, which was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = 0.76$, $P = 0.03$). However, there was no significant correlation between the total number of Nissl-positive pallidal neurons and overall volume of the GPe in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50$, $P = 0.22$).

B: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the average pallidal soma volume in control ($r = 0.07$, $P = 0.88$) and HD ($r = 0.60$, $P = 0.13$) cases, according to a two-tailed Spearman’s correlation analysis.

C: There was a strong positive correlation between the total number of Nissl-positive pallidal neurons and the density of pallidal neurons in control ($r = 0.95$, $P = **0.001$) and HD ($r = 0.95$, $P = **0.001$) cases, which was statistically significant according to a two-tailed Spearman’s correlation analysis.
4.3.5 The pattern of pallidal neuron loss in the HD globus pallidus external segment compared with symptomatology and clinical assessments (Table 4.4, Figure 4.13, 4.14)

It was next investigated whether the loss of Nissl-positive pallidal neurons was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 4.13 highlights the correlation between the total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and all variables examined. However, there is a general trend as shown in panel E, which shows that the total number of pallidal neurons decreased with a reduced outward irritability score. This was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), although the highly positive regression value of $r = 0.9$ highlights a very strong trend.

Figure 4.14 shows the correlation between the total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and all variables examined.
Figure 4.13 Comparison between the total number of pallidal neurons in the HD globus pallidus external segment with measures of Total Functional Capacity, cognition and mood

This figure shows the correlation between the total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total number of pallidal neurons and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.40$, $P = 0.52$).

B: There was no significant correlation between the total number of pallidal neurons and Mini-Mental State Examination scores in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.10$, $P = 0.85$).

C: There was no significant correlation between the total number of pallidal neurons and the Hospital Anxiety scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.30$, $P = 0.69$).

D: There was no significant correlation between the total number of pallidal neurons and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.15$, $P = 0.83$).

E: There is a clear trend that the total number of pallidal neurons decreased with the SNAITH outward irritability score, although this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$). However, the large positive regression value of $r = 0.90$ highlights a very strong trend.

F: There was no significant correlation between total number of pallidal neurons and the Inward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.47$, $P = 0.47$).
Figure 4.13 Comparison between the total number of pallidal neurons in the HD globus pallidus external segment with measures of Total Functional Capacity, cognition and mood.
Figure 4.14 Comparison between the total number of pallidal neurons in the HD globus pallidus external segment with clinical measures of motor impairment and chorea

This figure shows the correlation between the total number of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) with the Quantitative Neurological Exam (QNE) motor impairment and chorea scores, in addition to the Unified Huntington's Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total number of pallidal neurons and QNE motor impairment score in HD cases according to a two-tailed Spearman's correlation analysis ($r = -0.10$, $P = 0.95$).

B: There was no significant correlation between the total number of pallidal neurons and the QNE chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.30$, $P = 0.68$).

C: There was no significant correlation between the total number of pallidal neurons and the UHDRS motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.10$, $P = 0.95$).

D: There was no significant correlation between the total number of pallidal neurons and the UHDRS chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.35$, $P = 0.50$).
4.3.6 Overall summary of pallidal neuron loss in the globus pallidus external segment compared with all variables (Table 4.4)

A summary of the comparisons between the total number of Nissl-positive pallidal neurons in the GPe for all cases and all variables discussed in sections 4.3.3, 4.3.4 and 4.3.5 is highlighted in Table 4.4. Key findings include:

(1) The total number of pallidal neurons decreased with increasing disease duration for HD cases only
(2) The total number of pallidal neurons decreased with a reduced outward irritability score for HD cases only
Table 4.4 Summary of comparisons between the total number of pallidal neurons in the GPe and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size (n)</th>
<th>Relationship</th>
<th>r value (GPe pallidal neuron number)</th>
<th>Two-tailed P value (GPe pallidal neuron number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>HD</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>HD CAG repeat length</td>
<td>- 8</td>
<td></td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>8 8</td>
<td></td>
<td>Inverse</td>
<td>Direct</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>6 7</td>
<td></td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8 8</td>
<td></td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>- 8</td>
<td></td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>- 8</td>
<td></td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>Overall GPe volume (mm³)</td>
<td>8 8</td>
<td></td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Average parvalbumin-positive pallidal soma volume (µm³)</td>
<td>8 8</td>
<td></td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Density of Nissl-positive pallidal neurons</td>
<td>8 8</td>
<td></td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Total Functional Capacity</td>
<td>- 5</td>
<td></td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Mini-Mental State Examination score</td>
<td>- 5</td>
<td></td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Mood Hospital Anxiety Scale</td>
<td>- 5</td>
<td></td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Mood Hospital Depression Scale</td>
<td>- 5</td>
<td></td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>SNAITH Outward Irritability Score</td>
<td>- 5</td>
<td></td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>SNAITH Inward Irritability Score</td>
<td>- 5</td>
<td></td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>QNE Motor Impairment score</td>
<td>- 5</td>
<td></td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>QNE Chorea Score</td>
<td>- 5</td>
<td></td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>UHDRS Motor Assessment Score</td>
<td>- 5</td>
<td></td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>UHDRS Chorea Assessment Score</td>
<td>- 5</td>
<td></td>
<td>Inverse</td>
<td>-</td>
</tr>
</tbody>
</table>

* denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: *P < 0.05, **P < 0.01, ***P < 0.001. Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
4.4 The pattern of pallidal neuron cell soma size changes in the globus pallidus external segment in Huntington’s disease using the Isotropic Nucleator

In order to study changes in pallidal neuron cell soma volume in the globus pallidus external segment (GPe) in detail, first, immunohistochemistry using standard single peroxidise labelling techniques was performed on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire GPe (1 in every 40th section) using an antibody directed against the calcium-binding protein parvalbumin, to delineate the entire cell soma of large pallidal neurons. Parvalbumin is a calcium binding protein expressed in 80-90% of pallidal neurons in the human globus pallidus (Waldvogel et al., 1999), labelling all of the large pallidal neurons (types 1 and 2) in the GPe. Secondly, an unbiased design-based stereological technique to measure the cross-sectional area and volume of pallidal neurons in the GPe was performed using StereoInvestigator. The Isotropic Nucleator is a 3-dimensional systematic random sampling method which uses the intersection of rays to measure the cross-sectional area to obtain an estimate of mean pallidal soma volume in the GPe, and was run using the StereoInvestigator software on 8 Huntington’s disease (HD) and 8 control cases (see Chapter 3 on detailed methodology).

4.4.1 The pattern of pallidal soma volume changes in the globus pallidus external segment in all HD and control cases (Table 4.5, Figure 4.15, 4.16)

The results of stereological pallidal soma volume quantification using the Isotropic Nucleator shows that there is a **35% reduction in the mean pallidal soma volume in the GPe in HD**, when all 8 HD cases are grouped together and compared with 8 controls (Figure 4.15). The mean cell soma volume ± SEM of 8 controls was 17,945 ± 2,053 µm³ compared to the mean cell soma volume ± SEM of all 8 HD cases which was 11,725 ± 778.5 µm³ (Table 4.3). The mean changes in pallidal cell soma volume in HD compared to control were significant according to the two-sided Mann-Whitney test, with the *P*-value being < 0.05 (*P = 0.02).

It was also interesting to note that there was considerable **variation** in mean pallidal soma volume in the GPe **within** the control cohort as shown by large standard deviations (SD). Control cases had a larger standard deviation (SD = 5,806 µm³) compared to the HD cohort.
where the cases show a smaller standard deviation from the mean (SD = 2,202 µm³) (Table 4.5). This is also reinforced by the larger range within the control cohort as shown by Figure 4.15, with the difference between case H204R (largest cell volume of 26,765 µm³) and case H230R (smallest cell volume of 12,119 µm³) being 14,646 µm³. In comparison, the HD cohort has a smaller range with lower variability, with the difference between case HC125L (largest cell volume of 14,236 µm³) and case HC120R (smallest cell volume of 8,793 µm³) being 5,443 µm³. Figure 4.16 qualitatively describes the reduction in cell soma observed in HD through the comparison of cell soma cross-sectional delineation between a representative control and HD case.

For the stereological analysis of control and HD groups carried out using the *Isotropic Nucleator*, the average coefficient of error (CE) for the mean pallidal neuron soma volume in the GPe was always less than 0.10. The average CE for estimates of mean pallidal neuron soma volume for the control and HD cases was 0.006 and 0.02 respectively, which are ≤ 0.10 and therefore a generally reliable estimation of pallidal soma volume (Table 4.5) (Gundersen and Jensen, 1987, Slomianka and West, 2005). This reinforces that any variability observed is due to a true difference between cases in mean pallidal neuron soma volume rather than a lack of precision in terms of the stereological technique used (see Chapter 3, Materials and Methods) (Slomianka and West, 2005).

Table 4.5 Variation in the mean pallidal neuron soma volume in the globus pallidus external segment between control and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell soma volume of Parvalbumin-positive pallidal neurons (µm³)</td>
<td>17,945</td>
<td>11,725</td>
<td>13,147</td>
<td>10,872</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>5,806</td>
<td>2,202</td>
<td>835</td>
<td>2,390</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>2,053</td>
<td>778.5</td>
<td>482.0</td>
<td>1,069</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage reduction (%) as compared to control</td>
<td>-</td>
<td>*35% reduced</td>
<td>27% reduced</td>
<td>*39% reduced</td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.006</td>
<td>0.02</td>
<td>0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *P < 0.05, **P < 0.01, ***P < 0.001
Figure 4.15 Mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) in HD cases compared to all control cases in the human brain

The graph shows the variation in mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) in 8 control and 8 HD brains, determined using design-based stereological methods involving the Isotropic Nucleator. Each blue diamond indicates the mean cell soma volume of parvalbumin-positive neurons in the GPe (in $\mu m^3$) for each control case, and each red diamond indicates the mean cell soma volume of parvalbumin-positive neurons in the GPe (in $\mu m^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 35% reduction in mean cell soma volume of parvalbumin-positive neurons in the GPe in Huntington’s disease, which is statistically significant based on a 2-tailed $P$-value from the Mann-Whitney test ($^*P = 0.02$). See Table 4.5 for detailed results.
Figure 4.16 Representative photomicrographs showing the cell soma cross-sectional size of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) from a control (A-F) and advanced grade HD case (G-M)

This figure shows the morphological changes of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) in a representative control (H204R) and HD case (HC119R). Note the generally smaller morphology of the delineated pallidal neuron cell bodies in the HD case. Dotted lines encircle the cell soma.

A-C: Three representative high power (20x) photomicrographs of parvalbumin-positive pallidal neurons taken from a middle section within the GPe of the control case H204.

D-F: Higher magnification (60x oil) inserts of panels A-C. The pallidal neuron cell soma cross-section is delineated as shown.

G-I: Three representative high power (20x) photomicrographs of parvalbumin-positive pallidal neurons taken from a middle section within the GPe of the HD case HC119.

J-M: Higher magnification (60x oil) inserts of panels D-F. The pallidal neuron cell soma cross-section is delineated as shown. Note the smaller morphology of the HD pallidal neuron soma as indicated by the smaller delineated cross sections

Scale bar: A-C and G-I = 100 µm

Scale bar: D-F and J-M = 20 µm
Figure 4.16 Representative photomicrographs showing the cell soma cross-sectional size of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) from a control (A-F) and advanced grade HD case (G-M)
4.4.2 The pattern of pallidal soma volume changes in the globus pallidus external segment compared with striatal neuropathological grade (Table 4.5, Figure 4.17)

In order to investigate whether the reduction in pallidal soma size in the globus pallidus external segment (GPe) is related to striatal pathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological pallidal soma measurements using the Isotropic Nucleator, shows that there is a **27% reduction in the mean soma volume of pallidal neurons in the GPe in grade 0-1 HD**, when compared with 8 controls (Figure 4.17). The mean ± SEM of 8 controls was 17,945 ± 2,053 µm³, compared to the mean ± SEM of the grade 0-1 cases which was 13,147 ± 482 µm³ (Table 4.5). However, the mean changes in pallidal soma volume in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted \( P \)-value of \( > 0.05 (P = 0.42) \).

Analysis of the grade 2-3 group showed a **39% reduction in the mean pallidal soma volume compared to 8 controls** (Figure 4.17), highlighted by the mean ± SEM of 10,872 ± 1,069 µm³ (Table 4.5). This decline in pallidal soma volume in HD grade 2-3 compared to control was significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted \( P \)-value of \( < 0.05 (*P = 0.01) \).

In summary, it was observed that the **mean pallidal soma volume in the GPe decreased with advancing HD striatal neuropathological grade**. However, with a small cohort of HD cases in the grade 0-1 group, the reduction observed was not statistically significant. This reduction was even greater beyond grade 2, with the **mean pallidal soma volume for the grade 2-3 group reduced by 39%**, reinforcing that the decline in pallidal neuron soma volume coincides with striatal pathology.
Figure 4.17 Mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) in HD cases grouped according to striatal neuropathological grade compared to control cases.

The graph shows the variation in mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) in 8 control brains compared with 3 HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the Isotropic Nucleator. Each blue diamond indicates mean cell soma volume of parvalbumin-positive pallidal neurons in the GPe for each control case, each red diamond indicates the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPe for each grade 0-1 HD case, and each green diamond indicates the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPe for each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars.

The graph shows a 27% reduction in the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPe in HD grade 0-1, compared to control, though this was not statistically significant (*P = 0.42). By HD grade 2-3, a 39% reduction in mean cell soma volume of parvalbumin-positive pallidal neurons was found compared to control, and is statistically significant (*P = 0.03) based on a multiplicity adjusted P-value from a Kruskal-Wallis test combined with Dunn's multiple comparisons post-test. See Table 4.5 for detailed results.
4.4.3 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration (Table 4.6, Figure 4.18)

It was next investigated whether the pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the GPe was correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both control and HD cases for which all this information was available (Figure 4.18). In general terms, no significant correlation was found between the mean cell soma volume of parvalbumin-positive pallidal neurons and CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration for all control and HD cases.

4.4.4 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) compared with total GPe volume, pallidal neuron number, and pallidal neuron density (Table 4.6, Figure 4.19)

It was also explored whether the cell soma volume changes of parvalbumin-positive pallidal neurons in the GPe was correlated with overall GPe volume, total pallidal neuron number, and pallidal neuron density in both control and HD cases as determined using design-based stereology (Figure 4.19). In general terms, no significant correlation was found between the mean cell soma volume of parvalbumin-positive pallidal neurons and all stereological variables examined.
Figure 4.18 Comparison between mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration, in both HD and control cases

This figure shows the correlation of the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe), with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset and disease duration for all HD and control cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the CAG repeat number in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.04, P = 0.94$).

B: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the post-mortem delay in control ($r = -0.49, P = 0.21$) and HD ($r = 0.24, P = 0.55$) cases, according to a two-tailed Spearman’s correlation analysis.

C: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the post-mortem brain weight in control ($r = 0.37, P = 0.49$) and HD ($r = -0.32, P = 0.54$) cases according to a two-tailed Spearman’s correlation analysis.

D: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the age of death for control ($r = 0.60, P = 0.13$) and HD ($r = -0.04, P = 0.92$) cases, according to a two-tailed Spearman’s correlation analysis.

E: There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and the age of disease onset in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.00, P = 0.99$).

F: There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and the age of disease onset in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.01, P = 0.96$).
Figure 4.18 Comparison between mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration, in both HD and control cases.

A

Average Parvalbumin Pallidal Soma Volume in the GPe (x10^4 μm^2)

HD CAG Repeat Length

B

PM Delay (Hours)

Average Parvalbumin Pallidal Soma Volume in the GPe (x10^4 μm^2)

C

Brain Weight (g)

Average Parvalbumin Pallidal Soma Volume in the GPe (x10^4 μm^2)

D

Age Of Death (Years)

Average Parvalbumin Pallidal Soma Volume in the GPe (x10^4 μm^2)

E

Age Of Onset (Years)

Average Parvalbumin Pallidal Soma Volume in the GPe (x10^4 μm^2)

F

Age Of Death - Age Of Onset (Disease Duration)
Figure 4.19 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) with overall GPe volume, total pallidal neuron number, and pallidal neuron density, in both HD and control cases.

This figure shows the correlation of the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe), with overall GPe volume, total pallidal neuron number, and pallidal neuron density for all control and HD cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and overall GPe volume in control \( (r = -0.12, P = 0.79) \) and HD \( (r = 0.45, P = 0.27) \) cases, according to a two-tailed Spearman's correlation analysis.

B: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the total number of Nissl-positive pallidal neurons in control \( (r = 0.07, P = 0.88) \) and HD \( (r = 0.59, P = 0.13) \) cases, according to a two-tailed Spearman's correlation analysis.

C: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the density of pallidal neurons in control \( (r = 0.12, P = 0.79) \) and HD \( (r = 0.45, P = 0.27) \) cases, according to a two-tailed Spearman's correlation analysis.
4.4.5 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) compared with symptomatology and clinical assessments (Table 4.6, Figure 4.20, 4.21)

It was next investigated whether cell soma volume changes of parvalbumin-positive pallidal neurons in the GPe was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 4.20 shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe), with Total Functional Capacity, Mini-Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between the mean cell soma volume of parvalbumin-positive pallidal neurons and all variables examined.

Figure 4.21 shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe), with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between the mean cell soma volume of parvalbumin-positive pallidal neurons and all variables examined.

4.4.6 Overall summary of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus external segment compared with all variables (Table 4.6)

A summary of the comparisons between the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPe for all cases and all variables discussed in sections 4.4.3, 4.4.4 and 4.4.5 is highlighted in Table 4.6.
Figure 4.20 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the HD globus pallidus external segment (GPe), with measures of Total Functional Capacity, cognition and mood

This figure shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe), with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.10, P = 0.95$).

B: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and Mini-Mental State Examination scores in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.40, P = 0.52$).

C: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Hospital Anxiety scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.30, P = 0.68$).

D: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.15, P = 0.77$).

E: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the outward irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.15, P = 0.77$).

F: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Inward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.00, P = 0.80$).
Figure 4.20 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the HD globus pallidus external segment (GPe), with measures of Total Functional Capacity, cognition and mood.
Figure 4.21 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the HD globus pallidus external segment (GPe), with clinical measures of motor impairment and chorea

This figure shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe), with the Quantitative Neurological Exam (QNE) motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and QNE motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.40, P = 0.52$).

B: There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and the QNE chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.30, P = 0.68$).

C: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the UHDRS motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.40, P = 0.52$).

D: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the UHDRS chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.41, P = 0.43$).
Table 4.6 Summary of correlations between the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPe and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size (n)</th>
<th>Relationship</th>
<th>( r ) value (GPe pallidal neuron soma volume)</th>
<th>Two-tailed ( P ) value (GPe pallidal neuron soma volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>HD</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>HD CAG repeat length</td>
<td></td>
<td>8</td>
<td>Direct</td>
<td></td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>8</td>
<td>8</td>
<td>Inverse</td>
<td>Inverse</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>6</td>
<td>7</td>
<td>Direct</td>
<td>Inverse</td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8</td>
<td>8</td>
<td>Direct</td>
<td>Inverse</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td></td>
<td>8</td>
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<tr>
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<td>8</td>
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<td>Direct</td>
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<tr>
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<tr>
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<td>Inverse</td>
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</table>

*denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \). Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
4.5 The density of pallidal neurons in the globus pallidus external segment in Huntington’s disease

In order to study pallidal neuron density in the globus pallidus external segment in detail, the stereological cell counting data obtained using the *Optical Fractionator* (the total number of Nissl-positive pallidal neurons, see 4.3.1) was divided by the GPe volume estimate data obtain using the *Cavalieri Estimator* (GPe volume in mm³, see 4.2.1) in order to obtain a density estimate of pallidal neurons per mm³. The pallidal neuron density measures were obtained for 8 Huntington’s disease (HD) and 8 control cases.

4.5.1 The density of pallidal neurons in the globus pallidus external segment in all HD and control cases (Table 4.7, Figure 4.22)

After assessment of the density of pallidal neurons in the GPe, the mean cell density of Nissl-positive pallidal neurons was slightly reduced by 11%, when all 8 HD cases are grouped together and compared with 8 controls (Figure 4.22). The mean cell density ± SEM of 8 controls was 509 ± 70 cells/mm³ compared to the mean cell density ± SEM of all 8 HD cases which was 452 ± 86 cells/mm³ (Table 4.7). The mean changes in cell density in HD compared to control were not significant according to the two-sided Mann-Whitney test, with the *P*-value being >0.05 (*P* = 0.6).

| Table 4.7 Variation in mean cell density of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) between control and neuropathological grades in Huntington’s disease |
|---------------------------------|-------------|-------------|-------------|
| Mean cell density of Nissl-positive pallidal neurons (cells/mm³) | Control | HD (all grades combined) | HD grade 0-1 | HD grade 2-3 |
| Standard deviation of the mean (SD) | 198 | 243 | 380 | 125 |
| Standard error of the mean (SEM) | 70 | 86 | 220 | 56 |
| Sample size (n) | 8 | 8 | 3 | 5 |
| Percentage reduction (%) as compared to control | - | 11% reduced | 11% increased | 25% reduced |

*denotes significant difference compared with control: *P* < 0.05, **P* < 0.01, ***P* < 0.001
The graph shows the variation in mean cell density of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) in 8 control and 8 HD brains, determined from data obtained using the Optical Fractionator (total number of cells) divided by the data obtained using the Cavalieri Estimator (overall volume in mm$^3$) for each case. Each blue diamond indicates the cell density of Nissl-positive pallidal neurons in the GPe (cells/mm$^3$) for each control case, and each red diamond indicates the cell density of Nissl-positive pallidal neurons in the GPe (cells/mm$^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a negligible 11% reduction in mean cell density of Nissl-positive pallidal neurons in the GPe in Huntington’s disease, which is not significant based on a 2-tailed $P$-value from the Mann-Whitney test ($P = 0.6$). See Table 4.7 for detailed results.
**4.5.2** The pattern of pallidal neuron density changes in the globus pallidus external segment did not correlate with striatal neuropathological grade (Table 4.7, Figure 4.23)

In order to investigate the relationship between pallidal neuron density in the GPe and striatal neuropathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. After assessment of the density of pallidal neurons in the GPe, the data shows a slight 11% increase in the mean density of Nissl-positive pallidal neurons in the GPe in grade 0-1 HD, when compared with 8 controls (Figure 4.23). The mean ± SEM of 8 controls was 509 ± 70 cells/mm³, compared to the mean ± SEM of the grade 0-1 cases which was 565 ± 220 cells/mm³ (Table 4.7). However, the mean changes in cell density of pallidal neurons in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $> 0.05$ ($P > 0.99$). It was also important to highlight the extreme variability amongst the grade 0-1 HD cases, with an SD of 380 cells/mm³, almost double the SD of the control cohort.

**Analysis of the grade 2-3 group showed a 25% reduction in the density of pallidal neurons** compared to 8 controls (Figure 4.23), highlighted by the mean ± SEM of 384 ± 56 cells/mm³ (Table 4.7). This reduction in pallidal neuron density in HD grade 2-3 compared to control was not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $> 0.05$ ($P = 0.6$).

In summary, it was observed that pallidal neuron density did not correlate with striatal neuropathological grade, and did not show any statistically significant changes when the data from control cases were compared with the entire HD cohort or neuropathological grade subgroups.
Figure 4.23 Mean cell density of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) in HD cases grouped according to striatal neuropathological grade compared to control cases.

The graph shows the variation in mean cell density of Nissl-positive pallidal neurons in the globus pallidus external segment (GPe) in 8 control brains compared with three HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined from data obtained using the Optical Fractionator (total number of cells) divided by the data obtained using the Cavalieri Estimator (overall volume in mm$^3$) for each case. Each blue diamond indicates the cell density of Nissl-positive pallidal neurons in the GPe (cells/mm$^3$) for each control case, each red diamond indicates the cell density of Nissl-positive pallidal neurons in the GPe (cells/mm$^3$) for each HD grade 0-1 case, and each green diamond indicates the cell density of Nissl-positive pallidal neurons in the GPe (cells/mm$^3$) for each HD grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 11% increase in the mean cell density of Nissl-positive pallidal neurons in the GPe in HD grade 0-1, compared to control, though this was not statistically significant ($P > 0.99$). By HD grade 2-3, a 25% reduction in mean cell density of Nissl-positive pallidal neurons in the GPe was found compared to control, but was not statistically significant ($P = 0.6$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn's multiple comparisons post-test. See Table 4.7 for detailed results.
4.6. Discussion

This chapter provides a discussion of the results presented in this thesis on the degeneration of the globus pallidus external segment (GPe) in Huntington’s disease (HD), using histology, immunohistochemistry, and unbiased design-based stereological techniques. Three different stereological probes were used to measure GPe volume, pallidal neuron loss, and pallidal soma volume. These results show that there was a significant striking reduction in overall GPe volume (54%), pallidal neurons (59%), and pallidal cell soma volume (35%), when all HD cases were grouped together ($N = 8$) and compared with control cases ($N = 8$) (Figures 4.1, 4.8, 4.15). These results considerably extend on the previous study on HD degeneration in the GPe (Lange et al., 1976), which used non-stereological morphometric methods and reported a 57% reduction in GPe volume, and 47% reduction in pallidal neurons in 6 HD cases.

4.6.1 The pattern of volumetric changes in the globus pallidus external segment in Huntington’s disease using the Cavalieri Estimator

The results of stereological volume quantification using the *Cavalieri Estimator*, shows that there is a striking 54% reduction in the mean volume of the GPe in HD, when all 8 HD cases are grouped together and compared with 8 controls ($***P = 0.0002$) (Figure 4.1). This finding is consistent with findings in other *post-mortem* studies (Lange et al., 1976, de la Monte et al., 1988, Mann et al., 1993) although none of these studies used design-based stereological techniques. One study, (Halliday et al., 1998) used stereological techniques to delineate the external and internal pallidum segments separately (although detailed delineation with immunohistochemical techniques were not carried out), and used the *Cavalieri Estimator* protocol to obtain a volume estimate. This study reported a 42% reduction in mean GPe volume based on analysis of seven HD cases, which is generally consistent with our detailed results.

Region-of-interest based MRI studies have also reported a loss of volume in the globus pallidus of HD patients (Aylward et al., 1997, Rosas et al., 2003, Fennema-Notestine et al., 2004). A study using ROI-based combined with voxel based morphometry (VBM), reported a 56% loss in mean volume of the globus pallidus, demonstrating that recent improvements in MRI technology can illustrate pallidal atrophy in a non-invasive manner. However, ROI-based MRI combined with VBM is operator dependent and time-consuming, and although the globus pallidus was
observed and quantified using this method, there is currently no way to manually distinguish the GPe from the GPi, thereby restricting the amount of data available using this technique (Douaud et al., 2006).

It has long been known that atrophy of the striatum in HD is mainly due to the severe loss of medium spiny neurons, whereas GP atrophy has been thought to be due to neuropil loss, resulting from striatal fibres and terminals, and to a lesser extent the loss of neurons (Reiner et al., 1988, Albin et al., 1990, Storey and Beal, 1993). It has been thought that the loss of white matter traversing the GP might be a contributor to the reduction in overall GPe volume. Many bundles of myelinated fibres traverse the GP in fresh preparations, accounting for a paler colour than the neighbouring striatum (Nieuwenhuys et al., 2008). As highlighted by Douaud et al, the atypical striatal and pallidal increase in fractional isotropy as determined using diffusive tensor imaging (DTI), was concurrent to a decrease of the dispersion of the fibre orientation, unambiguously characterising a preferential loss of connections along specific radiating directions from these structures, while others are comparatively spared (Douaud et al., 2009). Further DTI analysis of striatal and pallidal white matter tracts by this same group, revealed that striato-pallidal projections are most affected compared to other subcortical projections. This suggests that a loss of myelinated afferent and efferent connections of the GPe could be accounting for the major decline in GPe volume in HD.

However, other studies have hypothesised that a loss of intrinsic neurons also results in GPe atrophy (Dom et al., 1976, Lange et al., 1976). This will be addressed further in the chapter (section 4.6.5).

**4.6.2 The pattern of volumetric changes in the globus pallidus external segment compared with striatal neuropathological grade**

In order to investigate if the volumetric reduction of the globus pallidus external segment (GPe) is related to striatal pathology, the 8 HD cases were independently graded based on striatal neuropathology according to the Vonsattel grading system (Vonsattel et al., 1985, Vonsattel et al., 2008). The grades of the 8 HD cases ranged from 1 to 3. There were three grade 0-1 cases and five grade 2-3 cases (case details are listed in Table 3.2, Chapter 3). The results of stereological volume quantification using the *Cavalieri Estimator* shows that there is a 45% reduction in the mean volume of the GPe in grade 0-1 HD when compared with 8 controls.
However, a clear statistical association was not evident ($P > 0.05$). **Analysis of the grade 2-3 group showed a 60% reduction in mean GPe volume** compared to 8 controls which was significant (**$P = 0.001$**) (Figure 4.2). It has been reported by other studies that there is a preferential shrinkage of the GPe compared to the GPi for HD grades 0-4 (Roos, 1986). This finding was confirmed by a more recent study, which involved the measurement of GPe and GPi cross-sectional areas within tissue sections (Deng et al., 2004). It was concluded that while the GPe and GPi shrunk to 30-40% of controls by HD grade 4, the shrinkage was slightly but significantly greater for the GPe than GPi (Deng et al., 2004). This suggests that the degree of atrophy of the globus pallidus, increases with advancing grades of striatal degeneration in HD, indicating that degeneration in the GP and striatum are linked and share a similar pattern (de la Monte et al., 1988).

The larger volume reduction of the GPe with increasing HD grade could mirror the pace of striatal-GPe projection loss in HD. The boundaries of the GPe for volumetric measurements in this study were outlined with enkephalin immunoreactivity (see Chapter 3, Materials and Methods). Within the GPe, enkephalin immunoreactivity is localised in punctate terminals and fibres described as “woolly fibres” in earlier studies (Haber and Elde, 1981) that outlines the dense network of long branching pallidal dendrites (Allen et al., 2009). In previous studies of the GP, in HD grades 1-4, enkephalin positive fibres in the GPe were dramatically reduced compared to control. Although the loss is severe by grade 1, it progresses with advancing grade (Deng et al., 2004, Allen et al., 2009). These results have been confirmed in a previous study from our group which established that enkephalin striatal-pallidal terminals are progressively reduced in HD in grades from 0 to 4; enkephalin density showing an average 23% reduction at grades 0-1, 33% for grade 2, and 59% for grades 3-4 compared to controls (Allen et al., 2009).

Such immunohistochemical observations of the loss of enkephalin positive efferent terminals in the GPe have reinforced the notion that the enkephalin-containing **indirect** pathway projecting from the striatum to the GPe is involved earlier and more predominantly than the substance-P/dynorphin-containing **direct** pathway to the GPi (Reiner et al., 1988, Sapp et al., 1995). Therefore, the greater vulnerability of the striatal-GPe projections, and the progressive grade-wise degeneration of striatal enkephalin projections in the GPe, could be a major contributor to the progressive GPe volume reduction with advancing HD grade, therefore highlighting the link between striatal pathology and GPe volume loss.
4.6.3 The pattern of volumetric changes in the globus pallidus external segment compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration

This study also investigated the differences in CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset and disease duration, which may also be contributors to the dramatic volumetric reduction of the GPe. In general terms, the overall GPe volume decreased with brain weight for HD cases only, but not control cases (*P = 0.03) (Figure 4.4). No significant correlation was found between GPe total volume and repeat length, post-mortem delay, age of death, age of disease onset and disease duration for all control and HD cases.

Striatal pathology and volume loss has shown to be closely associated with the number of CAG repeats in HD (Penney et al., 1997, Rosas et al., 2001, Vonsattel et al., 2008). However, as shown by the lack of statistical association between CAG repeat length and GPe volume, it appears that the reduction in GPe volume does not follow the same association according to Spearman’s correlation analysis (P > 0.05). The analysis of post-mortem delay showed no overall correlation with GPe volume for both control and HD cases, thereby suggesting that the differences in time interval between death and tissue processing did not influence the volume reduction of the eight HD cases in this study.

It has been long known that the overall brain weight of HD patients is decreased compared to control brains (Vonsattel et al., 1985, de la Monte et al., 1988, Vonsattel et al., 2008). Therefore, it was important to evaluate the relationship between brain weight and GPe volume. There was no significant correlation between the total volume of the GPe and the post-mortem brain weight in control cases (P > 0.5). However, there was a strong positive correlation between the total volume of the GPe and the post-mortem brain weight in HD cases, (*P = 0.03). This suggests that the extent in which the GPe degenerates in volume coincides with the extent of brain weight loss, which is reinforced by many studies reporting that the globus pallidus is a major contributor to the loss in overall brain volume (de la Monte et al., 1988, Halliday et al., 1998). Halliday et al analysed the proportional volume (i.e. the regional volume expressed as a percentage of the total cerebrum volume in HD cases compared with control), and found that along with a 42% and 33% reduction in proportional atrophy of the caudate and putamen respectively, the GPe was also selectively affected, showing a 33% greater volume loss proportionally compared to the rest of the cerebrum in HD cases. However, the proportional volumes of all other brain structures
were not significantly reduced, thereby highlighting the GPe as a major contributor to brain weight reduction.

Quantitative analysis of several post-mortem and neuroimaging studies analysed by Raz et al, (Raz et al., 1995), have suggested that there are age-related declines in the volume of the striatum. However, there is no data available on the aging of the globus pallidus. Rosa’s et al (Rosas et al., 2001) demonstrated a correlation between striatal volume and normal aging, although there was no information provided about neighbouring structures. The correlation analysis of age of death with GPe volume showed no overall relationship for both control and Huntington’s cases ($P > 0.05$), suggesting that age of death does not relate to volume decline. There was also no correlation between GPe volume and age of disease onset, or disease duration ($P > 0.05$) (Figure 4.4). The lack of association between GP volume decline and both disease onset and duration has also been noted in a longitudinal MRI study (Aylward et al., 1997).

4.6.4 The pattern of volumetric changes in the globus pallidus external segment compared with symptomatology and clinical assessments

It was next investigated whether the total volume of the GPe was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined independently by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh). Their research have previously contributed to other HD neuropathological studies from our lab on the striatum and cerebral cortex, published in *Brain* (Tippett et al., 2007, Thu et al., 2010).

In general terms, there was no significant correlation between total GPe volume and clinical measurements of mood, cognition and total functional capacity for the HD cases examined (Figure 4.6). However, there was a general trend as shown in panel B, which shows that the overall GPe volume decreased with Mini-Mental State Examination score, but with a small sample size, this did not reach significance using a two-tailed $P$-value ($P = 0.08$), although, the highly positive regression value of $r = 0.9$ suggests an almost perfect correlation. The Mini-Mental State Examination is a useful assessment to quantitatively document cognitive change (Folstein et al., 1975), and it has been found that similarly to the correlation with GPe volume
presented here, caudate atrophy has also been shown to correlate with a measured decline in MMSE score (Bäckman et al., 1997, Halliday et al., 1998).

As the GPe is a crucial component of the basal ganglia motor circuitry, it was essential to look at the relationships between GPe volume and motor symptoms. In general terms, there was no significant correlation between GPe volume and both Quantitative Neurological Exam (QNE) and Unified Huntington’s Disease Rating Scale (UHDRS) scores of chorea (Figure 4.7). However, there was a general trend as shown in panel A and C, which shows that the overall GPe volume decreased with increased QNE and UHDRS motor impairment scores. Although, this relationship did not reach statistical significance with a two-tailed $P$-value ($P = 0.08$), despite the highly negative inverse regression value of $r = -0.9$, which suggests an almost perfect inverse correlation. This result is paralleled by findings from Guo et al (Guo et al., 2012), who reported using design-based stereological techniques, that the overall reduction in putamen volume is correlated with the severity of QNE motor impairment, but not QNE chorea. This suggests the GPe atrophy, like striatal atrophy is more related to the pathogenesis of motor dysfunction, as opposed to chorea, reinforcing the hypothesis that chorea may be a manifestation of cellular dysfunction within the region, not degeneration itself (Ross and Tabrizi, 2011, Guo et al., 2012).

4.6.5 The pattern of pallidal neuron loss in the globus pallidus external segment in Huntington’s disease using the Optical Fractionator

The results of stereological pallidal neuron quantification, using the Optical Fractionator, shows that there is a striking 59% reduction in the mean total pallidal neuron number in the GPe in HD, when all 8 HD cases are grouped together and compared with 8 controls ($**P = 0.007$) (Figure 4.8). These findings are consistent with a morphometric study carried out on 6 HD post-mortem brains of unreported grades by Lange et al, who found a 44% decrease in absolute number of pallidal neurons in the GPe (Lange et al., 1976).

There has been considerable conflict as to whether or not pallidal neuron loss accounts for the massive volume reduction of the GPe in HD, as most studies suggest that GPe atrophy is apparently chiefly neuropil loss, resulting from striatal fibres and terminals, and to a lesser extent the loss of neurons (Reiner et al., 1988, Albin et al., 1992, Storey and Beal, 1993, Vonsattel et al., 2008). However, these studies have concentrated on striato-pallidal afferents rather than
pallidal neuron degeneration. Only a few investigators, including Lange et al and Roos et al, concluded that neuronal depletion occurs in the GP in HD and therefore GP atrophy should be attributed not only to striato-pallidal fibre loss, but also pallidal neuron loss (Lange et al., 1976, Roos, 1986). Lange et al further suggested that pallidal neuronal loss was due to primary degeneration, rather than the consequence of striatal degeneration. The stereological assessment in the present study revealing striking pallidal neuron loss, combined with a lack of change in surviving pallidal neuron density, supports the hypothesis that pallidal neuron depletion is occurring, and thereby is important in terms of overall GPe volume degeneration in HD.

There are several sources which provide insight into the mechanisms behind the major loss of GPe pallidal neurons, based on neurochemical evidence. Several studies have reported a preferential pattern of cell loss of striatal projection neurons based on their neurochemistry and connectivity. These studies indicate that the most vulnerable group of GABAergic projection neurons are those projecting to the GPe (indirect pathway), which are rich in enkephalin (Graveland et al., 1985, Reiner et al., 1988, Glass et al., 2000, Deng et al., 2004). Findings from our laboratory reinforce that the degeneration of these striatal efferent neurons result in the upregulation of the various subunits of both GABA A and GABA B receptors in the GPe, indicative of receptor-based remodelling in response to the loss of GABAergic input from enkephalin positive medium spiny striatal neurons (Allen et al., 2009). It is also known that pallidal neurons are 100 times less numerous than spiny striatal neurons, which suggests that there is major numeric convergence of striatal input onto pallidal neurons. Combined with this, the large dendritic arborisations of pallidal neurons which are discoidal in shape and disposed perpendicularly to striatal afferent axons, also suggests a geometric convergence of striatal input onto pallidal neurons (Yelnik, 2002). Thus, because of the major loss of medium spiny neurons (50% loss by HD grade 1 in the caudate, and 95% loss by grade 4) (Vonsattel et al., 1985), pallidal neurons will lose their major striatal input, which means, that even in grade 1, half of the striatal input on to pallidal neurons are lost, which will have a great impact in the striatal-GPe projection. This anterograde transneuronal degenerative mechanism may play a role in any decrease in GPe volume and number of GPe pallidal neurons.

Another possible mechanism for the loss of pallidal neurons could be based on a more cell autonomous degenerative process, possibly due to the direct action of the HD gene on pallidal neurons. Sapp et al, 1997 found a near-complete loss of huntingtin expression in the GP in low-grade HD post-mortem tissue, long before the majority of pallidal neurons are lost and before
major neurodegeneration was detected (Sapp et al., 1997). It was important to note that neuronal labelling declined more in the GPe than the internal segment of the globus pallidus (Sapp et al., 1997). Landwehrmyer et al found reduced mRNA levels for the HD gene, IT15, slightly reduced in GPe pallidal neurons of HD brain compared with other HD brain areas and control pallidal neurons (Landwehrmeyer et al., 1995). This early decline in huntingtin expression in HD pallidal neurons may possibly contribute to the loss of pallidal neurons, as the changes in huntingtin expression precede chronic neurodegeneration.

4.6.6 The relationship between pallidal neuron loss in the globus pallidus external segment with striatal neuropathological grade

After grouping the results of stereological pallidal neuron quantification using the Optical Fractionator based on striatal neuropathological grade, the data showed that there is a 37% reduction in the mean total number of pallidal neurons in the GPe in grade 0-1 HD, when compared with 8 controls (Figure 4.9). However, the mean changes in total cell number in HD grade 0-1 compared to control were not significant due to high case variability and a limited sample size ($P > 0.05$). Analysis of the grade 2-3 group showed a significant 72% reduction in the mean total number of pallidal neurons compared to 8 controls (Figure 4.9), (**$P = 0.001$). Therefore, it is clear that the extent of pallidal neuron loss in the GPe parallels the Vonsattel grade of neuropathology which is related to striatal degeneration.

The progressive reduction in pallidal neurons in the GPe with increased striatal neuropathological grade could possibly be a by-product of the progressive loss of striato-pallidal afferent input onto GPe neurons. Several studies have noted that while striatal projection neurons are the most vulnerable cell types to be lost as the disease progresses, the striato-GPe neurons are lost more rapidly in HD than other striatal projection systems, including striatal-GPi projections. Several studies of post-mortem tissue using immunohistochemistry have noted the grade wise reduction in enkephalin positive GABAergic medium spiny neurons as shown by the progressive loss of enkephalin positive striatal terminals (Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009). By HD grade 1, enkephalin positive MSN’s are reduced by 65% compared to controls. By grade 2, the reduction is by 75% (Sapp et al., 1995, Deng et al., 2004). By grade 3 and 4, the reduction is 80% to 95% compared to control (Deng et al., 2004). Diffusion Tensor Imaging confirms the massive loss of striatal projections into the GP, indicating that the immunolabelling changes reflect real fibre loss and not just staining loss (Douaud et al., 2009).
Therefore, it is possible that the grade-wise reduction in pallidal neurons in the GPe is occurring in parallel with the loss of striato-pallidal projection neurons, as both factors have shown to decline with advancing striatal neuropathological grade.

4.6.7 The pattern of pallidal neuron loss in the globus pallidus external segment compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration

It was also investigated whether the pattern of pallidal neuron loss in the GPe was correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both HD and control cases for which all this information was available (Figure 4.11). In general terms, the total number of Nissl-positive pallidal neurons decreased with increasing disease duration for HD cases (panel F), but did not reach statistical significance according to a two-tailed $P$-value ($P = 0.07$). No significant correlation was found between the total number of Nissl-positive pallidal neurons and CAG repeat length, post-mortem delay, brain weight, age of death, and age of disease onset for all control and HD cases. Previous studies have suggested that atrophy of the caudate and putamen increase progressively with HD disease duration, which was based on the observation that higher HD grades were found in patients with longer disease duration (de la Monte et al., 1988, Myers et al., 1988). Since heavy cell loss is observed in the striatum, and striato-pallidal projection loss is well documented, it may be possible that subsequent pallidal neuron loss could also parallel with increased disease duration.

4.6.8 The pattern of pallidal neuron loss in the globus pallidus external segment compared with symptomatology and clinical assessments

It was also examined whether the GPe pallidal neuron loss was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and measurements of total functional capacity and cognition (Figure 4.13). However, there is a general trend as shown in panel E, Figure 4.13, which shows that the total number of pallidal neurons decreased with a reduced outward irritability score, but with a small sample size, this did not reach significance using a two-tailed $P$-value ($P = 0.08$), although the highly positive regression value of $r = 0.9$ suggests an almost perfect correlation. The outwardly
directed irritability scale is a 9 point questionnaire to assess whether a person will attack, or fear they will attack another person physically or verbally (Snaith et al., 1978). In HD, irritability has been reported in over 50% of patients (Paulsen et al., 2001). Recent research has also suggested that psychiatric symptoms such as irritability may be the first manifestation of HD in up to 79% of patients (Morris and Scourfield, 1996), with irritability also being reported in the prediagnostic phase (Walker, 2007). This could explain why irritability scores decline as pallidal neurons are lost and motor aspects of the disease become a more predominant feature as the motor circuits of the basal ganglia become progressively disrupted.

As the GPe is a crucial component of the basal ganglia motor circuitry, it was essential to look at the relationships between pallidal neuron loss in the GPe and motor symptoms. In general terms, there was no significant correlation between GPe pallidal neuron numbers and both Quantitative Neurological Exam (QNE) and Unified Huntington’s Disease Rating Scale (UHDRS) scores of chorea and motor impairment (Figure 4.14). This was an unexpected result, as GPe volume did show a correlation with motor impairment, and not chorea (Figure 4.7). It may be possible that the lack of correlation with motor impairment could be due to the high levels of variability in terms of cell counts between HD cases, as the variation between GPe volume measurements were much smaller and therefore correlations were more detectable. However, it could also be possible that motor impairment is simply not related to the loss of pallidal neurons, and might only be directly related to the loss of DARPP-32 positive striatal neurons (Guo et al., 2012).

4.6.9 The pattern of pallidal neuron cell soma size changes in the globus pallidus external segment in Huntington’s disease using the Isotropic Nucleator

The results of stereological pallidal soma volume quantification of parvalbumin positive pallidal neurons using the Isotropic Nucleator, shows that there is a 35% reduction in the pallidal soma volume in the GPe in HD, when all 8 HD cases are grouped together and compared with 8 controls (*P = 0.02) (Figure 4.15). Early studies from Vonsattel have reported that pallidal neurons are smaller compared to control in late grade HD cases, although this was not quantified (Vonsattel et al., 1985, Vonsattel et al., 2008). In a murine model of HD, heterozygotes with the Hdh<sup>ex5</sup> mutation (equivalent to a mutation in the IT15 gene in the human), the mean profile area of neuronal cell bodies in the globus pallidus were reduced by 21% compared to controls, and it was found that the significant decrease in mean profile area was due to an overall decrease in neuron size, rather than simply the loss of the largest neurons (O’Kusky et al., 1999). It is
interesting to see that recently a similar finding has been reported by Guo et al, using design-based stereology, where remaining DARPP-32 positive striatal neurons were found to be reduced in volume by 40%, as determined using the Isotropic Nucleator (Guo et al., 2012). This suggests that pallidal neuron atrophy could parallel striatal neuron atrophy in Huntington’s disease.

The atrophy of remaining pallidal neurons could be indicative of ongoing neuronal dysfunction which proceeds cell death in Huntington’s disease (Ross and Tabrizi, 2011). In many neurodegenerative diseases, the remaining cells may show degenerative changes in terms of size, shape, and morphology. Although these changes are not specific, they might represent definite ongoing pathogenesis of neurons (Kanazawa, 2001, Graeber and Moran, 2002). In Parkinson’s disease, nearly 50% of remaining nigral neurons exhibit cellular shrinkage (Kanazawa, 2001), and in Amyotrophic lateral sclerosis, remaining spinal motor neurons shrink to ~70-80% in size (Kiernan and Hudson, 1993). It has been thought that dysfunctional “sick” neurons might be maintained at a lower level than control in terms of metabolism and function of neurons, providing a protracted survival for more than 3-10 years in an atrophic state (Kanazawa, 2001).

In terms of HD, Guo et al hypothesised that the shrinkage of DARPP-32 positive striatal neurons may represent neurons at different stages of neuronal degeneration, which could be the same case for the shrinkage of pallidal neurons.

4.6.10 The pattern of pallidal soma volume changes in the globus pallidus external segment compared with striatal neuropathological grade

In order to investigate if the reduction in pallidal soma size in the globus pallidus external segment (GPe) is related to striatal pathology, the pallidal soma volume measurements obtained with the Isotropic Nucleator were grouped according to striatal neuropathological grade and compared with controls. The results revealed a 27% reduction in the mean soma volume of pallidal neurons in the GPe in grade 0-1 HD, when compared with 8 controls (Figure 4.17). However the reduction in mean pallidal soma volume in HD grade 0-1 were not significant due to a limited sample size ($P > 0.05$). Analysis of the grade 2-3 group showed a significant 39% reduction in the mean pallidal soma volume compared to 8 controls (Figure 4.14) ($*P = 0.01$). Early studies from Vonsattel et al have reported that pallidal neurons are smaller compared to control in HD grade 3, and even more so in grade 4, although this was not quantified (Vonsattel et al., 1985, Vonsattel et al., 2008). This present study is the first quantitative evidence of pallidal cell soma reduction and its direct relationship with striatal neuropathological grade.
4.6.11 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration

This study also investigated the differences in CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset and disease duration, which may also be contributors to decline in pallidal soma volume in HD. In general terms, no significant correlation was found between pallidal soma volume and repeat length, post-mortem delay, brain weight, age of death, age of disease onset and disease duration for all control and HD cases (Figure 4.18). This highlights that there is no apparent relationship between these demographic variables and the size of pallidal neuron soma.

4.6.12 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus external segment (GPe) compared with symptomatology and clinical assessments

It was next investigated whether the pattern of cell soma volume changes was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh). As shown in Figures 4.20 and 4.21, there was no correlation between pallidal cell soma size, and measures of Total Functional Capacity, mood, or motor symptom scores.

4.6.13 The density of pallidal neurons in the globus pallidus external segment in Huntington’s disease

In order to study pallidal neuron density in the globus pallidus external segment in detail, the stereological cell counting data obtained using the Optical Fractionator (the total number of Nissl-positive pallidal neurons, see 4.3.1) was divided by the GPe volume estimate data obtain using the Cavalieri Estimator (GPe volume in mm$^3$, see 4.2.1) in order to obtain a density estimate of pallidal neurons per mm$^3$. After assessment of the density of pallidal neurons in the GPe, the mean cell density of Nissl-positive pallidal neurons was slightly reduced by 11%, when all 8 HD cases are grouped together and compared with 8 controls (Figure 4.22). The mean
changes in cell density in HD compared to control were not significant, \( P = 0.6 \), possibly suggesting that this rather small reduction is negligible.

According to Lange et al (Lange et al., 1976), although a dramatic loss of pallidal neurons in the GPe was found in HD, pallidal neuronal density was up to 42% higher in HD cases compared to control. Although stereology was not used by Lange et al, this finding was reinforced by Vonsattel et al, who stated that neurons in the GP were more densely packed by grade 4 HD, suggesting that neurons are relatively preserved while tissue bulk decreases (Vonsattel et al., 1985). A more recent study by Wakai et al, using a non-stereological method, concluded that there was no significant loss of pallidal neurons in the GPe in HD. However, a significant increase in neuronal cell density was found, leading to the suggestion that pallidal neuron loss does not contribute to GPe atrophy (Wakai et al., 1993). Therefore, because of this dramatic pallidal neuron density increase seen in previous studies, it was thought that GPe atrophy is mainly due to the loss of neuropil, resulting from the loss of striatal fibres and terminals, and to a lesser extent the loss of neurons (Vonsattel et al., 2008).

Contrary to this, our studies show that there was not a statistically significant increase in pallidal neuron density in HD (Figure 4.22), which emphasises the importance of using a stereological-based estimate, because the histochemical method utilised by Lange et al did not take into account the fact that cell density varies along the antero-posterior axis of the brain (Wakai et al., 1993). Wakai et al attempted to overcome this problem by taking five selected areas from the antero-posterior axis and determining separate density values for each area. However, biased selection of areas, combined with not taking the full region of interest into account when quantifying cell number and volume could result in a biased estimate of cell number, volume, and subsequently density. Therefore, the lack of change in pallidal neuron density in HD suggests that the number of neurons per mm\(^3\) of tissue does not increase in HD, and that the loss of neurons in HD must be contributing in a major way to the overall atrophy of the GPe.
4.6.14 The pattern of pallidal neuron density changes in the globus pallidus external segment did not correlate with striatal neuropathological grade

It was also investigated if pallidal neuron density in the GPe is related to striatal pathology. After assessment of the density of pallidal neurons in the GPe, the data shows a slight 11% increase in the mean density of Nissl-positive pallidal neurons in the GPe in grade 0-1 HD, when compared with 8 controls (Figure 4.23). However, this was not statistically significant ($P > 0.99$), making this increase negligible. **Analysis of the grade 2-3 group showed a 25% reduction in the density of pallidal neurons** compared to 8 controls (Figure 4.23). This reduction in pallidal neuron density in HD grade 2-3 compared to control was not significant ($P = 0.6$). The trend towards a reduction in pallidal neuron density is likely to be reflective of the major loss of pallidal neurons by HD grade 2-3, generating fewer cells per mm$^3$ of tissue by this stage. Since density is a reflection of both pallidal neuron number and overall GPe volume, the lack of density change observed at grade 0-1 highlights that the ratio of cell number to unit volume of tissue is the same as control; that is, GPe atrophy and reduction in pallidal neuron number are occurring at the same rate, leaving no change in density as a result. However, the extent of pallidal neuron loss by grade 2-3 results in a decline in overall density, as fewer pallidal neurons occupy the volume of tissue available, thereby reinforcing the extent of cell death occurring.
4.7 Conclusion

The major findings of this present study is a striking 54% reduction in overall GPe volume, accompanied with a striking 59% loss of pallidal neurons, and, a significant 35% decline in pallidal soma volume in post-mortem Huntington’s disease cases. There is a major reduction in overall volume of the globus pallidus external segment in Huntington’s disease, which was shown to parallel advancing striatal neuropathological grade, and, for the first time, demonstrate a relationship with motor impairment. In addition, the major loss of pallidal neurons also coincided with striatal pathology, and surviving pallidal neurons were smaller in volume, suggesting ongoing neuronal dysfunction. Furthermore, the overall density of pallidal neurons did not show the same pattern of increase in HD as proposed by early studies, thereby challenging the current view that GPe atrophy only results from striato-pallidal projection loss, and highlighting the importance of cell-autonomous pallidal neuron loss as a contributor to basal ganglia dysfunction in Huntington’s disease.
CHAPTER 5: RESULTS

The Ventral Pallidum

5.1 Introduction

Huntington’s disease (HD), a neurodegenerative disorder, involves generalised loss of brain tissue, but the most prominent pathology is in the basal ganglia. The most pronounced neuropathology in HD occurs within the striatal part of the basal ganglia, in which there is gross atrophy. This is principally due to the loss of medium spiny GABAergic projection neurons in the striatum (Vonsattel et al., 1985, Vonsattel et al., 2008). An interesting fact, however, is that the ventral (limbic) striatum, which comprises of the nucleus accumbens as its major component, appears to be relatively preserved in HD compared to the dorsal (motor) striatum (Vonsattel et al., 1985, Kassubek et al., 2004). Functionally, the nucleus accumbens is related to somatic motor and limbic functions such as reward processing (Heimer et al., 2007, Haber and Knutson, 2010).

The ventral pallidum (VP) was first identified as a primary GABAergic output for the ventral striatum, located immediately ventral to the anterior commissure (Heimer and Wilson, 1975, Haber et al., 1990b). Notions of the VP as a striatal output for movement, comparable to the GP, contributed originally from the view that it functioned as a motor expression region (Heimer et al., 1982). For example, based on a series of behavioural studies, Mogenson et al proposed that the nucleus accumbens projections to the VP translated limbic motivation signals into motor output (Mogenson and Yang, 1991). This account attributed the “limbic-motor integration” to the accumbens-pallidal system, and specifically identified ventral pallidal projections to the brainstem (i.e. pedunculopontine tegmentum) as a primary motor output for limbic motivation signals. The VP also receives glutamatergic input from the subthalamic nucleus (Kita and Kitai, 1987, Turner et al., 2001). Likewise, dopaminergic projections from both the substantia nigra and ventral tegmental area (midbrain regions associated with the basal ganglia and limbic systems, respectively) terminate in the VP (Napier et al., 1991, Klitenick et al., 1992).

The VP projects back to nearly all of its input sources, including the nucleus accumbens for reciprocal information exchange (Spooren et al., 1996). The VP also projects to the subthalamic nucleus and hypothalamus, as well as midbrain structures including the substantia nigra and
ventral tegmental area (Haber et al., 1990b, Haber et al., 1993). Fibres also innervate the pedunculopontine nucleus, a key structure involved in the reward circuit (Kobayashi and Okada, 2007). Furthermore, VP outputs re-enter corticolimbic loops via direct projections to the medial prefrontal cortex, and dense projections to the mediodorsal nucleus of the thalamus (Zahm et al., 1987, Haber et al., 1993, Pirot et al., 1994, Parent et al., 1999). The VP also projects back to both internal and external segments of the dorsal pallidum (globus pallidus). Parts of the VP also project to the lateral habenular nucleus, a structure now considered to be part of the reward circuit (Matsumoto and Hikosaka, 2007, Morissette and Boye, 2008). Such limbic-related anatomical connectivity sets the stage for the VP to mediate reward and motivation functions at many levels of the brain, beyond merely aiding translation to movement (Smith et al., 2009). The close relation between the ventral striatum and VP within the limbic loop of the basal ganglia coined the term “ventral striatopallidum” (Petrasch-Parwez et al., 2012).

The human VP, like the GPe and GPi can be divided chemoarchitectonically into areas based on the extent of substance-P and enkephalin innervation (Haber and Watson, 1985). Staining for these peptides has been useful in defining the boundaries of the VP (Fox et al., 1974, Difiglia et al., 1982, Haber and Watson, 1985, Mai et al., 1986, Reiner et al., 1999). It has also been shown that the VP contains heterogeneous cell types, including cholinergic and GABAergic projection neurons (Smith et al., 2009). Although pallidal neuron subtyping in terms of calcium-binding proteins, which has been well characterised in the dorsal pallidum, has not been determined in the VP.

Currently, neuropathological analyses of limbic associated regions in human HD brains are relatively sparse, with no currently reported studies in HD post-mortem tissue which documents cellular changes within the VP. Many neuroimaging studies that document ventral striatum activation also document overlapping VP activation. However, until recently, these imaging methods lacked sufficient spatial resolution to distinguish the VP from the ventral striatum (Haber and Knutson, 2010). With the increased resolution of modern day human brain imaging technology, preclinical studies are being substantiated, and the role of the VP in the human emotional repertoire and psychiatric disorders is being clarified. In one such study of Parkinson’s disease patients with pathological gambling, single-photon emission computed tomography showed enhanced resting state activity (regional cerebral blood flow) in the VP of these individuals compared with non-gambling PD patients (Cilia et al., 2008). It could therefore be possible that Huntington’s disease, being a neurodegenerative disorder involving both the limbic
system and basal ganglia, like Parkinson’s disease, could also have an association with changes in VP activity.

Although psychiatric symptoms have been reported to affect 35-75% of HD individuals (van Duijin et al., 2007), nothing is known about the role of the VP in HD, despite its role as part of the ventral striatopallidum as an integrator of emotional, cognitive and sensory information, and in linking motivation to behaviour (Waraczynski, 2006, Heimer et al., 2007). The morphological correlates of psychiatric-associated symptoms is poorly defined in HD, but there is increasing evidence that the ventral striatum is involved in the psychiatric affection of the disease (Majid et al., 2011). Largely garnered from studies in laboratory animals, the VP, being a major output of the ventral striatum, is recognised as an integrator of sensory, emotional, and cognitive information with appropriate motoric responses (Smith et al., 2009). These functional complexities are reflected in human and primate behaviours associated with pain experiences (Zubieta et al., 2002), reward-motivated function (Cilia et al., 2008), social interaction and affiliation (Bales et al., 2007). Therefore, a study into the role of the VP in such a dynamic disease such as HD, with the triad of motor, cognitive, and psychiatric impairments, is warranted.

As there is currently no literature which implicates the VP in HD, a stereological examination of the VP in HD post-mortem human tissue is a very novel endeavour, and would provide an interesting first examination of VP involvement in HD. Thus, the major aims of this section of the thesis are to investigate:

(1) The overall volumetric changes of the VP in HD with regard to striatal pathological grade and symptom heterogeneity; and
(2) the extent of pallidal neuron loss in HD, with regard to striatal pathological grade and symptom heterogeneity;

These aspects will be investigated using design-based stereological techniques, including an unbiased method of quantifying VP regional volume (Cavalieri Estimator) and total pallidal neuron number (Optical Fractionator) using the whole region of interest. The research performed in this study is novel, as very few laboratories have access to tissue which is extensively characterised in terms of symptom and clinical information.
For each stereological technique used, the data was obtained using the StereoInvestigator software on 8 Huntington’s disease (HD) (HC101L, HC119R, HC120R, HC125L, HC132L, HC137R, HC139R, HC140R) and 8 control (H153R, H170R, H186R, H204R, H226R, H227R, H230R and H231R) cases (see Chapter 3 on detailed methodology). The stereological estimates for overall volume (in mm$^3$) and total pallidal neuron number (total number of Nissl-positive pallidal neurons) for the VP within this chapter are presented for each HD case ($N=8$) and each control case ($N=8$) in the form of a scatter plot with the standard error of the mean expressed as (mean ± SEM). An estimation of cell density was also derived based on the quotient of the Optical Fractionator data and Cavalieri Estimator data (cells/mm$^3$). The standard deviation (SD) was also reported to assess variation amongst the samples. The mean changes in total regional volume, total pallidal neuron number, and pallidal neuron density in HD compared to control were assessed using a two-sided Mann-Whitney test, where P-values <0.05 were considered statistically significant (statistical significance expressed as *$P<0.05$, **$P<0.01$, ***$P<0.001$).

In order to investigate if stereological estimates for overall volume and total pallidal neuron number for the VP within this chapter is related to striatal pathology, the 8 HD cases were also independently examined by a neuropathologist (Dr B Synek) and each case was designated a grade based on striatal neuropathology criteria according to the Vonsattel grading system (Vonsattel et al., 1985, Vonsattel et al., 2008). The grades of the 8 HD cases ranged from 1 to 3. There were three grade 0-1 cases (HC101L, HC132L and HC137R) one grade 2 case (HC120R) and four grade 3 cases (HC119R, HC125L, HC139R, HC140R) (case details are listed in Table 3.2, Chapter 3). For the purpose of this investigation, the only grade 2 case was pooled with the grade 3 cases, with analyses conducted with and without this case to ensure it did not skew the data.

Grade-wise comparisons were conducted for the stereological estimates for overall volume (in mm$^3$) and total pallidal neuron number (total number of Nissl-positive pallidal neurons) for the VP within this chapter. The mean changes were presented for each HD grade 0-1 case ($N=3$), each HD grade 2-3 case ($N=5$) and each control case ($N=8$) in the form of a scatter plot with the standard error of the mean expressed as mean ± SEM. The mean changes were grouped according to striatal neuropathological grade and compared to control using a Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value. $P$-values <0.05 were considered statistically significant (statistical significance expressed as *$P<0.05$, **$P<0.01$, ***$P<0.001$).
5.2 The pattern of volumetric changes in the ventral pallidum in Huntington’s disease using the *Cavalieri Estimator*

In order to study volumetric changes of the ventral pallidum in detail, immunohistochemistry was performed using standard single peroxidise labelling techniques on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire VP (1 in every 5th section) using antibodies to the neuropeptide enkephalin, which allows accurate delineation of the VP from other structures in the basal ganglia. Secondly, an unbiased design-based stereological technique to quantify the volume of the VP was performed using StereoInvestigator. The *Cavalieri Estimator* is a design-based stereological probe for determining the total volume of the entire ventral pallidum using a point-counting method, and was run using the StereoInvestigator software (see Chapter 3 on detailed methodology).

5.2.1 The pattern of volumetric changes in the ventral pallidum in all HD and control cases (Table 5.1, Figure 5.1)

The results of stereological volume quantification using the *Cavalieri Estimator*, shows that there is a 31% reduction in the mean volume of the VP in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 5.1). The mean VP volume ± SEM of 8 controls was 51.43 ± 5.04 mm³, compared to the mean VP volume ± SEM of all 8 HD cases which was 35.52 ± 5.87 mm³ (Table 5.1). However, the mean changes in total regional volume in HD compared to control were not significant according to the two-sided Mann-Whitney test, with the *P*-value being > 0.05 (*P* = 0.08).

It was also interesting to note that there was considerable variation in the total regional volume of the VP within the control and HD cohorts as shown by the differential standard deviations (SD). The mean for all control cases has a smaller standard deviation (SD = 14.26 mm³) compared to the HD cohort where the cases show a larger standard deviation from the mean (SD = 16.61 mm³) (Table 5.1). It is possible that H226R was considered an outlier in the control cohort. However, there was no methodological reason for exclusion. The two-sided Mann-Whitney test was run with and without the suspected outlier, with the same statistical conclusion being met.
For the stereological analysis of HD and control groups carried out using the *Cavalieri Estimator*, the average coefficient of error (CE) for the total volume of the VP was always less than 0.10. The average CE for mean estimates of volume for both HD and control cases was 0.01, which is ≤ 0.10, and is therefore a generally reliable estimation of volume (Table 5.1) (Gundersen and Jensen, 1987, Slomianka and West, 2005). This reinforces that any variability observed is due to a true difference between cases in the total volume rather than a lack of precision in terms of the stereological technique used (see Chapter 3, Materials and Methods) (Slomianka and West, 2005).

### Table 5.1 Variation in the mean overall volume of the ventral pallidum (VP) between control cases and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean total volume of the ventral pallidum (mm³)</td>
<td>51.43</td>
<td>35.52</td>
<td>50.75</td>
<td>26.38</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>14.26</td>
<td>16.61</td>
<td>15.61</td>
<td>9.108</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>5.041</td>
<td>5.874</td>
<td>9.014</td>
<td>4.073</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage reduction (%) as compared to control</td>
<td>-</td>
<td>31% reduced</td>
<td>1% reduced</td>
<td>*49% reduced</td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.009</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
The graph shows the variation of the total volume of the ventral pallidum (VP) (defined by the Enkephalin immunoreactivity) in 8 HD and 8 control brains, determined using design-based stereological methods involving the Cavalieri Estimator protocol. Each blue diamond indicates the total volume of the VP (in mm$^3$) for each control case, and each red diamond indicates the total volume of the VP (in mm$^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 31% reduction in the mean total volume of the VP in Huntington’s disease, which was not significant based on a 2-tailed $P$-value from the Mann-Whitney test ($P = 0.08$). See Table 5.1 for detailed results.
5.2.2 The pattern of volumetric changes in the ventral pallidum in HD correlated with striatal neuropathological grade (Table 5.1, Figure 5.2, 5.3)

To investigate if the volumetric reduction of the ventral pallidum (VP) is related to striatal pathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological volume quantification using the *Cavalieri Estimator*, shows that there is a negligible 1% reduction in the mean volume of the VP in grade 0-1 HD, when compared with 8 controls (Figure 5.2). The mean VP volume ± SEM of 8 controls was 51.43.0 ± 5.041 mm³, compared to the mean VP volume ± SEM of the grade 0-1 cases which was 50.75 ± 9.041 mm³ (Table 5.1). However, the mean changes in total VP volume in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of >0.05 ($P > 0.99$). Analysis of the grade 2-3 group showed a 49% reduction in mean VP volume compared to 8 controls (Figure 5.2), highlighted in Table 5.1 by the mean ± SEM of 26.38 ± 4.073 mm³. This reduction in mean VP volume in HD grade 2-3 compared to control was significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of < 0.05 (*$P = 0.02$). Qualitative descriptions showcase coronal sections of the VP at striatal neuropathological grades 1 and 3 as highlighted in Figure 5.3.

In summary, it was observed that the overall mean volume of the VP was reduced at HD striatal neuropathological grade 2-3, and not grade 1. With a small cohort of HD cases in the grade 0-1 group, no significant differences in VP volume compared to control were identified. Therefore, in terms of striatal pathology, it appears that VP atrophy occurs when striatal pathology is beyond grade 2 in HD.
Figure 5.2 Overall volume of the ventral pallidum (VP) in HD cases grouped according to striatal neuropathological grade compared to control cases

The graph shows the variation of the total volume of the ventral pallidum (VP) defined by the Enkephalin immunoreactivity in 8 control brains compared with three HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the Cavalieri Estimator protocol. Each blue diamond indicates the total volume of the VP (in mm$^3$) for each control case, each red diamond indicates the total volume of the VP (in mm$^3$) for each grade 0-1 HD case, and each green diamond indicates the total volume of the VP (in mm$^3$) of each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a negligible 1% reduction in the mean total volume of the VP in HD grade 0-1 compared to control, which was not statistically significant ($P > 0.99$). However, by HD grade 2-3, the mean VP volume was reduced by 49% compared to control, and is statistically significant ($*P = 0.02$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn's multiple comparisons post-test. See Table 5.1 for detailed results.
This figure shows representative macroscopic images at 3 coronal levels (rostral, middle, caudal) through the ventral pallidum (VP) defined by enkephalin immunoreactivity and stained with Nissl. Images are taken of each 70 µm coronal section at low power from the rostral, middle and caudal areas of the VP from a control (H226R), HD grade 1 (HC101L), and grade 3 (HC125L) case respectively. Note the progressive reduction of the delineated VP cross-section with HD grade.

A-C: Three representative coronal sections immunohistochemically stained with enkephalin (cross-section delineated) at rostral (A), middle (B) and caudal (C) levels of the VP of control case H226R.

D-F: Three representative coronal sections immunohistochemically stained with enkephalin (cross-section delineated) at rostral (D), middle (E) and caudal (F) levels of the VP of HD grade 1 case HC101L. Note the reduction in the outlined VP cross-section observed at all levels in comparison to the control (H226R) equivalent levels.

G-I: Three representative coronal sections immunohistochemically stained with enkephalin (cross-section delineated) at rostral (D), middle (E) and caudal (F) levels of the VP of HD grade 3 case HC125L. Note the reduction in the outlined VP cross-section observed at all levels in comparison to case HC101L, and H226R.

Abbreviations: Put, putamen; VP, ventral pallidum; GPe, globus pallidus external segment; CN, caudate nucleus; ac, anterior commissure

Scale bar: A-I = 5000 µm
Figure 5.3 Macroscopic cross-sectional examination of the ventral pallidum (VP) from HD cases of striatal neuropathological grade 1 and 3 compared to a representative control case.
5.2.3 The pattern of volumetric changes in the ventral pallidum compared with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration (Table 5.2, Figure 5.4)

The total volume of the ventral pallidum (VP), was next correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset) in both HD and control cases for which all this information was available (Figure 5.4). In general terms, the overall VP volume decreased with brain weight for HD cases only (panel C), which is statistically significant according to a two-tailed Spearman’s correlation (*$P = 0.048$). No significant correlation was found between VP total volume and CAG repeat length, post-mortem delay, age of death, age of disease onset, and disease duration for all control and HD cases.

5.2.4 The pattern of volumetric changes in the ventral pallidum compared with pallidal neuron number, average pallidal soma volume, and pallidal neuron density (Table 5.2, Figure 5.5)

It was next investigated whether the total volume of the VP was correlated with total pallidal neuron number and pallidal neuron density in both control and HD cases as determined using design-based stereology (Figure 5.5). In general terms, the density of pallidal neurons is higher at smaller VP volumes for control cases only (panel B), which is statistically significant according to a two-tailed Spearman’s correlation (*$P = 0.02$). No significant correlation was found between VP total volume and the total number of pallidal neurons for all control and HD cases.
Figure 5.4 Comparison between the total volume of the ventral pallidum with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration in both HD and control cases

This figure shows the correlation of the total volume of the ventral pallidum (VP) with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, disease duration for all control and HD cases which have the information available. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total volume of the VP and the CAG repeat number in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.06, P = 0.90$).

B: There was no significant correlation between the total volume of the VP and the post-mortem delay in control ($r = 0.41, P = 0.31$) and HD ($r = 0.46, P = 0.26$) cases, according to a two-tailed Spearman’s correlation analysis.

C: There was no significant correlation between the total volume of the VP and the post-mortem brain weight in control cases according to a two-tailed Spearman’s correlation analysis ($r = -0.37, P = 0.50$). **However, there was a strong positive correlation between the total volume of the VP and the post-mortem brain weight in HD cases**, which was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = 0.79, P = *0.048$).

D: There was no significant correlation between the total volume of the VP and the age of death for control ($r = -0.26, P = 0.54$) and HD ($r = -0.48, P = 0.22$) cases, according to a two-tailed Spearman’s correlation analysis.

E: There was no significant correlation between the total volume of the VP and the age of disease onset in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.60, P = 0.11$).

F: There was no significant correlation between the total volume of the VP and the duration of disease from age of onset to death in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.44, P = 0.26$).
Figure 5.4 Comparison between the total volume of the ventral pallidum with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration in both HD and control cases.
Figure 5.5 Comparison between the total volume of the ventral pallidum with total pallidal neuron number, and pallidal neuron density in both HD and control cases

This figure shows the correlation of the total volume of the ventral pallidum (VP) with total pallidal neuron number and pallidal neuron density for all HD and control cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total volume of the VP and the total number of Nissl-positive pallidal neurons in control ($r = -0.33$, $P = 0.42$) and HD ($r = 0.53$, $P = 0.20$) cases according to a two-tailed Spearman’s correlation analysis.

B: There was an inverse correlation between the total volume of the VP and the density of pallidal neurons in control cases ($r = -0.81$, *$P = 0.02$). However, there was no correlation between the total volume of the VP and the density of pallidal neurons in HD cases, according to a two-tailed Spearman’s correlation analysis ($r = 0.24$, $P = 0.58$).
5.2.5 The pattern of volumetric changes in the HD ventral pallidum compared with symptomatology and clinical assessments (Table 5.2, Figure 5.6, 5.7)

It was next investigated whether the total volume of the VP was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 5.6 highlights the correlation between the total volume of the VP with Total Functional Capacity, Mini-Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between VP total volume and all variables examined. However, as shown in panel B, the overall VP volume decreased with Mini-Mental State Examination score. This was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), despite the highly positive regression value of $r = 0.9$, which highlights a very strong trend.

Figure 5.7 shows the correlation between the total volume of the ventral pallidum (VP) with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between VP total volume and all variables examined. However, as shown in panel A and C, the overall VP volume decreased with increased QNE and UHDRS motor impairment scores. This was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), despite the highly negative inverse regression value of $r = -0.9$, which highlights a very strong trend.
This figure shows the correlation between the total volume of the ventral pallidum (VP) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases.

A: There was no significant correlation between the total volume of the VP and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.40, P = 0.52$).

B: There is a clear trend that the overall VP volume decreased with Mini-Mental State Examination score. However, this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$), however the highly positive regression value of $r = 0.9$ highlights a very strong trend.

C: There was no significant correlation between the total volume of the VP and the Hospital Anxiety scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50, P = 0.45$).

D: There was no significant correlation between the total volume of the VP and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.67, P = 0.27$).

E: There was no significant correlation between the total volume of the VP and the Outward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50, P = 0.45$).

F: There was no significant correlation between the total volume of the VP and the Inward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.79, P = 0.20$).
Figure 5.6 Comparison between the total volume of the HD ventral pallidum with measures of Total Functional Capacity, cognition and mood.
Figure 5.7 Comparison between the total volume of the HD ventral pallidum with clinical measures of motor impairment and chorea

This figure shows the correlation between the total volume of the ventral pallidum (VP) with the Quantitative Neurological Exam (QNE) motor impairment and chorea scores, in addition to the Unified Huntington's Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case. In general terms, no significant correlation was found between VP total volume and all variables examined.

A: There is a clear trend that the overall VP volume decreased with increased QNE motor impairment score, although this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$). However, the large negative inverse regression value of $r = -0.9$ highlights a very strong trend.

B: There was no significant correlation between the total volume of the VP and the QNE chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.30$, $P = 0.68$).

C: There is a clear trend that the overall VP volume decreased with increased UHDRS motor impairment score, although this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$). However, the large negative inverse regression value of $r = -0.9$ highlights a very strong trend.

D: There was no significant correlation between the total volume of the VP and the UHDRS chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.36$, $P = 0.50$).
5.2.6 Overall summary of volumetric changes in the ventral pallidum compared with all variables (Table 5.2)

A summary of the comparisons between the total volume of the VP for all cases and all variables discussed in sections 5.2.3, 5.2.4 and 5.2.5 is highlighted in table 5.2. Key findings include:

(1) The overall VP volume decreased with brain weight for HD cases only
(2) The density of pallidal neurons is higher at smaller VP volumes for control cases only
(3) The overall VP volume decreased with Mini-Mental State Examination score for HD cases
(4) The overall VP volume decreased with increased QNE and UHDRS motor impairment scores for HD cases
Table 5.2 Summary of comparisons between the ventral pallidum volume and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size ((n))</th>
<th>Relationship</th>
<th>Relationship</th>
<th>(r) value (VP volume)</th>
<th>Two-tailed (P) value (VP volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD CAG repeat length</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>0.06</td>
<td>0.90</td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>Control 8 HD 8</td>
<td>Direct</td>
<td>Direct</td>
<td>0.41</td>
<td>0.46</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>Control 6 HD 7</td>
<td>Inverse</td>
<td>Direct</td>
<td>-0.37</td>
<td>0.79</td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>Control 8 HD 8</td>
<td>Inverse</td>
<td>Inverse</td>
<td>-0.26</td>
<td>0.48</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>Control 8 HD -</td>
<td>Inverse</td>
<td>Inverse</td>
<td>-0.60</td>
<td>0.11</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>Control 8 HD -</td>
<td>Inverse</td>
<td>Inverse</td>
<td>-0.44</td>
<td>0.26</td>
</tr>
<tr>
<td>Total number of Nissl-positive pallidal neurons</td>
<td>Control 8 HD 8</td>
<td>Inverse</td>
<td>Direct</td>
<td>-0.33</td>
<td>0.53</td>
</tr>
<tr>
<td>Density of Nissl-positive pallidal neurons</td>
<td>Control 8 HD 8</td>
<td>Inverse</td>
<td>Direct</td>
<td>-0.81</td>
<td>*0.02</td>
</tr>
<tr>
<td>Total Functional Capacity</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>0.40</td>
<td>0.52</td>
</tr>
<tr>
<td>Mini-Mental State Examination score</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>0.90</td>
<td>0.08</td>
</tr>
<tr>
<td>Mood Hospital Anxiety Scale</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>Mood Hospital Depression Scale</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>0.67</td>
<td>0.27</td>
</tr>
<tr>
<td>SNAITH Outward Irritability Score</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>SNAITH Inward Irritability Score</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>0.79</td>
<td>0.20</td>
</tr>
<tr>
<td>QNE Motor Impairment score</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>-0.90</td>
<td>0.08</td>
</tr>
<tr>
<td>QNE Chorea Score</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>-0.30</td>
<td>0.68</td>
</tr>
<tr>
<td>UHDRS Motor Assessment Score</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>-0.90</td>
<td>0.08</td>
</tr>
<tr>
<td>UHDRS Chorea Assessment Score</td>
<td>Control 8 HD -</td>
<td>Direct</td>
<td>Direct</td>
<td>-0.36</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: \(*P < 0.05\), \(**P < 0.01\), \(***P < 0.001\). Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
5.3 The pattern of pallidal neuron loss in the ventral pallidum in Huntington’s disease using the Optical Fractionator

In order to study pallidal neuron loss in the ventral pallidum in detail, first, immunohistochemistry using standard single peroxidise labelling techniques was performed on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire VP (1 in every 5th section). Accurate delineation of the VP from other structures in the basal ganglia was achieved using antibodies to the neuropeptide enkephalin. Secondly, a Nissl stain with cresyl violet was used to stain all cells within the ventral pallidum. Pallidal neurons were morphologically identified on Nissl-stained sections as large, ellipsoid-shaped, nucleolated cells. Thirdly, an unbiased design-based stereological technique to quantify pallidal neurons in the VP was performed using StereoInvestigator. The Optical Fractionator is a 3-dimensional systematic random sampling method used to estimate the total number of Nissl-positive pallidal neurons in the VP and was run using the StereoInvestigator software on 8 Huntington’s disease (HD) and 8 control cases (see Chapter 3 on detailed methodology).

5.3.1 The pattern of pallidal neuron loss in the ventral pallidum in all HD and control cases (Table 5.3, Figure 5.8)

The results of stereological pallidal neuron quantification, using the Optical Fractionator, show that there is 24% reduction in the mean total pallidal neuron number in the VP in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 5.8). The mean cell number ± SEM of 8 controls was 31,742 ± 2,673 pallidal neurons compared to the mean cell number ± SEM of all 8 HD cases which was 24,069 ± 8,779 pallidal neurons (Table 5.3). The mean changes in total pallidal neuron number in HD compared to control were not significant according to the two-sided Mann-Whitney test, with the $P$-value being $> 0.05$ ($P = 0.10$).

It was also interesting to note that there was considerable variation in the total pallidal neuron number in the VP within the HD cohort as shown by the considerably larger standard deviation (SD) compared to the control group. HD cases had a standard deviation which was almost equal to the mean itself (SD = 24,830 cells) compared to the control cohort where the cases show a much smaller standard deviation from the mean (SD = 7,560 cells) (Table 5.3). This is reinforced by the larger range within the HD cohort as shown by Figure 5.8, with the difference between
case HC132L (highest count of 76,738 cells) and case HC139R (lowest count of 4,020 cells) being 72,718 cells. In comparison, the control cohort has a smaller range, with the difference between case H231R (highest count of 42,658 cells) and case H227R (lowest count of 22,888 cells) being 19,770 cells. It can also be noted that HC101L and HC132L produced cell counts which were well above the HD group mean (Figure 5.8), and with these cases both being classified with low striatal neuropathological grades of 0-1, it is not surprising that these cases produced counts higher than the HD cluster of cases with more severe pathology (i.e., HD grade > 2).

It must be acknowledged that **HC132L** could be a potential outlier within the HD cohort, with a returned cell count considerably higher than the control cohort mean. However, because the data set is not assumed to be from a normally distributed population, it was not reliable to perform a test for outliers, and otherwise, there was no methodological reason for exclusion of this case. Biologically, it was interesting to note that the total number of sections which contained VP at a 1/5 sampling interval, was 15 for HC132L. This was, in fact, the highest number of sections quantified for a single case for both the *Cavalieri Estimator* and *Optical Fractionator*, in both the HD and normal cohort. This suggests that the VP was more elongated coronally compared to all other cases analysed from both the control and HD cohort, which would influence the number of sampling sites within the case, and therefore the area sampling fraction (*asf*) component of the *Optical Fractionator* Equation to obtain cell number (Chapter 3, Equation 3.1). The two-sided Mann-Whitney test was run with and without the suspected outlier, and it is important to note that exclusion of this suspected outlier would produce a statistically significant difference between both cohorts of *P = 0.02*. However, the data presented in figure 5.8 includes HC132L to highlight biological case variation.

For the stereological analysis of control and HD groups carried out using the *Optical Fractionator*, the average coefficient of error (CE) for the total number of pallidal neurons in the VP was always less than 0.10. The average CE for mean estimates of total cell number for the control and HD cases was 0.05 and 0.08 respectively, which are ≤ 0.10 and therefore a generally reliable estimation of pallidal neuron number (Table 5.3) (Gundersen and Jensen, 1987, Slomianka and West, 2005). This reinforces that any variability observed is due to a true difference between cases in total pallidal neuron number rather than a lack of precision in terms of the stereological technique used (see Chapter 3 Materials and Methods) (Slomianka and West, 2005).
Table 5.3 Variation in the mean total pallidal neuron number in the ventral pallidum between control and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of Nissl-positive pallidal neurons</td>
<td>31,742</td>
<td>24,069</td>
<td>41,634</td>
<td>13,531</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>7,560</td>
<td>24,830</td>
<td>36,286</td>
<td>7,098</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>2,673</td>
<td>8,779</td>
<td>20,950</td>
<td>3,174</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage loss (%) as compared to normal</td>
<td>-</td>
<td>24% loss</td>
<td>31% increase</td>
<td>*57% loss</td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.05</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with normal: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
Figure 5.8 Total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) in all HD cases compared to control cases in the human brain.

The graph shows the variation in the total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) in 8 HD and 8 control brains, determined using design-based stereological methods involving the Optical Fractionator. Each blue diamond indicates the total number of Nissl-positive neurons in the VP for each control case, and each red diamond indicates the total number of Nissl-positive neurons in the VP for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 24% reduction in the mean total number of Nissl-positive pallidal neurons in the VP in Huntington’s disease, which was not statistically significant based on a 2-tailed P-value from the Mann-Whitney test (P = 0.10). It is also important to note the suspected outlier within the HD cohort, case HC132L. See Table 5.3 for detailed results.
5.3.2 The pattern of pallidal neuron loss in the ventral pallidum compared with striatal neuropathological grade (Table 5.3, Figure 5.9, 5.10)

To investigate if the loss of pallidal neurons in the ventral pallidum (VP) is related to striatal pathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological pallidal neuron quantification, using the Optical Fractionator, shows that there is a 31% increase in the mean total number of pallidal neurons in the VP in grade 0-1 HD, when compared with 8 controls (Figure 5.9). The mean total cell number ± SEM of 8 controls was 31,742 ± 2,673 cells, compared to the mean total cell number ± SEM of the grade 0-1 cases which was 41,634 ± 20,950 cells (Table 5.3). However, the mean changes in total cell number in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted P-value of > 0.05 (P > 0.99).

It is important to note that there is considerable variability amongst cases in the grade 0-1 group, as highlighted by the very large standard deviation of 36,286 cells, which is almost as large as the mean of the group itself (Table 5.3). It is likely that the level of variability is highly attributable to the potential outlier within the HD cohort (HC132L) as described in section 5.3.1. Analysis of the grade 2-3 group showed a 57% reduction in the mean total number of pallidal neurons compared to 8 controls (Figure 5.9), highlighted by the mean ± SEM of 13,531 ± 3,174 cells (Table 5.3). This striking loss of pallidal neurons in HD grade 2-3 compared to control was significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted P-value of < 0.05 (*P = 0.02). Figure 5.10 highlights these findings qualitatively through microscopic examination of pallidal neuron distribution in a representative control, HD grade 1, and HD grade 3 case.

In summary, it was observed that the total number of pallidal neurons in the VP decreased significantly at grades 2-3. However, with a small cohort of HD cases in the grade 0-1 group, and the extreme case variation within this subgroup, it is clear that the overall HD cohort variability is attributable to the case variation within the grade 0-1 subgroup.
Figure 5.9 Total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) in HD cases grouped according to striatal neuropathological grade compared to control cases

The graph shows the variation in the total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) in 8 control brains compared with three HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the Optical Fractionator. Each blue diamond indicates total number of Nissl-positive neurons in the VP for each control case, each red diamond indicates the total number of Nissl-positive neurons in the VP for each grade 0-1 HD case, and each green diamond indicates the total number of Nissl-positive neurons in the VP for each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 31% increase in the mean total number of Nissl-positive pallidal neurons in the VP in HD grade 0-1, compared to control, though this was not statistically significant ($P > 0.99$). **By HD grade 2-3, a striking 57% reduction in the mean total number of Nissl-positive pallidal neurons was found** compared to control, and is statistically significant (*$P = 0.02$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn’s multiple comparisons post-test. See Table 5.3 for detailed results.
This figure shows high power photomicrographs through the ventral pallidum (VP), at the level of the anterior commissure, highlighting the overall distribution of Nissl-positive pallidal neurons from a control (H170R), HD grade 1 (HC137R) and grade 3 (HC125L) case respectively. Red arrows mark pallidal neurons. Note the major reduction of pallidal neurons observed at grade 3. It is also interesting to note the increase in small punctate Nissl-positive glial cells with progressive HD grade, which are likely to be a collection of astrocytes, oligodendrocytes and microglia.

A: A representative coronal section of a control case (H170R) stained with Nissl, showing the distribution of pallidal neurons within the subcommissural VP. Red arrows denote representative pallidal neurons.

B: A representative coronal section of a HD grade 1 case (HC137R) stained with Nissl, showing the distribution of pallidal neurons within the subcommissural VP. Note the distribution of pallidal neurons (as marked with red arrows) in HD grade 1 (HC137R) is comparable to the distribution of these neurons in control (H170R, panel A). There is also an increase in the number of small punctate glial cells compared to H170R.

C: A representative coronal section of a HD grade 3 case (HC125L) stained with Nissl, showing the distribution of pallidal neurons within the subcommissural VP. Note the major reduction in the number of Nissl-positive pallidal neurons (as marked with red arrows) observed at all levels in comparison to the control case (H170R), and the grade 1 HD case (HC137R). This is accompanied by an increase in the number of small punctate glial cells compared to HC137R.
5.3.3 The pattern of pallidal neuron loss in the ventral pallidum compared with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration (Table 5.4, Figure 5.11)

It was next investigated whether the pattern of pallidal neuron loss in the VP was correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset) in both HD and control cases, for which all this information was available (Figure 5.11). In general terms, a positive correlation was found between the total number of Nissl-positive pallidal neurons and CAG repeat length (Panel A). However, this correlation was not statistically significant according to a two-tailed Spearman’s correlation upon removal of the potential outlier (HC132L), as highlighted by the drop in P-value from 0.02 to > 0.05. In contrast, a striking inverse correlation was found between the total number of Nissl-positive pallidal neurons and age of death (**P = 0.002), age of onset (**P = 0.007), and disease duration (**P = 0.003) in HD as shown by the considerable reduction in pallidal neuron number with increased age of death (Panel D), greater age of onset (Panel E) and longer disease duration (Panel F). It is important to note that the correlations in Panels D-F remained significant after the removal of the potential outlier (HC132L). No significant correlation was found between the total number of Nissl-positive pallidal neurons and post-mortem delay and brain weight for all control and HD cases.

5.3.4 The pattern of pallidal neuron loss in the ventral pallidum compared with overall VP volume and pallidal neuron density (Table 5.4, Figure 5.12)

It was also explored whether the total number of Nissl-positive pallidal neurons correlated with overall VP volume and pallidal neuron density in both control and HD cases, which were variables measured using design-based stereology (Figure 5.12). In general terms, the loss of Nissl-positive pallidal neurons coincided with a reduction in overall pallidal neuron density, as shown by strong positive correlations for HD cases only (**P = 0.004). No significant correlation was found between the total number of Nissl-positive pallidal neurons and overall VP volume.
This figure shows the correlation of total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) with CAG repeat length, *post-mortem* delay, brain weight, age of death, age of disease onset and disease duration for all HD and control cases which have the information available. Each triangular symbol indicates a control case, and each square symbol indicates an HD case. The red circle outlines the potential outlier (HC132L) as mentioned in section 5.3.1.

**A:** There was a significant correlation between the total number of Nissl-positive pallidal neurons and the CAG repeat number in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.80, *P = 0.02$). However, it must be noted that the correlation was not significant after the removal of the potential outlier (HC132L) ($P > 0.05$).

**B:** There was no significant correlation between the total number of Nissl-positive pallidal neurons and the *post-mortem* delay in control ($r = -0.10, P = 0.78$) and HD ($r = 0.57, P = 0.14$) cases, according to a two-tailed Spearman’s correlation analysis.

**C:** There was no significant correlation between the total number of Nissl-positive pallidal neurons and the *post-mortem* brain weight in control ($r = 0.31, P = 0.56$) and HD ($r = 0.75, P = 0.07$) cases according to a two-tailed Spearman’s correlation analysis.

**D:** There was no significant correlation between the total number of Nissl-positive pallidal neurons and the age of death for control cases according to a two-tailed Spearman’s correlation analysis ($r = -0.21, P = 0.62$). However, there was a strong inverse correlation between the total number of Nissl-positive pallidal neurons and the age of death for the HD cohort ($r = -0.92, **P = 0.002$), which was still significant (**$P = 0.008$) after the removal of the potential outlier (HC132L).

**E:** There was a strong inverse correlation between the total number of Nissl-positive pallidal neurons and the age of onset for the HD cohort ($r = -0.87, **P = 0.007$), according to a two-tailed Spearman’s correlation analysis, which was still significant ($*P = 0.03$) after the removal of the potential outlier (HC132L).

**F:** There was a strong inverse correlation between the total number of Nissl-positive pallidal neurons and the duration of disease for the HD cohort ($r = -0.90, **P = 0.003$), according to a two-tailed Spearman’s correlation analysis, which was still significant ($*P = 0.01$) after the removal of the potential outlier (HC132L).
Figure 5.11 Comparison between the total number of Nissl-positive pallidal neurons in the ventral pallidum with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset and disease duration in both HD and control cases.
Figure 5.12 Comparison between the total number of pallidal neurons in the ventral pallidum with overall VP volume, and pallidal neuron density in HD and control cases

This figure shows the correlation of total number of Nissl-positive pallidal neurons with overall VP volume and pallidal neuron density for all HD and control cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case. The red circle outlines the potential outlier (HC132L) as mentioned in section 5.3.1.

A: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the total volume of the VP in control ($r = -0.33$, $P = 0.43$) and HD ($r = 0.52$, $P = 0.20$) cases, according to a two-tailed Spearman’s correlation analysis.

B: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the density of pallidal neurons in control cases according to a two-tailed Spearman’s correlation analysis ($r = 0.71$, $P = 0.06$). However, there was a strong positive correlation between the total number of Nissl-positive pallidal neurons and the density of pallidal neurons in the HD cohort ($r = 0.90$, $P = **0.004$), which was statistically significant according to a two-tailed Spearman’s correlation analysis. This correlation was still significant ($^*P = 0.02$) after the removal of the potential outlier (HC132L).
5.3.5 The pattern of pallidal neuron loss in the HD ventral pallidum compared with symptomatology and clinical assessments (Table 5.4, Figure 5.13, 5.14)

It was next investigated whether the loss of Nissl-positive pallidal neurons correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 5.13 highlights the correlation between the total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and all variables examined. **However, there is a general trend as shown in panel E, which shows that the total number of pallidal neurons decreased with a reduced outward irritability score.** This was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), although the highly positive regression value of $r = 0.9$ highlights a very strong trend.

Figure 5.14 shows the correlation between the total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and all variables examined.
Figure 5.13 Comparisons between the total number of pallidal neurons in the HD ventral pallidum with measures of Total Functional Capacity, cognition and mood

This figure shows the correlation between the total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total number of pallidal neurons and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.10, P = 0.95$).

B: There was no significant correlation between the total number of pallidal neurons and Mini-Mental State Examination scores in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.40, P = 0.52$).

C: There was no significant correlation between the total number of pallidal neurons and the Hospital Anxiety scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.30, P = 0.68$).

D: There was no significant correlation between the total number of pallidal neurons and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.67, P = 0.27$).

E: There is a clear trend that the total number of pallidal neurons decreased with the SNAITH outward irritability score. This was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$). However, the large positive regression value of $r = 0.90$ highlights a very strong trend.

F: There was no significant correlation between total number of pallidal neurons and the Inward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.79, P = 0.20$).
Figure 5.13 Comparison between the total number of pallidal neurons in the HD ventral pallidum with measures of Total Functional Capacity, cognition and mood.
This figure shows the correlation between the total number of Nissl-positive pallidal neurons in the ventral pallidum (VP) with the Quantitative Neurological Exam (QNE) motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total number of pallidal neurons and QNE motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.40$, $P = 0.52$).

B: There was no significant correlation between the total number of pallidal neurons and the QNE chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.20$, $P = 0.78$).

C: There was no significant correlation between the total number of pallidal neurons and the UHDRS motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.40$, $P = 0.52$).

D: There was no significant correlation between the total number of pallidal neurons and the UHDRS chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.36$, $P = 0.50$).
5.3.6 Overall summary of pallidal neuron loss in the ventral pallidum compared with all variables (Table 5.4)

A summary of the comparisons between the total number of Nissl-positive pallidal neurons in the VP for all cases and all variables discussed in sections 5.3.3, 5.3.4 and 5.3.5 is highlighted in Table 5.4. Key findings include:

1. A striking inverse correlation was found between the total number of Nissl-positive pallidal neurons and age of death, age of onset, and disease duration in HD
2. The loss of Nissl-positive pallidal neurons coincided with a reduction in overall pallidal neuron density, as shown by strong positive correlations for HD cases
3. The total number of pallidal neurons decreased with a reduced outward irritability score
Table 5.4 Summary of comparisons between the total number of pallidal neurons in the VP and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size (n)</th>
<th>Relationship</th>
<th>r value (VP pallidal neuron number)</th>
<th>Two-tailed P value (VP pallidal neuron number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HD</td>
<td>Control</td>
<td>HD</td>
</tr>
<tr>
<td>HD CAG repeat length</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>Direct</td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>8</td>
<td>8</td>
<td>Inverse</td>
<td>Direct</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>6</td>
<td>7</td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8</td>
<td>8</td>
<td>Inverse</td>
<td>Inverse</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>Inverse</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>-</td>
<td>8</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>Overall VP volume (mm³)</td>
<td>8</td>
<td>8</td>
<td>Inverse</td>
<td>Direct</td>
</tr>
<tr>
<td>Density of Nissl-positive pallidal neurons</td>
<td>8</td>
<td>8</td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Total Functional Capacity</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Mini-Mental State Examination score</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Mood Hospital Anxiety Scale</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Mood Hospital Depression Scale</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>SNAITH Outward Irritability Score</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>SNAITH Inward Irritability Score</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>QNE Motor Impairment score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>QNE Chorea Score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>UHDRS Motor Assessment Score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>UHDRS Chorea Assessment Score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-</td>
</tr>
</tbody>
</table>

*denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: *P < 0.05, **P < 0.01, ***P < 0.001. Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
5.4 The density of pallidal neurons in the ventral pallidum in Huntington’s disease

In order to study pallidal neuron density in the ventral pallidum in detail, the stereological cell counting data obtained using the *Optical Fractionator* (the total number of Nissl-positive pallidal neurons, see 5.3.1) was divided by the VP volume estimate data obtained using the *Cavalieri Estimator* (VP volume in mm³, see 5.2.1). The pallidal neuron density measures were obtained for 8 Huntington’s disease (HD) and 8 control cases.

5.4.1 The density of pallidal neurons in the ventral pallidum in all HD and control cases (Table 5.5, Figure 5.15)

After assessment of the density of pallidal neurons in the VP, the mean cell density of Nissl-positive pallidal neurons was slightly reduced by 7%, when all 8 HD cases are grouped together and compared with 8 controls (Figure 5.15). The mean cell density ± SEM of 8 controls was 662 ± 84 cells/mm³ compared to the mean cell density ± SEM of all 8 HD cases which was 617 ± 143 cells/mm³ (Table 5.5). The mean changes in cell density in HD compared to control were not significant according to the two-sided Mann-Whitney test, with the *P*-value being >0.05 (*P* = 0.7).

Table 5.5 Variation in mean cell density of Nissl-positive pallidal neurons in the ventral pallidum (VP) between control and neuropathological grades in Huntington's disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell density of Nissl-positive pallidal neurons (cells/mm³)</td>
<td>662</td>
<td>617</td>
<td>711</td>
<td>560</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>238</td>
<td>406</td>
<td>535</td>
<td>365</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>84</td>
<td>143</td>
<td>309</td>
<td>163</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage reduction (%) as compared to control</td>
<td>-</td>
<td>7% reduced</td>
<td>7% increased</td>
<td>15% reduced</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *P < 0.05, **P < 0.01, ***P < 0.001
Figure 5.15 Mean cell density of Nissl-positive pallidal neurons in the ventral pallidum (VP) in all HD cases compared to control cases in the human brain

The graph shows the variation in mean cell density of Nissl-positive pallidal neurons in the ventral pallidum (VP) in 8 control and 8 HD brains, determined from data obtained using the Optical Fractionator (total number of cells) divided by the data obtained using the Cavalieri Estimator (overall volume in mm$^3$) for each case. Each blue diamond indicates the cell density of Nissl-positive pallidal neurons in the VP (cells/mm$^3$) for each control case, and each red diamond indicates the cell density of Nissl-positive pallidal neurons in the VP (cells/mm$^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a negligible 7% reduction in mean cell density of Nissl-positive pallidal neurons in the VP in Huntington's disease, which is not significant based on a 2-tailed P-value from the Mann-Whitney test ($P = 0.7$). See Table 5.5 for detailed results.
5.4.2 The pattern of pallidal neuron density changes in the ventral pallidum did not correlate with striatal neuropathological grade (Table 5.5, Figure 5.16)

In order to investigate the relationship between pallidal neuron density in the VP and striatal neuropathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. After assessment of the density of pallidal neurons in the VP, the data shows a slight 7% increase in the mean density of Nissl-positive pallidal neurons in the VP in grade 0-1 HD, when compared with 8 controls (Figure 5.16). The mean ± SEM of 8 controls was 662 ± 84 cells/mm³ compared to the mean ± SEM of the grade 0-1 cases which was 711 ± 309 cells/mm³ (Table 5.5). However, the mean changes in cell density of pallidal neurons in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $> 0.05$ ($P > 0.99$). It is also important to highlight the extreme variability amongst the grade 0-1 HD cases, with an SD of 535 cells/mm³, which is more than double the SD of the control cohort and almost the size of the grade 0-1 group mean. Analysis of the grade 2-3 group showed a 15% reduction in the density of pallidal neurons compared to 8 controls (Figure 5.16), highlighted by the mean ± SEM of 560 ± 163 cells/mm³ (Table 5.5). This reduction in pallidal neuron density in HD grade 2-3 compared to control was not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $> 0.05$ ($P > 0.99$). It is also important to note that there is considerable case variability within the grade 2-3 cohort, with an SD of 365 cells/mm³ (Table 5.5), which is more than half of the cohort mean.

In summary, it was observed that pallidal neuron density did not correlate with striatal neuropathological grade. Furthermore, no statistically significant changes were found when the control cases were compared with the entire HD cohort, or neuropathological grade subgroups.
Figure 5.16 Mean cell density of Nissl-positive pallidal neurons in the ventral pallidum (VP) in HD cases grouped according to striatal neuropathological grade compared to control cases

The graph shows the variation in mean cell density of Nissl-positive pallidal neurons in the ventral pallidum (VP) in 8 control brains compared with 3 HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined from data obtained using the Optical Fractionator (total number of cells) divided by the data obtained using the Cavalieri Estimator (overall volume in mm$^3$) for each case. Each blue diamond indicates the cell density of Nissl-positive pallidal neurons in the VP (cells/mm$^3$) for each control case, each red diamond indicates the cell density of Nissl-positive pallidal neurons in the VP (cells/mm$^3$) for each HD grade 0-1 case, and each green diamond indicates the cell density of Nissl-positive pallidal neurons in the VP (cells/mm$^3$) for each HD grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 7% increase in the mean cell density of Nissl-positive pallidal neurons in the VP in HD grade 0-1, compared to control, though this was not statistically significant ($P > 0.99$). By HD grade 2-3, a 15% reduction in mean cell density of Nissl-positive pallidal neurons in the VP was found compared to control, but was not statistically significant ($P > 0.99$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn's multiple comparisons post-test. See Table 5.5 for detailed results.
5.5 Discussion

This chapter provides a discussion of the results presented in this thesis on the degeneration of the ventral pallidum (VP) in Huntington’s disease (HD), using histology, immunohistochemistry, and unbiased design-based stereological techniques. Two different stereological probes were used to measure VP volume and pallidal neuron loss. These results show that there was a reduction in overall VP volume (31%) and total number of pallidal neurons (24%) when all HD cases were grouped together (N = 8) and compared with control cases (N = 8) (Figures 5.1 and 5.8). These findings provide the first reported evidence of volumetric decline and cell loss in the VP in HD.

5.5.1 The pattern of volumetric changes in the ventral pallidum in Huntington’s disease using the Cavalieri Estimator

The results of stereological volume quantification using the Cavalieri Estimator, shows that there is a 31% reduction in the mean volume of the VP in HD, when all 8 HD cases were grouped together and compared with 8 controls (Figure 5.1). However, the reduction was not significant (P = 0.08), which is attributable to the variation in total regional volume within the control and HD cohorts.

Currently, there are no imaging studies to date which have documented any volumetric changes occurring specifically to the VP in humans in any disease state, which is mainly due to the lack of sufficient spatial resolution to distinguish the VP from the neighbouring ventral striatum (also known as the nucleus accumbens) (Haber and Knutson, 2010). However, many neuroimaging studies that document ventral striatum activation also document ventral pallidum activation (Haber and Knutson, 2010). The close relationship between the ventral striatum and ventral pallidum coined the term the ventral striato-pallidum (VSP) (Petrasch-Parwez et al., 2012). Since the VP is a primary output of the nucleus accumbens (Heimer and Wilson, 1975, Heimer, 1978) with reciprocal projections between two structures (Spooren et al., 1996) that share common neuromodulators such as GABA and opioids including substance-P and enkephalin (Difiglia et al., 1982, Haber and Watson, 1985, Mai et al., 1986, Chrobak and Napier, 1993, Churchill and Kalivas, 1994, Reiner et al., 1999) it is not surprising that the ventral striatum and ventral pallidum are collectively termed the VSP, consisting of the nucleus accumbens, olfactory...
tubercle, the ventral caudate nucleus and putamen and the VP which integrates emotional, cognitive and sensory information and is implicated in linking motivation to behaviour (Waraczynski, 2006, Heimer et al., 2007).

The dorsal-ventral gradient of striatum pathology in HD could possibly account for the lesser extent of VP atrophy compared to its external and internal pallidum counterparts. It has recently been shown that when compared with control brains, the ventral striatum/nucleus accumbens shows significant volume shrinkage already at pre-HD stages as detected by MRI and voxel based morphometry (Majid et al., 2011, van den Bogaard et al., 2011a, van den Bogaard et al., 2011b). A combined ROI-based and voxel-based morphometric study of MRI images acquired from 20 patients with early HD found an overall 38% loss in volume of the ventral striatum, compared to a 57% loss dorsally and 51.5% loss from medial striatum respectively (Douaud et al., 2006), reinforcing the dorsal-ventral gradient of striatal atrophy in HD which is in line with other voxel-based morphometric studies (Kassubek et al., 2004), other MRI imaging studies (Fennema-Notestine et al., 2004) and histopathological studies in HD patients (Vonsattel and DiFiglia, 1998). Post-mortem non-stereological studies of the ventral striatum have shown that the structure has the least degree of degeneration compared to the dorsal and medial components of the striatum (Bots and Bruyn, 1981, de la Monte et al., 1988). Therefore, the lesser extent of VP atrophy in HD as highlighted by the 31% reduction in volume in this study reflects the lesser extent of ventral striatum atrophy in HD, which is its main source of input. This is reinforced by the greater extent ofglobus pallidus externus (54%) and internus (40%) atrophy in HD as reported in chapters 4 and 6, which both receive input predominantly from the more vulnerable dorsal striatum, thereby highlighting that the dorsal-ventral gradient of atrophy seen in the dorsal striatum also applies to the pallidum.

5.5.2 The pattern of volumetric changes in the ventral pallidum correlated with striatal neuropathological grade

To investigate if the volumetric reduction of the ventral pallidum (VP) is related to striatal pathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological volume quantification using the Cavalieri Estimator, shows that there is a negligible 1% reduction in the mean volume of the VP in grade 0-1 HD, when compared with 8 controls (Figure 5.2). However, the mean changes in total VP volume in HD grade 0-1 were not significant ($P > 0.99$). Analysis of the grade 2-3
group showed a 49% reduction in mean VP volume compared to 8 controls (Figure 5.2), (*\(P = 0.02\)). Therefore, in terms of striatal pathology, it appears that VP atrophy occurs when striatal pathology is beyond grade 2 in HD.

The pronounced volume reduction of the VP exclusively at striatal neuropathological grades greater than 2 mirrors the pace of ventral striatum/nucleus accumbens degeneration in HD. As reported by the pioneering study from Vonsattel et al, involving striatal neuropathological grade characterisation of 163 post-mortem HD cases, there was no reported neuronal death or fibrillary astrocytosis within the nucleus accumbens at grades 0-2 of HD (Vonsattel et al., 1985). By grade 3 however, there is slight neuronal depletion and astrocytosis, with shrinkage of the region visible at grade 4 (Vonsattel et al., 1985). Therefore, the major atrophy of the VP in cases which are exclusively classified at a striatal neuropathological grade of 2 and above, combined with a lack of change in the grade 0-1 group, reflects the minimal impact of HD on the ventral striatum/nucleus accumbens until higher HD grades, which is thereby reflected in its main output structure, the VP.

The relative sparing of the VP in early grades of striatal neuropathology compared to the dorsal pallidum (GPe) could be a reflection of the differential vulnerability of the striatal input into these regions. The boundaries of the VP for volumetric measurements in this study were outlined with enkephalin immunoreactivity similarly to the GPe (see Chapter 3, Materials and Methods). However, it is important to note that although the same peptide was used to delineate these two regions, the GPe was shown to be more atrophic than the VP, with a 45% volume reduction at HD grade 0-1, and a 60% reduction at HD grade 2-3 (Chapter 4, Globus Pallidus External Segment). The VP is innervated by a large anteroventromedial striatal region (also known as the nucleus accumbens), receiving substantial inputs from a variety of limbic and limbic-associated structures. One the other hand, the GPe receives striatal input from an anterodorsolateral striatal sector, receiving only sparse limbic afferents but instead heavily innervated by the sensorimotor cortex (Haber et al., 1985). As presented in Chapter 4, the vulnerability of the striato-GPe projections, and the progressive grade-wise degeneration of striatal projections immunoreactive for enkephalin in the GPe, could be major contributors to the progressive GPe volume reduction with increasing HD-grade, therefore highlighting the link between striatal pathology and GPe volume loss. This was reinforced by the progressive loss of enkephalin immunoreactivity in the GPe in HD grades 0 to 4, showing an average 23% reduction at grades 0-1, 33% for grade 2, and 59% for grades 3-4 compared to controls (Allen et al., 2009). No such study has been carried out
in the ventral pallidum to date. However, it can be hypothesised, based on the sparing of enkephalin immunoreactivity in the ventral ‘limbic’ striatum in all grades of HD, compared to the progressive and pronounced loss of enkephalin immunoreactivity in the dorsal ‘somatomotor’ striatum from early grades of HD (Ferrante et al., 1986), this could translate to the possible sparing of striatal-VP projections until > grade 2. This is reflected in later VP atrophy, compared to the earlier and greater decline in GPe volume which was seen at grade 0-1 (Chapter 4), which relates to the early loss of striato-GPe enkephalinergic projections even proceeding grade 1 (Deng et al., 2004). Thus, it can be suggested that the projections from the ventral striatum to the VP are more resistant to HD than the dorsal striatum projections into the GPe.

5.5.3 The pattern of volumetric changes in the ventral pallidum compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration

The total volume of the ventral pallidum (VP) was next compared with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both control and HD cases for which all this information was available (Figure 5.4). In general terms, the overall VP volume decreased with brain weight for HD cases only (*P = 0.048). No significant correlation was found between VP total volume and repeat length, post-mortem delay, age of death, age of disease onset, and disease duration for all control and HD cases.

Bulk striatal pathology and volume loss has shown to be closely associated with the number of CAG repeats in HD (Penney et al., 1997, Rosas et al., 2001, Vonsattel et al., 2008). However, there are no such reported studies of the VP, and studies exploring the relationship between ventral striatum volume (the main input source into the VP) and CAG repeat are limited. One such study found a clear association between the dorsal striatum with CAG repeat length, however, the ventral striatum region was spared even in the high-CAG repeat group (Kassubek et al., 2004). In this study, the lack of statistical association between CAG repeat length and VP volume suggests that the reduction in VP volume is not associated with the length of CAG repeats, much like the ventral striatum. The analysis of post-mortem delay showed no overall correlation with VP volume for both control and HD cases, thereby suggesting that the
differences in time interval between death and tissue processing did not influence the volume reduction of HD cases in this study.

It has been long known that the overall brain weight of HD patients declines when compared to control brains (Vonsattel et al., 1985, de la Monte et al., 1988, Vonsattel et al., 2008). Therefore, it was important to evaluate the relationship between brain weight and VP volume. There was no significant correlation between the total volume of the VP and the post-mortem brain weight in control cases ($P > 0.5$). However, there was a strong positive correlation between the total volume of the VP and the post-mortem brain weight in HD cases, ($*P = 0.048$). This result suggests that the extent which the VP degenerates in volume in HD coincides with the degree of brain weight loss, similarly to its dorsal counterpart, the GPe (Chapter 4). This is reinforced by many studies showing the globus pallidus as a major contributor to a loss in overall brain volume (de la Monte et al., 1988, Halliday et al., 1998).

Quantitative analysis of several post-mortem and neuroimaging studies analysed by Raz et al, (Raz et al., 1995), have suggested that there are age-related declines in the volume of the striatum, however, no data is available with regard to the VP. Rosas et al demonstrated a correlation between striatal volume and normal aging, although there was no information provided about neighbouring structures (Rosas et al., 2001). The correlation analysis of age of death with VP volume showed no overall relationship for both control and Huntington’s cases ($P > 0.05$), suggesting that age of death does not relate to VP volume decline. There was also no correlation between VP volume and age of disease onset, or disease duration ($P > 0.05$). The lack of association between overall pallidum volume decline and both disease onset and duration has been noted in a longitudinal MRI study (Aylward et al., 1997), which is consistent with the results of this study.

5.5.4 The pattern of volumetric changes in the ventral pallidum correlated with symptomatology and clinical assessments

It was next investigated whether the total volume of the VP was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh). Their research have previously contributed to other HD
neuropathological studies from our lab on the striatum and cerebral cortex, published in *Brain* (Tippett et al., 2007, Thu et al., 2010).

In general terms, there was no significant correlation between total VP volume and clinical measurements of mood, cognition and Total Functional Capacity for the HD cases examined (Figure 5.6). However, there was a general trend, which shows that the **overall VP volume decreased with Mini-Mental State Examination score**, but with a small sample size, this did not reach significance using a two-tailed *P*-value (*P* = 0.08). Although the highly positive regression value of *r* = 0.9 suggests an almost perfect correlation. The Mini-Mental State Examination is a useful assessment to quantitatively document cognitive changes (Folstein et al., 1975). In HD, caudate atrophy has also been shown to correlate with a measured decline in MMSE score (Bäckman et al., 1997, Halliday et al., 1998, Montoya et al., 2006). However, this is the first study to report a correlation between VP volume and MMSE, which is consistent with the reported findings in the caudate nucleus.

In studies of other movement disorders, such as Parkinson’s disease (PD), brain structures implicated in the development of cognitive decline include the “limbic loop,” namely involving the prefrontal neocortex, ventral striato-pallidum and mediodorsal thalamus (Braak et al., 2006). It has been found that bilateral impairment of this loop almost certainly contributes to the appearance of amnestic dysfunctions and cognitive decline in PD, with a similar conclusion drawn from studies in Alzheimer’s disease (Hyman et al., 1990, Braak et al., 2006). In HD, reversal learning (a measurement of cognition) deficits have been reported. Reversal learning implicates the “affective loop,” which constitutes the orbitofrontal cortex/anterior cingulate cortex/hippocampus/basolateral amygdala – ventral striatum – ventral pallidum – mediodorsal thalamus (structures similarly implicated in the limbic circuit). (Oscar-Berman and Zola-Morgan, 1980, Lawrence et al., 1998). Therefore, it can be possible that the volumetric decline in the VP in HD, as a component of the limbic pathway which shares common connectivity to the affective loop, could have an association with cognitive decline as highlighted by the correlation with MMSE, which could relate to deficits in cognitive tasks such as reversal learning.

As the VP is located in very close proximity to structures involved in the basal ganglia motor circuitry, including the GPe, it was essential to look at the relationships between VP volume and motor symptoms. In general terms, there was no significant correlation between VP volume and both Quantitative Neurological Exam (QNE) and Unified Huntington’s Disease Rating Scale
(UHDRS) scores of chorea (Figure 5.7). However, there was a general trend as shown in panel A and C, which shows that the **overall VP volume decreased with increased QNE and UHDRS motor impairment scores**. This did not reach statistical significance ($P = 0.08$), despite a regression value of $r = -0.9$ suggesting an almost perfect inverse correlation. This unexpected relationship between VP volume and motor impairment could indicate that the pattern of atrophy in the VP may share some commonalities to its dorsal motor counterpart, the GPe (Chapter 4). Notions of the VP as a striatal output for movement, comparable to the GP, contributed originally from the view that it functioned as a motor expression site (Heimer et al., 1982). For example, based on a series of behavioural studies, Mogenson et al proposed that the nucleus accumbens projections to the VP translated limbic motivation signals into motor output (Mogenson and Yang, 1991). This account attributed the “limbic-motor integration” to accumbens-pallidal systems, and specifically identified ventral pallidal projections to the brainstem (i.e. pedunculopontine tegmentum) as a primary motor output for limbic motivation signals. Therefore, in addition to the predominant roles of the VP in reward and motivation, it must be noted that the VP also plays a role in transferring input from accumbens to brainstem motor-related targets (Mogenson and Yang, 1991, Smith et al., 2009). Therefore, it could be possible that the relationship between VP atrophy and motor impairment is a reflection of processing and interaction of reward and motor circuits, possibly through a “motivation-to-movement” interface through the basal ganglia (Haber and Knutson, 2010).

5.5.5 The pattern of pallidal neuron loss in the ventral pallidum in Huntington’s disease using the **Optical Fractionator**

The results of stereological pallidal neuron quantification, using the **Optical Fractionator**, shows that **there is a 24% reduction in the mean total pallidal neuron number in the VP in HD**, when all 8 HD cases are grouped together and compared with 8 controls (Figure 5.8). The mean changes in total pallidal neuron number in HD were not significant ($P = 0.10$). The lack of significance is likely to be attributable to the considerable variability in pallidal neuron number in the VP within the HD cohort, and a presence of a potential outlier (case HC132L), which was described in section 5.3.1. It can be noted that upon removal of this potential outlier, the difference between the control and HD group means were statistically significant. To date, there is no literature which quantifies cell loss in the VP in HD post-mortem human tissue, and there is very limited information in relation to neurochemical changes in the VP which could be indicative of cellular changes in HD. Furthermore, although considerable literature has focused
on striato-pallidal projection loss within the GPe and GPi in HD through the study of terminal labelling (Graveland et al., 1985, Reiner et al., 1988, Glass et al., 2000, Deng et al., 2004), no such study has been undertaken in the VP. This is the first study of its kind to document pallidal neuron loss separately in the VP in HD.

It is clear that the extent of cell loss in the VP is much smaller in comparison to its dorsal counterpart, the GPe, which was shown to have a striking 59% reduction in pallidal neurons in HD (Chapter 4). This major difference could possibly be due to the greater vulnerability of the sensorimotor dorsal striatal input to the GPe, compared to the limbic ventral striatal input to the VP. The pathological process in HD shows a dorso-ventral gradient, with more extensive neuronal loss and astrogliosis in the dorsal than in the ventral striatum. The ventral striatum is relatively spared, being affected in only severe cases (Vonsattel et al., 1985). These findings are reflected in the preservation of both enkephalin and substance-P in the ventral striatum in HD, with major loss dorsally (Ferrante et al., 1986). Although substance-P and enkephalin have not been assessed in the HD VP compared to controls, it is known that the VP contains substance-P, enkephalin, and GABAergic projection neurons similarly to other segments of the pallidum (Smith et al., 2009). It is well documented that the major loss in dorsal striatum projection neurons, which parallels the loss of enkephalin in the GPe, could contribute to the loss of pallidal neurons (Chapter 4). Therefore, based on the preservation of cells in the ventral striatum which would mainly project into the VP (Vonsattel et al., 1985), and the preservation of enkephalin immunoreactivity (Ferrante et al., 1986), it can be hypothesised that the preservation of striatal input into the VP is the major reason behind the observed lack of pallidal neuron loss.

5.5.6 The relationship between pallidal neuron loss in the ventral pallidum with striatal neuropathological grade

After grouping the results of stereological pallidal neuron quantification, using the Optical Fractionator, based on striatal neuropathological grade, the data showed that there was a 31% increase in the mean total number of pallidal neurons in the VP in grade 0-1 HD, when compared with 8 controls (Figure 5.9). However, the mean changes in total cell number in HD grade 0-1 compared to control were not significant due to high case variability and a limited sample size ($P > 0.99$). It is likely that the level of variability is highly attributable to the potential outlier within the HD cohort (HC132L), as described in section 5.3.1. Analysis of the grade 2-3 group showed a significant 57% reduction in the mean total number of pallidal neurons compared to 8 controls.
As mentioned in section 5.5.2, similarly to VP atrophy, it is likely that pronounced cell loss in the VP exclusively at striatal neuropathological grades greater than 2, mirrors the pace of ventral striatum/nucleus accumbens degeneration in HD. As reported by Vonsattel et al, there was no reported neuronal death or fibrillary astrocytosis within the nucleus accumbens at grades 0-2 of HD (Vonsattel et al., 1985). By grade 3, however, there is slight neuronal depletion and astrocytosis (Vonsattel et al., 1985). Therefore, the reported cell loss in the VP in cases which are exclusively classified at a striatal neuropathological grade of 2 and above, combined with a lack of change in the grade 0-1 group, reflects the minimal impact of HD on the ventral striatum/nucleus accumbens until higher HD grades, which is thereby reflected in its main output structure, the VP.

Also mentioned in section 5.5.2, it can be hypothesised, based on the sparing of enkephalin immunoreactivity in the ventral ‘limbic’ striatum in all grades of HD (Ferrante et al., 1986), this could translate to the possible sparing of striato-VP projections until >grade 2, which is reflected in later VP atrophy, and thereby latter loss of pallidal neurons until >grade 2. It is known that the ventral striatum exhibits much higher levels of enkephalin immunostaining compared to the dorsal striatum in normal *post-mortem* tissue (Holt et al., 1997). With evidence suggesting that peptides such as enkephalin may influence the excitability of striatal neurons and control the release of neurotransmitters from striatal neurons into the pallidum (Maneuf et al., 1994), it could be possible that the higher levels of enkephalin in the ventral striatum might contribute to greater control of neurotransmitter release during disease processes occurring in HD, and thereby contribute to greater cell preservation in the ventral striatum. This could subsequently lead to the preservation of projections into the VP, and therefore maintain input into the VP until later grades of HD, accounting for subsequent later cell loss.

Furthermore, the fact that the VP receives inputs from substance-P positive medium spiny projection neurons (MSN’s), in addition to enkephalinergic MSN’s, could account for the preservation of VP pallidal neurons until > grade 2 HD. It is widely known that the GPe is distinguished from other structures in the basal ganglia by enkephalin immunoreactivity, whereas the GPI is distinguished based on substance-P labelling (Haber and Elde, 1981, Haber et al., 1985). This complementary staining pattern is not absolute, but it is useful since it reflects
that afferents into these two pallidal segments originate from at least two different populations of MSN’s containing either enkephalin or substance-P. In comparison, the VP receives inputs from intermingling substance-P and enkephalin tubular profiles, suggesting that both types of MSN’s innervate the region (Haber et al., 1990a, Mitchell et al., 1999). It was well known that substance-P enriched projection neurons from the striatum to the GPi (direct pathway) are more resistant to HD pathogenesis until mid-late neuropathological grades, compared to indirect pathway striato-GPe enkephalinergic projection neurons which are more severely affected in the early and middle grades of HD (Graveland et al., 1985, Reiner et al., 1988, Glass et al., 2000, Deng et al., 2004). These findings have been supplemented by the findings in Chapter 4 (GPe) and 6 (GPi), which attributed the progressive grade-wise cell loss in the GPe in HD to the greater loss of enkephalinergic striato-GPe terminals, with preservation of GPi pallidal neurons being attributed to the greater preservation of striato-GPi substance-P terminals. Therefore, because the cell loss in the VP is apparent in cases classified as > grade 2 (similarly to the findings in the GPi, Chapter 6), it could possibly be a by-product of the more gradual loss of substance-P striato-VP afferents compared to the earlier, more extensive loss of enkephalin positive striato-VP afferents.

5.5.7 The pattern of pallidal neuron loss in the ventral pallidum compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration

It was next investigated whether the pattern of pallidal neuron loss in the VP correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both control and HD cases for which all this information was available (Figure 5.11). In general terms, a positive correlation was found between the total number of Nissl-positive pallidal neurons and CAG repeat length, however this correlation was not statistically significant upon removal of the potential outlier (HC132L), thereby implying that the potential outlier could have influenced the correlation between pallidal neuron number and CAG repeat.

However, in contrast, a striking inverse correlation was found between the total number of Nissl-positive pallidal neurons and age of death (**P = 0.002), age of onset (**P = 0.007), and disease duration (**P = 0.003) as shown by the considerable reduction in pallidal neuron number with increased age of death (Panel D), greater age of onset (Panel E) and longer disease
duration (Panel F). It is important to note that the correlations remained significant after the removal of the potential outlier (HC132L). No significant correlation was found between the total number of Nissl-positive pallidal neurons and post-mortem delay and brain weight for all control and HD cases. Previous studies have suggested that atrophy of the caudate and putamen increase progressively with HD disease duration, which was based on the observation that advanced HD grades were present in cases with longer disease duration (de la Monte et al., 1988, Myers et al., 1988). Since the VP is an output nucleus of the striatum, this relationship between VP pallidal neuron loss and disease duration could possibly be due to the pattern of degeneration in the VP reflecting disease duration, similarly to the striatum. There has also been a significant inverse correlation between striatal degeneration (represented by striatal pathological grade), and both age of onset and age of death in a study of 100 post-mortem cases, suggesting that these demographic variables could be related to degeneration of the striatum, and thereby downstream degeneration of the VP (Rosenblatt et al., 2003).

5.5.8 The pattern of pallidal neuron loss in the ventral pallidum compared with symptomatology and clinical assessments

It was also examined whether the VP pallidal neuron loss correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and measurements of total functional capacity and cognition (Figure 5.13). However, there is a general trend as shown in panel E, which shows that the total number of pallidal neurons decreased with a reduced outward irritability score, but with a small sample size, this did not reach significance using a two-tailed P-value (P = 0.08), although the highly positive regression value of $r = 0.9$ suggests an almost perfect correlation. The outwardly directed irritability scale is a 9 point questionnaire to assess whether a person will attack, or fear they will attack another person physically or verbally (Snaithe et al., 1978). In HD, irritability has been reported in over 50% of patients (Paulsen et al., 2001). Recent research has also suggested that psychiatric symptoms such as irritability may be the first manifestation of HD in up to 79% of patients (Morris and Scourfield, 1996), with irritability also being reported in the prediagnostic phase (Walker, 2007). Similar to the dorsal pallidum (Chapter 4 – GPe), the positive correlation with outward irritability suggests that prior to cell loss (i.e. in the presymptomatic stages of HD) patients are highly irritable (Klöppel et al., 2010), but as physical symptoms start to eventuate, and cell loss within the basal ganglia becomes progressive, such as in the case of the GPe
(Chapter 4) and the VP, psychiatric symptoms including outward irritability may stabilise, as highlighted by the reduction in score with cell loss.

As the VP is located in very close proximity to structures involved in the basal ganglia motor circuitry, including the GPe, it was essential to look at the relationships between pallidal neuron loss in the VP and motor symptoms. In general terms, there was no significant correlation between VP pallidal neuron numbers and both Quantitative Neurological Exam (QNE) and Unified Huntington’s Disease Rating Scale (UHDRS) scores of chorea and motor impairment (Figure 5.13). This was an unexpected result, as VP volume did show a correlation with motor impairment, and not chorea (Figure 5.6). It is possible that the lack of correlation with motor impairment could be due to the high levels of variability in terms of cell counts between HD cases, as the variation between VP volume measurements were much smaller and therefore correlations were more detectable. However, another possibility could be that motor impairment is simply not related to the loss of pallidal neurons, and might only be directly related to the loss of striatal neurons (Guo et al., 2012).

5.5.9 The density of pallidal neurons in the ventral pallidum in Huntington’s disease

In order to study pallidal neuron density in the ventral pallidum in detail, the stereological cell counting data obtained using the Optical Fractionator (the total number of Nissl-positive pallidal neurons, see 5.3.1) was divided by the VP volume estimate data obtain using the Cavalieri Estimator (VP volume in mm³, see 5.2.1) in order to obtain a density estimate of pallidal neurons per mm³. After assessment of the density of pallidal neurons in the VP, the mean cell density of Nissl-positive pallidal neurons was slightly reduced by 7%, when all 8 HD cases are grouped together and compared with 8 controls (Figure 5.15). The mean changes in cell density in HD compared to control were not significant, ($P = 0.7$), possibly suggesting that this rather small reduction is negligible.

This is the first line of evidence to suggest that the density of pallidal neurons in the VP does not significantly change in Huntington’s disease. These results are analogous to the findings in the dorsal motor counterpart of the VP, the GPe (Chapter 4 – GPe); thereby suggesting that pallidal neuron loss does in fact contribute to VP atrophy. Previous non-stereological studies have found that within the HD pallidum, pallidal neurons are preserved and more densely packed (Wakai et
suggesting that pallidum atrophy is mainly due to the loss of neuropil, striatal fibre connections, and fibres of passage, and to a lesser extent, the loss of neurons. However, as shown by these findings, a lack of change in pallidal neuron density in the VP in HD, highlights that the ratio of cell number to unit volume of tissue is not significantly different compared to control; that is, VP atrophy and reduction in pallidal neuron number are occurring at the same rate, leaving no change in density as a result, implying that the loss of pallidal neurons may be occurring in unison, if not contributing, to the overall atrophy of the VP in HD, similarly to the GPe (Chapter 4 – GPe results).

5.5.10 The pattern of pallidal neuron density changes in the ventral pallidum did not correlate with striatal neuropathological grade

It was also investigated if pallidal neuron density in the VP is related to striatal pathology. After assessment of the density of pallidal neurons in the VP, the data shows a slight 7% increase in the mean density of Nissl-positive pallidal neurons in the VP in grade 0-1 HD, when compared with 8 controls (Figure 5.16). However, this was not statistically significant ($P > 0.99$), making this increase negligible. **Analysis of the grade 2-3 group showed a 15% reduction in the density of pallidal neurons** compared to 8 controls (Figure 5.16). This reduction in pallidal neuron density in HD grade 2-3 compared to control was not significant ($P > 0.99$). The trend towards a reduction in pallidal neuron density is likely to be reflective of the major loss of pallidal neurons by HD grade 2-3, generating fewer cells per mm$^3$ of tissue by this stage. Since density is a reflection of both pallidal neuron number and overall VP volume, the lack of density change observed at grade 0-1 highlights that the ratio of cell number to unit volume of tissue is the same as control; that is, VP atrophy and reduction in pallidal neuron number are occurring at the same rate, leaving no change in density as a result. However, the extent of pallidal neuron loss by grade 2-3 results in a decline in overall density, as fewer pallidal neurons occupy the volume of tissue available, thereby reinforcing the extent of cell death occurring at advanced grades of HD.
5.6 Conclusion

As highlighted in this present study, there is a reduction in overall VP volume in Huntington’s disease, which was shown to be greater with increased striatal neuropathological grade. However, it is likely that dorsal-ventral gradient of striatal degeneration in HD, with the main source of input into the VP being the ventral striatum, could be a major contributing factor towards the lesser degree of subsequent VP atrophy compared to its dorsal counterpart, the GPe. The volumetric decline in the VP in HD, as a component of the limbic pathway, could have an association with cognitive decline as highlighted by the correlation with MMSE, which could relate to deficits in cognitive tasks such as reversal learning. Furthermore, the relationship between VP atrophy and motor impairment is a reflection of processing and interaction of reward and motor circuits, possibly through a “motivation-to-movement” interface through the basal ganglia. In terms of pallidal neuron loss, the extent of cell loss in the VP is much smaller in comparison to its dorsal counterpart, the GPe. This major difference could possibly be due to the greater vulnerability of the sensorimotor dorsal striatal input to the GPe, compared to the limbic ventral striatal input to the VP. Pallidal neuron loss was more predominant beyond grade 2 in terms of striatal neuropathological grade. In addition, the overall density of pallidal neurons in the VP did not follow the same pattern of increase in HD as proposed by early studies of the pallidum, thereby reinforcing that VP atrophy is not only a by-product of striato-pallidal projection loss, and highlighting the importance of VP pallidal neuron loss as a contributor to basal ganglia dysfunction in advanced stages of Huntington’s disease.
CHAPTER 6: RESULTS

The Globus Pallidus Internal Segment

6.1 Introduction

Huntington’s disease (HD), a neurodegenerative disorder, involves generalised loss of brain tissue, but the most prominent pathology is in the basal ganglia. The most pronounced neuropathology in HD occurs within the striatal part of the basal ganglia, in which there is gross atrophy. This is principally due to the loss of medium spiny GABAergic projection neurons in the striatum (Vonsattel et al., 1985, Vonsattel et al., 2008). The striatum sends rich GABAergic afferents to the globus pallidus internal segment (GPi), with about 70% of the total number of synaptic terminals in contact with GPi neurons originating from striatal spiny neurons (Shink and Smith, 1995). Located medially to the external segment (GPe), the GPi is separated from its larger counterpart by the internal medullary lamina, and is a final output station of the basal ganglia, through which body movements are controlled (DeLong, 1971, Nambu, 2007).

In addition to the striatum, the GPi also receives inputs from other basal ganglia nuclei, including glutamatergic information from the subthalamic nucleus (STN) and GABAergic information from the globus pallidus external segment (GPe) (Nambu, 2007). The main target of GPi output is the motor part of the thalamus, particularly the ventral anterior/ventral lateral thalamic complex, and centromedian nucleus (Kim et al., 1976), which in turn sends projections to the motor and premotor cortices (Nambu, 2007). In addition, the GPi projects to brainstem motor centres such as the pedunculopontine nucleus (Parent and De Bellefeuille, 1983). Therefore, the GPi has a major role in gathering movement-related activity from the striatum, GPe and STN, integrating this information, and finally, conveying this processed information outside the basal ganglia. One less-well-known fact is that the GPi also projects to the lateral habenular (LHb), which is a small nucleus located in the epithalamus and is involved principally in reward related signalling (Lecourtier and Kelly, 2007, Morissette and Boye, 2008). This is interesting considering the dominant motor role of the GPi. However, recent studies have shown that many neurons in the basal ganglia encode non-motor signals, especially with regard to expected rewards (Hikosaka et al., 2006). This raises the possibility that the projection from the GPi to
LHb might be a key to link the basal ganglia and the limbic system, relating to reward-related information and mood (Hong and Hikosaka, 2008).

The striatum projects to the GPi via two major projection systems, the **direct** and **indirect** pathways (Alexander and Crutcher, 1990). The **direct** pathway arises from GABAergic striatal neurons which contain substance-P and projects monosynaptically to the GPi. The **indirect** pathway arises from GABAergic striatal neurons containing enkephalin, and projects polysynaptically to the GPi by a sequence of connections involving the GPe and STN (Nambu, 2007). While many striatal projection neurons collateralize on to more than one target, striatal projection neurons can be subdivided into four major populations based on their primary projection target. One of the four includes those projecting mainly to the GPi, which are rich in substance-P but poor in enkephalin, and have cell bodies located in the striatal matrix compartment (Mai et al., 1986, Reiner et al., 1988, Graybiel, 1990, Deng et al., 2004).

Within the GPi, substance-P immunoreactivity is localised in terminals outlining the dendritic tree of pallidal neurons (Allen et al., 2009). In a study by Deng et al, quantification of substance-P immunolabelled terminals within the GPi, in a sample of HD cases ranging from 0-4, highlighted that the loss of striatal projections to the GPi proceeded far more gradually than the loss of enkephalin immunolabelled striatal terminals to the GPe (Deng et al., 2004). These results have been confirmed by a previous study from our group (Allen et al., 2009), that this substance-P immunoreactivity, characteristic of striato-pallidal terminals described as “woolly fibres” in earlier studies (Haber and Elde, 1981) is progressively reduced in HD grades from 0-4 (Allen et al., 2009). By HD grade 3, considerable loss of striato-GPi neurons may contribute to the dystonia, while the near complete loss of this projection system by grade 4 is associated with akinesia in terminal HD (Albin et al., 1989, Deng et al., 2004). Lesions to the GPi can lead to a variety of movement disorders including slowing of movements (Mink and Thach, 1991), dyskinesia (Crossman, 1987) and akinesia (Molinuevo et al., 2003). In short, the GPi is instrumental for the basal ganglia to control body movements.

Neuroimaging studies have reported severe atrophy of the globus pallidus in HD patients (Aylward et al., 1997, Rosas et al., 2003, Fennema-Notestine et al., 2004, Douaud et al., 2006). However, in vivo imaging studies have not isolated the internal segment and separately evaluated the extent of volumetric decline in HD. Post-mortem pathology studies have also reported that significant progressive atrophy of the globus pallidus internal segment occurs. Lange et al
reported a 50% decline in the fresh volume of the GPi in Huntington’s disease, although this was before the use of the striatal neuropathology grading system (Lange et al., 1976). However, the GPi has shown lesser atrophy and gliosis compared to the external segment, based on qualitative or morphometric studies (Lange et al., 1976, Vonsattel et al., 1985, Roos, 1986). Only one stereological approach has reported a decline in GPi volume specifically in HD, with Halliday et al reporting a decline of 21% relative to controls (Halliday et al., 1998). However, no data relating the extent of GPi volume loss to striatal neuropathological grade or clinical symptom information was presented.

The neuronal population in the GPi comprises of GABAergic pallidal neurons, which suggests that they have an inhibitory effect onto their target neurons (Albin et al., 1989). Pallidal neurons are sparse in distribution, compared to striatal neurons, and are 100 times less numerous than spiny striatal projection neurons (Yelnik, 2002). The cellular morphology of GPi neurons is similar to that of the GPe. GPi neurons have relatively large (20-60 µm) triagonal or polygonal soma with thick, sparsely-spined, poorly branching dendrites (Yelnik et al., 1984). The dendrites occupy a disk-like territory orientated perpendicular to incoming striatal fibres (Percheron et al., 1984). This topographic organisation of dendrites and fibres enables convergence of incoming information in the GPi, similarly to the GPe. However, unlike GPe pallidal neurons, GPi neurons do not have extensive local axon collaterals (Parent et al., 1999).

Three types of pallidal neurons have been identified based on neurochemical and morphology properties: Type 1 and 2 are the largest in nature and have been described in multiple mammalian species and make up 80-90% of the total population of pallidal neurons in the globus pallidus (Fox et al., 1974, Difiglia et al., 1982, Francois et al., 1984, Waldvogel et al., 1999). When stained with Nissl, the majority of these neuronal cell bodies in the GPi are large (35-70 µm), contain varying amounts of Nissl granules, and appear elongated with triangular or spindle shapes (Difiglia and Rafols, 1988). Type 1 pallidal neurons contain GABA (Smith et al., 1987) and the calcium binding protein parvalbumin (Kita, 1994) but are immunonegative for any other immunohistochemical marker and make up about ten percent of the large pallidal neuron population (Waldvogel et al., 1999). Type 2 cells are identical in cell morphology to type 1 cells, but are subdivided into “type 2” based on their double calcium binding protein immunoreactivity (Fortin and Parent, 1994). These large pallidal neurons are co-labelled with parvalbumin and calretinin, another calcium binding protein, on the same cells, and make up ninety percent of the large human pallidal neuron population (Waldvogel et al., 1999). However,
in the GPi, most of the type 2 pallidal neurons are weakly immunolabelled with parvalbumin compared to the GPe, and are surrounded by large parvalbumin-immunoreactive boutons. Medium-sized type 3 neurons in the globus pallidus have also been described in previous studies (Difiglia et al., 1982), and they are intensely immunoreactive for calretinin only (Fortin and Parent, 1994), are less than 25 µm in cell soma size, and in the human make up approximately 10-20% of the total number of neurons in the human globus pallidus (Waldvogel et al., 1999).

Currently, there has been no stereological study which has evaluated the degree of pallidal neuron loss in Huntington’s disease. However, two studies using non-stereological methods have reported conflicting conclusions in regards to GPi pallidal neuron involvement in HD. In one quantitative study by Lange et al in 1976, which compared 6 HD cases of unreported grades with 15 control cases, the absolute number of pallidal neurons was shown to decrease by up to 43% in the GPi, which was accompanied by a 50% reduction in pallidal volume (Lange et al., 1976). However, the neuronal density was up to 27% higher within the GPi, and no changes were found in relation to the soma volume of individual pallidal neurons in HD. This morphometric study suggested that pallidal neuronal loss was due to primary degeneration, rather than solely the consequence of striatal degeneration, thereby suggesting that pallidal neuron loss also contributes to GPi atrophy.

However, more recent studies have reached a conflicting conclusion, suggesting that the GP atrophy is mainly due to neuropil loss, resulting from striatal fibres and terminals, and to a lesser extent the loss of neurons (Reiner et al., 1988, Albin et al., 1990, Storey and Beal, 1993). Waikai et al examined 6 HD cases (of advanced striatal neuropathological grade) in comparison to 10 control cases histometrically. After examination of pallidal neurons in 5 selected regions of coronal sections taken along the rostral-caudal axis, using a non-stereological cell-counting method of large pallidal neurons, no cell loss was detected in HD (Wakai et al., 1993). This study supported the hypothesis that no pallidal neuronal depletion was recognised in HD despite marked atrophy of tissue bulk, thereby implicating overall GP atrophy to striato-pallidal fibre loss.

Thus, there is a disagreement in the literature about the extent of pallidal neuron loss in the GPi in Huntington’s disease. Furthermore, there is a lack of stereological knowledge detailing the relationship between GPi atrophy, with striatal neuropathological grade and symptom heterogeneity. There is also a current lack of understanding of pallidal neuron morphology.
changes in HD. Most importantly, since the GPi is involved in both the direct and indirect pathways implicated in HD, the major aims of this section of the thesis are to investigate:

(1) The overall volumetric changes of the GPi in HD with regard to striatal pathological grade and symptom heterogeneity;
(2) the extent of pallidal neuron loss in HD with regard to striatal pathological grade and symptom heterogeneity; and
(3) pallidal neuron soma volume changes in HD with regard to striatal pathological grade and symptom heterogeneity.

These aspects will be investigated using design-based stereological techniques, including an unbiased method of quantifying GPi regional volume (Cavalieri Estimator), total pallidal neuron number (Optical Fractionator), and pallidal neuron volume (Isotropic Nucleator) using the whole region of interest. The research performed in this study is novel, as very few laboratories have access to tissue which is extensively characterised in terms of symptom and clinical information.

For each stereological technique used, the data was obtained using the StereoInvestigator software on 8 Huntington’s disease (HD) (HC101L, HC119R, HC120R, HC125L, HC132L, HC137R, HC139R, HC140R) and 8 control (H153R, H170R, H186R, H204R, H226R, H227R, H230R and H231R) cases (see Chapter 3 on detailed methodology). The stereological estimates for overall volume (in mm³), total pallidal neuron number (total number of Nissl-positive pallidal neurons) and average pallidal cell soma volume (µm³) for the GPi within this chapter are presented for each HD case (N=8) and each control case (N=8) in the form of a scatter plot with the standard error of the mean expressed as mean ± SEM. An estimation of cell density was also derived based on the quotient of the Optical Fractionator data and Cavalieri Estimator data (cells/mm³). The standard deviation (SD) was also reported to assess variation amongst the samples. The mean changes in total regional volume, total pallidal neuron number, average pallidal cell soma volume, and pallidal neuron density in HD compared to control were assessed using a two-sided Mann-Whitney test, where P-values <0.05 were considered statistically significant (statistical significance expressed as *P < 0.05, **P < 0.01, ***P < 0.001).

In order to investigate if stereological estimates for overall volume, total pallidal neuron number and average pallidal cell soma volume for the GPi within this chapter is related to striatal
pathology, the 8 HD cases were also independently examined by a neuropathologist (Dr B Synek) and each case was designated a grade based on striatal neuropathology criteria according to the Vonsattel grading system (Vonsattel et al., 1985, Vonsattel et al., 2008). The grades of the 8 HD cases ranged from 1 to 3. There were three grade 0-1 cases (HC101L, HC132L and HC137R) one grade 2 case (HC120R) and four grade 3 cases (HC119R, HC125L, HC139R, HC140R) (case details are listed in Table 3.2, Chapter 3). For the purpose of this investigation, the only grade 2 case was pooled with the grade 3 cases, with analyses conducted with and without this case to ensure it did not skew the data.

Grade-wise comparisons were conducted for the stereological estimates for overall volume (in mm³), total pallidal neuron number (total number of Nissl-positive pallidal neurons), pallidal cell soma volume (µm³), and pallidal neuron density (cells/mm³) for the GPi within this chapter. The mean changes were presented for each HD grade 0-1 case (N=3), each HD grade 2-3 case (N=5) and each control case (N=8) in the form of a scatter plot with the standard error of the mean expressed as mean ± SEM. The mean changes were grouped according to striatal neuropathological grade and compared to control using a Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted P-value. P-values <0.05 were considered statistically significant (statistical significance expressed as *P < 0.05, **P < 0.01, ***P < 0.001).
6.2 The pattern of volumetric changes in the globus pallidus internal segment in Huntington’s disease using the Cavalieri Estimator

In order to study volumetric changes of the globus pallidus internal segment in detail, first, immunohistochemistry using standard single peroxidise labelling techniques was performed on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire GPi (1 in every 20\textsuperscript{th} section) using antibodies to the neuropeptide substance-P, which allows accurate delineation of the GPi from other structures in the basal ganglia. Secondly, an unbiased design-based stereological technique to quantify the volume of the GPi was performed using StereoInvestigator. The Cavalieri Estimator is a design-based stereological probe for determining the total volume of the entire globus pallidus internal segment using a point-counting method, and was run using the StereoInvestigator software on the 8 Huntington’s disease (HD) and 8 control cases (see Chapter 3 on detailed methodology).

6.2.1 The pattern of volumetric changes in the globus pallidus internal segment in all HD and control cases (Table 6.1, Figure 6.1)

The results of stereological volume quantification, using the Cavalieri Estimator, shows that there is a striking 40\% reduction in the mean volume of the GPi in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 6.1). The mean GPi volume ± SEM of 8 controls was 391.9 ± 25.7 mm\textsuperscript{3}, compared to the mean GPi volume ± SEM of all 8 HD cases which was 234.9 ± 22.0 mm\textsuperscript{3} (Table 6.1). The mean changes in total regional volume in HD compared to control were significant according to the two-sided Mann-Whitney test, with the $P$-value being < 0.05 (***$P$ = 0.0006).

For the stereological analysis of control and HD groups carried out using the Cavalieri Estimator, the average coefficient of error (CE) for the total volume of the GPi was always less than 0.10. The average CE for mean estimates of volume for the control and HD cases was 0.007 and 0.01 respectively, which is well below $\leq$ 0.10 and therefore a generally reliable estimation of volume (Table 6.1) (Gundersen and Jensen, 1987, Slomianka and West, 2005). This reinforces that any variability observed is due to a true difference between cases in the total volume rather than a lack of precision in terms of the stereological technique used (see Chapter 3, Materials and Methods) (Slomianka and West, 2005).
Table 6.1 Variation in the mean overall volume of the globus pallidus internal segment (GPI) between control cases and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean total volume of the globus pallidus internal segment (mm³)</td>
<td>391.9</td>
<td>234.9</td>
<td>276.8</td>
<td>209.8</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>72.77</td>
<td>62.30</td>
<td>62.17</td>
<td>52.47</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>25.73</td>
<td>22.03</td>
<td>35.90</td>
<td>23.46</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage reduction (%) as compared to control</td>
<td>-</td>
<td>***40% reduced</td>
<td>29% reduced</td>
<td>**46% reduced</td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.007</td>
<td>0.01</td>
<td>0.009</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
Figure 6.1 Overall volume of the globus pallidus internal segment (GPi) in all HD cases compared to control cases in the human brain

The graph shows the variation of the total volume of the globus pallidus internal segment (GPi) defined by the Substance-P immunoreactivity in 8 HD and 8 control brains, determined using design-based stereological methods involving the Cavalieri Estimator protocol. Each blue diamond indicates the total volume of the GPi (in mm$^3$) for each control case, and each red diamond indicates the total volume of the GPi (in mm$^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 40% reduction in the mean total volume of the GPi in Huntington's disease, which is statistically significant based on a 2-tailed $P$-value from the Mann-Whitney test (***)$P = 0.0006$). See Table 6.1 for detailed results.
6.2.2 The pattern of volumetric changes in the globus pallidus internal segment in HD compared with striatal neuropathological grade (Table 6.1, Figure 6.1, 6.3)

To investigate if the volumetric reduction of the globus pallidus internal segment (GPi) is related to striatal pathology, the HD results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological volume quantification, using the *Cavalieri Estimator*, shows that there is a **29% reduction in the mean volume of the GPi in grade 0-1 HD**, when compared with 8 controls (Figure 6.2). The mean GPi volume ± SEM of 8 controls was 391.9 ± 25.7 mm$^3$, compared to the mean GPi volume ± SEM of the grade 0-1 cases which was 276.8 ± 35.9 mm$^3$ (Table 6.1). However, the mean changes in total GPi volume in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $> 0.05$ ($P = 0.17$). It is important to note that this lack of significance can be attributable to the higher levels of variation between the grade 0-1 cases, as both the standard deviation and standard error of the mean for this sample is the highest within this group (Table 6.1).

**Analysis of the grade 2-3 group showed a 46% reduction in mean GPi volume** compared to 8 controls (Figure 6.2), highlighted by the mean ± SEM of 209.8 ± 23.5 mm$^3$ (Table 6.1). This reduction in mean GPi volume in HD grade 2-3 compared to control was significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of $< 0.05$ (**$P = 0.003$**). Qualitative descriptions showcase the cross-sectional reduction of the GPi with advancing striatal neuropathological grade as highlighted in Figure 6.3.

In summary, it was observed that the overall mean volume of the GPi decreased with increased HD striatal neuropathological grade. However, with a small cohort of HD cases in the grade 0-1 group, combined with the higher levels of case variation, the reduction observed was not statistically significant. This reduction was even greater beyond grade 2, with the mean GPi volume for the grade 2-3 group reduced by 46%, reinforcing that atrophy of the GPi coincides with striatal pathology.
Figure 6.2 Overall volume of the globus pallidus internal segment (GPI) in HD cases grouped according to striatal neuropathological grade compared to control cases

The graph shows the variation of the total volume of the globus pallidus internal segment (GPI) defined by the Substance-P immunoreactivity in 8 control brains compared with 3 HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the Cavalieri Estimator protocol. Each blue diamond indicates the total volume of the GPI (in mm³) for each control case, each red diamond indicates the total volume of the GPI (in mm³) for each grade 0-1 HD case, and each green diamond indicates the total volume of the GPI (in mm³) of each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 29% reduction in the mean total volume of the GPI in HD grade 0-1, compared to control, though this was not statistically significant ($P = 0.17$). By HD grade 2-3, the mean GPI volume reduced by 46% compared to control, and is statistically significant ($**P = 0.003$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn’s multiple comparisons post-test. See Table 6.1 for detailed results.
Figure 6.3 Macroscopic cross-sectional examination of the globus pallidus internal segment (GPI) in control and HD cases of striatal neuropathological grade 1 and 3

This figure shows representative macroscopic images at three coronal levels (rostral, middle, caudal) through the globus pallidus internal segment (GPI) defined by substance-P immunoreactivity and heavily counter-stained with Nissl. Images are taken of each 70 µm coronal section at low power from the rostral, middle and caudal areas of the GPI from a control (H231R), HD grade 1 (HC101L), and grade 3 (HC140R) case respectively. Note the progressive reduction of the delineated GPI cross-section with progressive HD grade, which is particularly noticeable at the rostral level.

A-C: Three representative coronal sections immunohistochemically stained with substance-P (cross-section delineated) at rostral (A), middle (B) and caudal (C) levels of the GPI of control case H231R.

D-F: Three representative coronal sections immunohistochemically stained with substance-P (cross-section delineated) at rostral (D), middle (E) and caudal (F) levels of the GPI of HD grade 1 case HC101L. Note the reduction in the outlined GPI cross-section observed at all levels in comparison to the control (H231R) equivalent levels. It can be noted that the reduction in cross-section is more evident at rostral and middle levels.

G-I: Three representative coronal sections immunohistochemically stained with substance-P (cross-section delineated) at rostral (D), middle (E) and caudal (F) levels of the GPI of HD grade 3 case HC140R. Note the reduction in the outlined GPI cross-section observed at all levels in comparison to case HC101L, and H231R. It can be noted that the reduction in cross-section is more evident at rostral and middle levels compared to HC101L and H231R.

Abbreviations: Put, putamen; GPe, globus pallidus external segment; GPI, globus pallidus internal segment; CN, caudate nucleus; ant. Thal, anterior thalamus; mid. Thal, middle thalamus; post. Thal, posterior thalamus.

Scale bar: A-I = 5000 µm
Figure 6.3 Macroscopic cross-sectional examination of the globus pallidus internal segment (GPI) of control and HD cases of striatal neuropathological grade 1 and 3

(A) H231R Rostral GPI
(B) H231R Middle GPI
(C) H231R Caudal GPI
(D) HC101L Rostral GPI (Grade 1)
(E) HC101L Middle GPI (Grade 1)
(F) HC101L Caudal GPI (Grade 1)
(G) HC140R Rostral GPI (Grade 3)
(H) HC140R Middle GPI (Grade 3)
(I) HC140R Caudal GPI (Grade 3)
6.2.3 The pattern of volumetric changes in the globus pallidus internal segment compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration (Table 6.2, Figure 6.4)

The total volume of the globus pallidus internal segment (GPi) was next correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both HD and control cases for which all this information was available (Figure 6.4). In general terms, no significant correlation was found between GPi total volume and all variables considered for all HD and control cases.

6.2.4 The pattern of volumetric changes in the globus pallidus internal segment compared with pallidal neuron number, average pallidal soma volume, and pallidal neuron density (Table 6.2, Figure 6.5)

It was next investigated whether the total volume of the GPi was correlated with total pallidal neuron number, average pallidal soma volume, and pallidal neuron density in both HD and control cases as determined using design-based stereology (Figure 6.5). In general terms, the overall GPi volume decreased with pallidal neuron number for HD cases only (panel A), which is statistically significant according to a two-tailed Spearman’s correlation (**P = 0.005). No significant correlation was found between GPi total volume and average pallidal soma volume for both control and HD cases (panel B). The density of pallidal neurons, in control cases only, was found to increase with lower GPi volumes (*P = 0.046) (panel C).
This figure shows the comparison of the total volume of globus pallidus internal segment (GPI) with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration for all HD and control cases which have the information available. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total volume of the GPI and the CAG repeat number in HD cases according to a two-tailed Spearman's correlation analysis ($r = -0.34, P = 0.74$).

B: There was no significant correlation between the total volume of the GPI and the post-mortem delay in control ($r = -0.36, P = 0.36$) and HD ($r = 0.22, P = 0.61$) cases, according to a two-tailed Spearman's correlation analysis.

C: There was no significant correlation between the total volume of the GPI and the post-mortem brain weight in control ($r = -0.83, P = 0.06$) and HD ($r = 0.68, P = 0.11$) cases, according to a two-tailed Spearman’s correlation analysis.

D: There was no significant correlation between the total volume of the GPI and the age of death for control ($r = 0.07, P = 0.88$) and HD ($r = -0.41, P = 0.30$) cases, according to a two-tailed Spearman’s correlation analysis.

E: There was no significant correlation between the total volume of the GPI and the age of disease onset in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.27, P = 0.50$).

F: There was no significant correlation between the total volume of the GPI and the duration of disease from age of onset to death in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.39, P = 0.31$).
Figure 6.4 Comparison between the total volume of the globus pallidus internal segment with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration in both HD and control cases
Figure 6.5 Comparison between the total volume of the globus pallidus internal segment with total pallidal neuron number, average pallidal soma volume, and pallidal neuron density in both HD and control cases

This figure shows the correlation of the total volume of globus pallidus internal segment (GPI) with total pallidal neuron number, average pallidal soma volume, and pallidal neuron density for all control and HD cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total volume of the GPI and the total number of Nissl-positive pallidal neurons in control cases according to a two-tailed Spearman’s correlation analysis ($r = -0.02$, $P = 0.98$). However, there was a strong positive correlation between the total volume of the GPI and the total number of Nissl-positive pallidal neurons in HD cases, which was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = 0.90$, $P = **0.005$).

B: There was no significant correlation between the total volume of the GPI and the average pallidal soma volume in control ($r = -0.38$, $P = 0.35$) and HD ($r = 0.38$, $P = 0.36$) cases, according to a two-tailed Spearman’s correlation analysis.

C: There was a significant inverse correlation between the total volume of the GPI and the density of pallidal neurons in control cases ($r = -0.73$, $^*P = 0.046$) with the density of pallidal neurons being higher in cases with smaller GPI volumes. However, no significant correlation was found between GPI total volume and pallidal neuron density in HD cases ($r = 0.55$, $P = 0.17$), according to a two-tailed Spearman’s correlation analysis.
6.2.5 The pattern of volumetric changes in the HD globus pallidus internal segment compared with symptomatology and clinical assessments (Table 6.2, Figure 6.6, 6.7)

It was next investigated whether the total volume of the GPi was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 6.6 highlights the correlation between the total volume of the GPi with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between the GPi total volume and all variables examined. However, there is a general trend as shown in panels E and F, which shows that the overall GPi volume increased with both of the SNAITH outward \( (r = 0.9, P = 0.08) \) and inward \( (r = 0.95, P = 0.07) \) irritability scores, although these correlations were not statistically significant according to a two-tailed Spearman’s correlation despite a highly positive regression value, indicative of a strong trend.

Figure 6.7 shows the correlation between the total volume of the GPi with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between GPi total volume and all variables examined according to a two-tailed Spearman’s correlation.
Figure 6.6 Comparison between the total volume of the HD globus pallidus internal segment with measures of Total Functional Capacity, cognition and mood

This figure shows the correlation between the total volume of globus pallidus internal segment (GPI) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total volume of the GPI and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50$, $P = 0.45$).

B: There was no significant correlation between the total volume of the GPI and the Mini-Mental State examination score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.60$, $P = 0.35$).

C: There was no significant correlation between the total volume of the GPI and the Hospital Anxiety scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.80$, $P = 0.13$).

D: There was no significant correlation between the total volume of the GPI and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.72$, $P = 0.17$).

E: There is a clear trend that the overall GPI volume reduced with Outward Irritability score, although this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$). However, the large positive regression value of $r = 0.9$ highlights a very strong trend.

F: There is a clear trend that the overall GPI volume reduced with Inward Irritability score, although this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.07$). However, the large positive regression value of $r = 0.95$ highlights a very strong trend.
Figure 6.6 Comparison between the total volume of the HD globus pallidus internal segment with measures of Total Functional Capacity, cognition and mood

A

B

C

D

E

F

HD (n=5)  
$r = 0.50$  
$P = 0.45$

HD (n=5)  
$r = 0.80$  
$P = 0.13$

HD (n=5)  
$r = 0.60$  
$P = 0.35$

HD (n=5)  
$r = 0.72$  
$P = 0.17$

HD (n=5)  
$r = 0.90$  
$P = 0.08$

HD (n=5)  
$r = 0.95$  
$P = 0.07$
Figure 6.7 Comparison between the total volume of the HD globus pallidus internal segment with clinical measures of motor impairment and chorea

This figure shows the correlation between the total volume of globus pallidus internal segment (GPI) with the Quantitative Neurological Exam (QNE) motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total volume of the GPI and the QNE motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.60, P = 0.35$).

B: There was no significant correlation between the total volume of the GPI and the QNE chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.00, P > 0.99$).

C: There was no significant correlation between the total volume of the GPI and the UHDRS motor assessment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.60, P = 0.35$).

D: There was no significant correlation between the total volume of the GPI and the UHDRS chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.05, P = 0.90$).
6.2.6 Overall summary of volumetric changes in the globus pallidus internal segment compared with all variables (Table 6.2)

A summary of the comparisons between the total volume of the GPi for all cases and all variables discussed in sections 6.2.3, 6.2.4 and 6.2.5 is highlighted in Table 6.2. Key findings include:

(1) The overall GPi volume decreased with pallidal neuron number for HD cases only
(2) The density of pallidal neurons in control cases only were found to increase with lower GPi volumes
(3) The overall GPi volume increased with both of the SNAITH outward and inward irritability scores
Table 6.2 Summary of comparisons between globus pallidus internal segment volume and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size (n)</th>
<th>Relationship</th>
<th>r value (GPi volume)</th>
<th>Two-tailed P value (GPi volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal HD</td>
<td>Normal HD</td>
<td>Normal HD</td>
<td>Normal HD</td>
</tr>
<tr>
<td>HD CAG repeat length</td>
<td>- 8</td>
<td>- Inverse</td>
<td>-0.13</td>
<td>- 0.74</td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>8 8</td>
<td>Inverse Direct -0.36 0.22</td>
<td>0.36 0.61</td>
<td></td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>6 7</td>
<td>Inverse Direct -0.83 0.68</td>
<td>0.06 0.11</td>
<td></td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8 8</td>
<td>Direct Inverse 0.07 -0.41</td>
<td>0.88 0.30</td>
<td></td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>- 8</td>
<td>Inverse -0.27</td>
<td>- 0.50</td>
<td></td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>- 8</td>
<td>Inverse -0.39</td>
<td>- 0.31</td>
<td></td>
</tr>
<tr>
<td>Normal HD</td>
<td>Normal HD</td>
<td>Relationship</td>
<td>r value (GPi volume)</td>
<td>Two-tailed P value (GPi volume)</td>
</tr>
<tr>
<td>HD CAG repeat length</td>
<td>- 8</td>
<td>Inverse Direct -0.02 0.90</td>
<td>0.98 **0.005</td>
<td></td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>8 8</td>
<td>Inverse Direct -0.38 0.38</td>
<td>0.35 0.36</td>
<td></td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>8 7</td>
<td>Inverse Direct -0.83 0.68</td>
<td>0.06 0.11</td>
<td></td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8 8</td>
<td>Direct Inverse 0.07 -0.41</td>
<td>0.88 0.30</td>
<td></td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>- 8</td>
<td>Inverse -0.27</td>
<td>- 0.50</td>
<td></td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>- 8</td>
<td>Inverse -0.39</td>
<td>- 0.31</td>
<td></td>
</tr>
<tr>
<td>Total number of Nissl-positive pallidal neurons</td>
<td>8 8</td>
<td>Inverse Direct -0.73 0.55</td>
<td>*0.046 0.17</td>
<td></td>
</tr>
<tr>
<td>Average parvalbumin-positive pallidal soma volume (µm³)</td>
<td>8 8</td>
<td>Inverse Direct -0.38 0.38</td>
<td>0.35 0.36</td>
<td></td>
</tr>
<tr>
<td>Density of Nissl-positive pallidal neurons</td>
<td>8 8</td>
<td>Inverse Direct -0.73 0.55</td>
<td>*0.046 0.17</td>
<td></td>
</tr>
<tr>
<td>Total Functional Capacity</td>
<td>- 5</td>
<td>Direct 0.50 0.45</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Mini-Mental State Examination score</td>
<td>- 5</td>
<td>Direct 0.60 0.35</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>Mood Hospital Anxiety Scale</td>
<td>- 5</td>
<td>Direct 0.80 0.13</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Mood Hospital Depression Scale</td>
<td>- 5</td>
<td>Direct 0.72 0.17</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>SNAITH Outward Irritability Score</td>
<td>- 5</td>
<td>Direct 0.90 0.08</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>SNAITH Inward Irritability Score</td>
<td>- 5</td>
<td>Direct 0.95 0.07</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>QNE Motor Impairment score</td>
<td>- 5</td>
<td>Inverse -0.60 0.35</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>QNE Chorea Score</td>
<td>- 5</td>
<td>None 0.00 &gt;0.99</td>
<td>&gt;0.99</td>
<td></td>
</tr>
<tr>
<td>UHDRS Motor Assessment Score</td>
<td>- 5</td>
<td>Inverse -0.60 0.35</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>UHDRS Chorea Assessment Score</td>
<td>- 5</td>
<td>Inverse -0.05 0.90</td>
<td>0.90</td>
<td></td>
</tr>
</tbody>
</table>

*denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: *P < 0.05, **P < 0.01, ***P < 0.001. Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
6.3 The pattern of pallidal neuron loss in the globus pallidus internal segment in Huntington’s disease using the Optical Fractionator

In order to study pallidal neuron loss in the globus pallidus internal segment in detail, first, immunohistochemistry using standard single peroxidise labelling techniques was performed on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire GPi (1 in every 20th section) using antibodies to the neuropeptide substance-P, allowing accurate delineation of the GPi from other structures in the basal ganglia. Secondly, a Nissl stain with cresyl violet was used to stain all cells within the globus pallidus. Pallidal neurons were morphologically identified on Nissl-stained sections as large, ellipsoid-shaped, nucleolated cells. Thirdly, an unbiased design-based stereological technique was performed to quantify pallidal neurons in the GPi, using StereoInvestigator. The Optical Fractionator is a 3-dimensional systematic random sampling method used to estimate the total number of Nissl-positive pallidal neurons in the GPi, and was run using the StereoInvestigator software on 8 Huntington’s disease (HD) and 8 control cases (see Chapter 3 on detailed methodology).

6.3.1 The pattern of pallidal neuron loss in the globus pallidus internal segment in all HD and control cases (Table 6.3, Figure 6.8)

The results of stereological pallidal neuron quantification, using the Optical Fractionator, shows that there is a 19% reduction in the mean total pallidal neuron number in the GPi in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 6.8). The mean cell number ± SEM of 8 controls was 166,600 ± 11,865 pallidal neurons compared to the mean cell number ± SEM of all 8 HD cases which was 135,018 ± 20,147 pallidal neurons (Table 6.3). The mean changes in total pallidal neuron number in HD compared to control were not significant according to the two-sided Mann-Whitney test, with the $P$-value being $> 0.05$ ($P = 0.193$).

It was also interesting to note that there was considerable variation in the total pallidal neuron number in the GPi within the control and HD cohorts as shown by the differential standard deviations (SD). The HD cohort had a larger standard deviation (SD = 56,983 cells) compared to the control cohort where the cases show a smaller standard deviation from the mean (SD = 33,558 cells) (Table 6.3). This is also reinforced by the larger range within the HD cohort as
shown by Figure 6.8, with the difference between case HC132L (highest count of 218,486 cells) and case HC137R (lowest count of 68,875 cells) being 149,611 cells. In comparison, the control cohort has a smaller range, with the difference between case H231R (highest count of 226,021 cells) and case H227R (lowest count of 123,249 cells) being 102,772 cells. Therefore, it can be seen that the HD cohort has considerably high levels of variability, which would affect the overall group mean.

For the stereological analysis of control and HD groups carried out, using the Optical Fractionator, the average coefficient of error (CE) for the total number of pallidal neurons in the GPi was always less than 0.10. The average CE for mean estimates of total cell number for the control and HD cases was 0.07 and 0.08 respectively, which are ≤ 0.10 and therefore a generally reliable estimation of pallidal neuron number (Table 6.3). This reinforces that any variability observed is due to a true difference between cases in total pallidal neuron number rather than a lack of precision in terms of the stereological technique used (see Chapter 3, Materials and Methods) (Slomianka and West, 2005).

**Table 6.3 Variation in the mean total pallidal neuron number in the globus pallidus internal segment between control and neuropathological grades in Huntington’s disease**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of Nissl-positive pallidal neurons</td>
<td>166,600</td>
<td>135,018</td>
<td>162,938</td>
<td>118,266</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>33,558</td>
<td>56,983</td>
<td>81,905</td>
<td>37,319</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>11,865</td>
<td>20,147</td>
<td>47,288</td>
<td>16,690</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage loss (%) as compared to control</td>
<td>-</td>
<td>19% loss</td>
<td>2% loss</td>
<td>29% loss</td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.07</td>
<td>0.08</td>
<td>0.07</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
Figure 6.8 Total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) in HD cases compared to all control cases in the human brain

The graph shows the variation in the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) in 8 HD and 8 control brains, determined using design-based stereological methods involving the Optical Fractionator. Each blue diamond indicates the total number of Nissl-positive neurons in the GPI for each control case, and each red diamond indicates the total number of Nissl-positive neurons in the GPI for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 19% reduction in the mean total number of Nissl-positive pallidal neurons in the GPI in Huntington’s disease, however this was not statistically significant based on a 2-tailed P-value from the Mann-Whitney test ($P = 0.193$). See Table 6.3 for detailed results.
6.3.2 The pattern of pallidal neuron loss in the globus pallidus internal segment compared with striatal neuropathological grade (Table 6.3, Figure 6.9, 6.10)

To investigate if pallidal neuronal number in the globus pallidus internal segment (GPi) is related to striatal pathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological pallidal neuron quantification, using the *Optical Fractionator*, shows that there is a **negligible 2% reduction in the mean total number of pallidal neurons in the GPi in grade 0-1 HD**, when compared with 8 controls (Figure 6.9). The mean total cell number ± SEM of 8 control cases was 166,600 ± 11,865 cells, compared to the mean total cell number ± SEM of the grade 0-1 cases which was 162,938 ± 47,288 cells (Table 6.3). The mean changes in total cell number in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted *P*-value of > 0.05 (*P* > 0.99). It is important to note that there is substantial variability amongst cases in the grade 0-1 group, as highlighted by the very large standard deviation of 81,905 cells, which is half the size of the mean (Table 6.3). Cases HC132L and HC101L returned counts which were in line with the control case H231R, suggesting that these cases contain a very high number of Nissl-positive pallidal neurons, comparative to control.

**Analysis of the grade 2-3 group showed a 29% reduction in the mean total number of pallidal neurons** compared to 8 controls (Figure 6.9), highlighted by the mean ± SEM of 118,266 ± 16,690 cells (Table 6.2). This loss of pallidal neurons by HD grade 2-3 compared to control was not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted *P*-value of > 0.05 (*P* = 0.12). Figure 6.10 highlights these findings qualitatively through microscopic examination of pallidal neuron distribution in a representative control, HD grade 1, and HD grade 3 case.

In summary, it was observed that the **total number of pallidal neurons in the GPi decreased with advancing HD striatal neuropathological grade**. However, with a small cohort of HD cases in the grade 0-1 group, combined with high levels of variability, the reduction observed is minor and not statistically significant. This reduction was greater beyond grade 2, with the mean total number of pallidal neurons for the **grade 2-3 group reduced by 29%**, suggesting that pallidal neuron loss occurs in the GPi at advanced striatal neuropathological grades.
Figure 6.9 Total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) in HD cases grouped according to striatal neuropathological grade compared to control cases

The graph shows the variation in the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) in 8 control brains compared with 3 HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the Optical Fractionator. Each blue diamond indicates the total number of Nissl-positive neurons in the GPI for each control case, each red diamond indicates the total number of Nissl-positive neurons in the GPI for each grade 0-1 HD case, and each green diamond indicates the total number of Nissl-positive neurons in the GPI for each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a negligible 2% reduction in the mean total number of Nissl-positive pallidal neurons in the GPI in HD grade 0-1, compared to control, which was not statistically significant ($P > 0.99$). By HD grade 2-3, there was a 29% reduction in the mean total number of Nissl-positive pallidal neurons compared to control, although this was not statistically significant ($P = 0.12$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn’s multiple comparisons post-test. See Table 6.3 for detailed results.
Figure 6.10 Representative photomicrographs showing the distribution of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) from control and HD cases of striatal neuropathological grades 1 and 3

This figure shows high magnification photomicrographs at 3 coronal levels (rostral, middle, caudal) through the globus pallidus internal segment (GPI), highlighting the overall distribution of Nissl-positive pallidal neurons from a control (H170R), HD grade 1 (HC137R) and grade 3 (HC125L) case respectively (red arrows). Despite the increase in gliosis observed at grade 3, pallidal neurons are found at all levels of the GPI in HD cases.

A-C: Three representative coronal sections of a control case (H170R) stained with Nissl at rostral (A), middle (B) and caudal (C) levels of the GPI, showing the distribution of pallidal neurons. Red arrows denote representative pallidal neurons.

D-F: Three representative coronal sections stained with Nissl at rostral (D), middle (E) and caudal (F) levels of the GPI, showing the distribution of pallidal neurons within the HD grade 1 case HC137R. Red arrows denote representative pallidal neurons. Note the slight reduction in the number of Nissl-positive pallidal neurons observed at all levels in comparison to the control (H170R) equivalent regional levels.

G-I: Three representative coronal sections immunohistochemically stained with Nissl at rostral (D), middle (E) and caudal (F) levels of the GPI of HD grade 3 case HC125L. Red arrows denote representative pallidal neurons. Note the slight reduction in the number of Nissl-positive pallidal neurons observed at all levels in comparison to the control case (H170R). Despite a lack of difference seen in comparison to the HD grade 1 (HC137R) case in terms of pallidal neuron count, an increase in glia can be clearly seen.

Scale bar: A-I = 100 µm
Figure 6.10 Representative photomicrographs showing the distribution of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) from control and HD cases of striatal neuropathological grades 1 and 3
6.3.3 The pattern of pallidal neuron loss in the globus pallidus internal segment compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration (Table 6.4, Figure 6.11)

It was next investigated whether the pattern of pallidal neuron loss in the GPI was correlated with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset) in both HD and control cases for which all this information was available (Figure 6.11). In general terms, the total number of Nissl-positive pallidal neurons was greater with increasing brain weight for control cases only (panel C), but was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.06$). No significant correlation was found between the total number of Nissl-positive pallidal neurons and CAG repeat length, post-mortem delay, age of death, age of disease onset and disease duration for all control and HD cases.

6.3.4 The pattern of pallidal neuron loss in the globus pallidus internal segment compared with overall GPI volume, average pallidal soma volume, and pallidal neuron density (Table 6.4, Figure 6.12)

It was also explored whether the total number of Nissl-positive pallidal neurons was correlated with overall GPI volume, average pallidal soma volume, and pallidal neuron density in both HD and control cases, which were variables measured using design-based stereology (Figure 6.12). In general terms, the loss of pallidal neurons was greater with reducing GPI volumes for HD cases only (panel A), which is statistically significant according to a two-tailed Spearman’s correlation (**$P = 0.005$). Furthermore, an inverse correlation was found between total pallidal neuron number and the average pallidal soma volume in control cases only, which is statistically significant according to a two-tailed Spearman’s correlation (*$P = 0.04$) (panel B). In addition to this, the loss of Nissl-positive pallidal neurons coincided with a reduction in overall pallidal neuron density, as shown by a positive correlation for HD cases (*$P = 0.04$) (panel C).
Figure 6.11 Comparison between the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration in both HD and control cases

This figure shows the correlation of total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset and disease duration for all HD and control cases which have the information available. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the CAG repeat number in HD cases according to a two-tailed Spearman's correlation analysis ($r = 0.05, P = 0.92$).

B: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the post-mortem delay in control ($r = 0.20, P = 0.63$) and HD ($r = 0.32, P = 0.43$) cases, according to a two-tailed Spearman's correlation analysis.

C: There is a clear trend that the total number of Nissl-positive pallidal neurons was greater with heavier brain weights in control cases, although this was not statistically significant according to a two-tailed Spearman's correlation analysis ($r = 0.83, P = 0.06$). There was no significant correlation between the total number of Nissl-positive pallidal neurons and the post-mortem brain weight in HD cases ($r = 0.68, P = 0.11$).

D: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the age of death for control ($r = -0.33, P = 0.43$) and HD ($r = -0.63, P = 0.09$) cases, according to a two-tailed Spearman's correlation analysis.

E: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the age of disease onset in HD cases according to a two-tailed Spearman's correlation analysis ($r = -0.34, P = 0.39$).

F: There was no significant correlation between the total number of Nissl-positive pallidal neurons and duration of disease from age of onset to death in HD cases ($r = -0.61, P = 0.11$), according to a two-tailed Spearman's correlation analysis.
Figure 6.11 Comparison between the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment with CAG repeat length, post-mortem (PM) delay, brain weight, age of death, age of disease onset, and disease duration in both HD and control cases.
Figure 6.12 Comparison between the total number of pallidal neurons in the globus pallidus internal segment with overall GPi volume, average pallidal soma volume, and pallidal neuron density in both HD and control cases

This figure shows the correlation of total number of Nissl-positive pallidal neurons with Overall GPi volume, average pallidal soma volume, and pallidal neuron density for all HD and control cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between the total number of Nissl-positive pallidal neurons and overall volume of the GPi in control cases, according to a two-tailed Spearman’s correlation analysis ($r = -0.02$, $P = 0.98$). There was a strong positive correlation between the total number of Nissl-positive pallidal neurons and the overall GPi volume in HD cases, which was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = 0.90$, **$P = 0.005$).

B: There was an inverse correlation between the total number of Nissl-positive pallidal neurons and the average pallidal soma volume in control cases only ($r = -0.74$), which was statistically significant according to a two-tailed Spearman’s correlation analysis (*$P = 0.04$). However, there was no significant correlation between the total number of Nissl-positive pallidal neurons and the average pallidal soma volume in HD cases ($r = 0.26$, $P = 0.54$).

C: There was no significant correlation between the total number of Nissl-positive pallidal neurons and the density of pallidal neurons in control cases ($r = 0.57$, $P = 0.15$). However, the density of pallidal neurons in HD cases appears to be higher in cases with a greater total number of Nissl-positive pallidal neurons. This was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = 0.76$, *$P = 0.04$).
6.3.5 The pattern of pallidal neuron loss in the globus pallidus internal segment compared with symptomatology and clinical assessments

It was next investigated whether the loss of Nissl-positive pallidal neurons was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 6.13 highlights the correlation between the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and all variables examined. However, there is a general trend as shown in panel C, which shows that the total number of pallidal neurons was greater in HD cases with a higher score for the Mood Hospital Anxiety Scale. This correlation was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), although the highly positive regression value of $r = 0.9$ highlights a very strong trend.

Figure 6.14 shows the correlation between the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and all variables examined.
Figure 6.13 Comparison between the total number of pallidal neurons in the HD globus pallidus internal segment with measures of Total Functional Capacity, cognition and mood

This figure shows the comparison between the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total number of pallidal neurons and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.80$, $P = 0.13$).

B: There was no significant correlation between the total number of pallidal neurons and Mini-Mental State Examination scores in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.70$, $P = 0.23$).

C: There is a clear trend that the total number of pallidal neurons was greater with the Hospital Anxiety Scale, although this was not statistically significant according to a two-tailed Spearman’s correlation analysis ($P = 0.08$). However, the large positive regression value of $r = 0.90$ highlights a very strong trend.

D: There was no significant correlation between the total number of pallidal neurons and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.46$, $P = 0.43$).

E: There was no significant correlation between the total number of pallidal neurons and the Outward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.70$, $P = 0.23$).

F: There was no significant correlation between total number of pallidal neurons and the Inward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.76$, $P = 0.20$).
Figure 6.13 Comparison between the total number of pallidal neurons in the HD globus pallidus internal segment with measures of Total Functional Capacity, cognition and mood.
Figure 6.14 Comparison between the total number of pallidal neurons in the HD globus pallidus internal segment with clinical measures of motor impairment and chorea

This figure shows the correlation between the total number of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) with the Quantitative Neurological Exam (QNE) motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the total number of pallidal neurons and QNE motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.70, P = 0.23$).

B: There was no significant correlation between the total number of pallidal neurons and the QNE chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.10, P = 0.95$).

C: There was no significant correlation between the total number of pallidal neurons and the UHDRS motor assessment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.70, P = 0.23$).

D: There was no significant correlation between the total number of pallidal neurons and the UHDRS chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.05, P = 0.90$).
6.3.6 Overall summary of pallidal neuron loss in the globus pallidus internal segment compared with all variables (Table 6.4)

A summary of the comparisons between the total number of Nissl-positive pallidal neurons in the GPi for all cases and all variables discussed in sections 6.3.3, 6.3.4 and 6.3.5 is highlighted in Table 6.4. Key findings include:

(1) The total number of pallidal neurons was greater with increasing brain weight for control cases only
(2) The loss of pallidal neurons was greater with reducing GPi volume for HD cases only
(3) The loss of pallidal neurons coincided with a reduction in overall pallidal neuron density, as shown by a positive correlation for HD cases
(4) The total number of pallidal neurons was greater in HD cases with a higher score for the Mood Hospital Anxiety Scale
Table 6.4 Summary of comparisons between the total number of pallidal neurons in the GPi and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size</th>
<th>Relationship</th>
<th>r value (GPi pallidal neuron number)</th>
<th>Two-tailed P value (GPi pallidal neuron number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>HD</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>HD CAG repeat length</td>
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<td>Direct</td>
<td>-</td>
</tr>
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<td>PM delay (hours)</td>
<td>8</td>
<td>8</td>
<td>Direct</td>
<td>Direct</td>
</tr>
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<td>Brain weight (g)</td>
<td>6</td>
<td>7</td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8</td>
<td>8</td>
<td>Inverse</td>
<td>Inverse</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>-</td>
<td>8</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>-</td>
<td>8</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>Overall GPI volume (mm³)</td>
<td>8</td>
<td>8</td>
<td>None</td>
<td>Direct</td>
</tr>
<tr>
<td>Average parvalbumin-positive pallidal soma volume (µm³)</td>
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<td>8</td>
<td>Inverse</td>
<td>Direct</td>
</tr>
<tr>
<td>Density of Nissl-positive pallidal neurons</td>
<td>8</td>
<td>8</td>
<td>Direct</td>
<td>Direct</td>
</tr>
<tr>
<td>Total Functional Capacity</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>0.80</td>
</tr>
<tr>
<td>Mini-Mental State Examination score</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>0.70</td>
</tr>
<tr>
<td>Mood Hospital Anxiety Scale</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>0.90</td>
</tr>
<tr>
<td>Mood Hospital Depression Scale</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>0.46</td>
</tr>
<tr>
<td>SNAITH Outward Irritability Score</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>0.70</td>
</tr>
<tr>
<td>SNAITH Inward Irritability Score</td>
<td>-</td>
<td>5</td>
<td>Direct</td>
<td>0.76</td>
</tr>
<tr>
<td>QNE Motor Impairment score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-0.70</td>
</tr>
<tr>
<td>QNE Chorea Score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-0.10</td>
</tr>
<tr>
<td>UHDRS Motor Assessment Score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-0.70</td>
</tr>
<tr>
<td>UHDRS Chorea Assessment Score</td>
<td>-</td>
<td>5</td>
<td>Inverse</td>
<td>-0.05</td>
</tr>
</tbody>
</table>

*denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: *P < 0.05, **P < 0.01, ***P < 0.001. Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
6.4 The size evaluation of parvalbumin-positive pallidal neuron soma in the globus pallidus internal segment in Huntington’s disease using the Isotropic Nucleator

In order to study changes in pallidal neuron cell soma volume in the globus pallidus internal segment (GPI) in detail, first, immunohistochemistry using standard single peroxidise labelling techniques was performed on a systematically and randomly sampled (SRS) series of coronal sections encompassing the entire GPI (1 in every 40th section) using antibodies to the calcium-binding protein parvalbumin, to delineate the entire cell soma of large pallidal neurons. Parvalbumin is a calcium-binding protein expressed in 80-90% of pallidal neurons in the human globus pallidus (Waldvogel et al., 1999), outlining the soma of large pallidal neurons with punctuate terminal labelling in the internal segment of the globus pallidus. Secondly, an unbiased design-based stereological technique to measure the cross-sectional area and volume of pallidal neurons in the GPI was performed using StereoInvestigator. The Isotropic Nucleator is a 3-dimensional systematic random sampling method which uses the intersection of rays to measure the cross-sectional area to obtain an estimate of mean pallidal soma volume in the GPI, and was run using the StereoInvestigator software on 8 Huntington’s disease (HD) and 8 control cases (see Chapter 3 on detailed methodology).

6.4.1 The size evaluation of parvalbumin-positive pallidal neuron soma in the globus pallidus internal segment in all HD and control cases (Table 6.5, Figure 6.15, 6.16)

The results of stereological pallidal soma volume quantification, using the Isotropic Nucleator, shows that there is a 21% reduction in the pallidal soma volume in the GPI in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 6.15). The mean cell soma volume ± SEM of 8 controls was 27,081 ± 3,193 μm³ compared to the mean cell soma volume ± SEM of all 8 HD cases which was 21,273 ± 1,170 μm³ (Table 6.5). These changes in pallidal neuron soma volume in HD compared to control were not significant according to the two-sided Mann-Whitney test, with the P-value being > 0.05 (P = 0.13).

It was also interesting to note that there was considerable variation in mean pallidal soma volume in the GPI within the control cohort as shown by a larger standard deviation (SD)
compared to the HD cohort. Control cases had a larger standard deviation (SD = 9,030 µm³) compared to the HD cohort where the cases show a smaller standard deviation from the mean (SD = 3,309 µm³) (Table 6.5). This is also reinforced by the larger range within the control cohort as shown by Figure 6.15, with the difference between case H204R (largest cell volume of 39,570 µm³) and case H231R (smallest cell volume of 12,727 µm³) being 26,843 µm³. In comparison, the HD cohort has a smaller range with lower variability, with the difference between case HC132L (largest cell volume of 26,249 µm³) and case HC119R (smallest cell volume of 15,689 µm³) being 10,560 µm³. Figure 6.16 qualitatively describes the reduction in cell soma observed in HD through the comparison of cell soma cross-sectional delineation between a representative control and HD case.

For the stereological analysis of control and HD groups carried out using the Isotropic Nucleator, the average coefficient of error (CE) for the mean pallidal neuron soma volume in the GPi was always less than 0.10. The average CE for estimates of mean pallidal neuron soma volume for the control and HD cases was 0.01 and 0.02 respectively, which are ≤ 0.10 and therefore a generally reliable estimation of pallidal soma volume (Table 6.5) (Gundersen and Jensen, 1987, Slomianka and West, 2005). This reinforces that any variability observed is due to a true difference between cases in mean pallidal neuron soma volume rather than a lack of precision in terms of the stereological technique used (see Chapter 3, Materials and Methods) (Slomianka and West, 2005).

### Table 6.5 Variation in the mean pallidal neuron soma volume in the globus pallidus internal segment between control and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell soma volume of Parvalbumin-positive pallidal neurons (µm³)</td>
<td>27,081</td>
<td>21,273</td>
<td>22,614</td>
<td>20,469</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>9,030</td>
<td>3,309</td>
<td>3,156</td>
<td>3,468</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>3,193</td>
<td>1,170</td>
<td>1,822</td>
<td>1,551</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage reduction (%) as compared to control</td>
<td>-</td>
<td>21% reduced</td>
<td>17% reduced</td>
<td>24% reduced</td>
</tr>
<tr>
<td>Average coefficient of error (CE)</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *P < 0.05, **P < 0.01, ***P < 0.001
Figure 6.15 Mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) in HD cases compared to all control cases in the human brain

The graph shows the variation in mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) in 8 HD and 8 control brains, determined using design-based stereological methods involving the Isotropic Nucleator. Each blue diamond indicates the mean cell soma volume of parvalbumin-positive neurons in the GPI (in µm$^3$) for each control case, and each red diamond indicates the mean cell soma volume of parvalbumin-positive neurons in the GPI (in µm$^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. **The graph shows a 21% reduction in mean cell soma volume of parvalbumin-positive neurons in the GPI in Huntington’s disease.** However, this was not statistically significant based on a 2-tailed $P$-value from the Mann-Whitney test ($P = 0.13$). See Table 6.5 for detailed results.
Figure 6.16 Representative photomicrographs showing the cell soma cross-sectional size of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) from a control (A-C) and advanced HD case (D-G).

This figure shows the morphological changes of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) in a representative control (H204R) and HD grade 3 case (HC119R). Dotted lines encircle the cell soma. Note the generally smaller morphology of the delineated pallidal neuron cell bodies in HC119R.

A: Representative high power (20x) photomicrograph of parvalbumin-positive pallidal neurons taken from a middle section within the GPI of the control case H204.

B-C: Higher magnification (60x oil) inserts of panel A. The pallidal neuron cell soma cross-section is delineated as shown.

D: Representative high power (20x) photomicrograph of parvalbumin-positive pallidal neurons taken from a middle section within the GPI of the HD case HC119.

E-F: Higher magnification (60x oil) inserts of panel D. The pallidal neuron cell soma cross-section is delineated as shown. Note the smaller morphology of the HD pallidal neuron soma as indicated by the smaller delineated cross sections.

Scale bar: A, D = 100 µm

Scale bar: B-C, E-G = 20 µm
6.4.2 The size evaluation of parvalbumin-positive pallidal neuron soma in the globus pallidus internal segment based on striatal neuropathological grade (Table 6.5, Figure 6.17)

In order to investigate if there is a relationship between pallidal soma size within the globus pallidus internal segment (GPi) and striatal neuropathological grade, the 8 HD case results were grouped according to striatal neuropathological grade and compared to the control group. The results of stereological pallidal soma measurements, using the Isotropic Nucleator, shows that there is a 17% reduction in the mean soma volume of pallidal neurons in the GPi in grade 0-1 HD, when compared with 8 controls (Figure 6.17). The mean ± SEM of 8 controls was 27,081 ± 3,193 µm³, compared to the mean ± SEM of the grade 0-1 cases which was 22,614 ± 1,822 µm³ (Table 6.5). However, the mean changes in pallidal soma volume in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of > 0.05 ($P > 0.99$). **Analysis of the grade 2-3 group showed a 24% reduction in the mean pallidal soma volume** compared to 8 controls (Figure 6.17), highlighted by the mean ± SEM of 20,469 ± 1,551 µm³ (Table 6.5). This decline in pallidal soma volume in HD grade 2-3 compared to control was not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted $P$-value of > 0.05 ($P = 0.157$).

In summary, it was observed that the **mean pallidal soma volume in the GPi decreased with advancing HD striatal neuropathological grade**. However, with a small cohort of HD cases in the grade 0-1 group, combined with the considerable variability amongst the control cases, the reduction observed was not statistically significant. **This reduction was even greater beyond grade 2, with the mean pallidal soma volume for the grade 2-3 group reduced by 24%**. However, despite the lack of statistical significance, this trending decline in pallidal neuron soma volume coincides with striatal pathology.
Figure 6.17 Mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) in HD cases grouped according to striatal neuropathological grade compared to control cases

The graph shows the variation in mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) in 8 control brains compared with 3 HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined using design-based stereological methods involving the *Isotropic Nucleator*. Each blue diamond indicates mean cell soma volume of parvalbumin-positive pallidal neurons in the GPI for each control case, each red diamond indicates the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPI for each grade 0-1 HD case, and each green diamond indicates the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPI for each grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 17% reduction in the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPI in HD grade 0-1, compared to control, though this was not statistically significant ($P > 0.99$). By HD grade 2-3, a 24% reduction in mean cell soma volume of parvalbumin-positive pallidal neurons was found compared to control. However this was not significant ($P = 0.157$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn’s multiple comparisons post-test. See Table 6.3 for detailed results.
6.4.3 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPi) compared with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration (Table 6.6, Figure 6.18)

It was next investigated whether the pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the GPi was compared with CAG repeat length in the HD gene, post-mortem delay, post-mortem brain weight, age of death, age of disease onset, and disease duration (taken from the difference between age of death and age of onset), in both control and HD cases for which all this information was available (Figure 6.18). In general terms, no significant correlation was found between the mean cell soma volume of parvalbumin-positive pallidal neurons and CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration for all HD and control cases.

6.4.4 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPi) compared with total GPi volume, pallidal neuron number, and pallidal neuron density (Table 6.6, Figure 6.19)

It was also explored whether the cell soma volume changes of parvalbumin-positive pallidal neurons in the GPi was compared with overall GPi volume, total pallidal neuron number, and pallidal neuron density in both control and HD cases as determined using design-based stereology (Figure 6.19). In general terms, the mean cell soma volume of parvalbumin-positive pallidal neurons were smaller in control cases with a greater total number of Nissl-positive pallidal neurons (panel B), which was statistically significant according to a two-tailed Spearman’s correlation analysis ($r = -0.74$, $*P = 0.046$). No significant correlation was found between mean pallidal neuron soma volume and either overall GPi volume or the density of pallidal neurons for all control and HD cases.
This figure shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI), with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset and disease duration for all control and HD cases which have the information available. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

**A:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the CAG repeat number in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.25, P = 0.55$).

**B:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the post-mortem delay in control ($r = -0.07, P = 0.85$) and HD ($r = -0.23, P = 0.57$) cases, according to a two-tailed Spearman’s correlation analysis.

**C:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the post-mortem brain weight in control ($r = 0.09, P = 0.92$) and HD ($r = -0.64, P = 0.14$) cases according to a two-tailed Spearman’s correlation analysis.

**D:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the age of death for control ($r = 0.26, P = 0.54$) and HD ($r = -0.30, P = 0.45$) cases, according to a two-tailed Spearman’s correlation analysis.

**E:** There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and the age of disease onset in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.20, P = 0.60$).

**F:** There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and the age of disease onset in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.36, P = 0.36$).
Figure 6.18 Comparison between mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) and CAG repeat length, *post-mortem* (PM) delay, brain weight, age of death, age of disease onset, and disease duration in both HD and control cases.

**A** HD (n=8)  
$r = 0.25$  
$P = 0.55$

**B** HD (n=8)  
$r = -0.23$  
$P = 0.57$

**C** HD (n=7)  
$r = -0.64$  
$P = 0.14$

**D** HD (n=8)  
$r = -0.30$  
$P = 0.26$

**E** HD (n=8)  
$r = -0.20$  
$P = 0.60$

**F** HD (n=8)  
$r = -0.36$  
$P = 0.36$
Figure 6.19 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) and overall GPI volume, total pallidal neuron number, and pallidal neuron density in both HD and control cases.

This figure shows the correlation of the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI), with overall GPI volume, total pallidal neuron number, and pallidal neuron density for all control and HD cases, as determined using stereology. Each triangular symbol indicates a control case, and each square symbol indicates an HD case.

A: There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and overall GPI volume in control ($r = -0.38, P = 0.36$) and HD ($r = 0.38, P = 0.36$) cases, according to a two-tailed Spearman’s correlation analysis.

B: An inverse correlation was found between mean cell soma volume of parvalbumin-positive pallidal neurons and the total number of Nissl-positive pallidal neurons in the GPI of control cases only, which was statistically significant according to a two-tailed Spearman’s correlation ($r = -0.74, *P = 0.046$). No significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the total number of Nissl-positive pallidal neurons in HD cases ($r = 0.26, P = 0.54$).

C: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the density of pallidal neurons in control ($r = -0.12, P = 0.79$) and HD ($r = -0.12, P = 0.79$) cases, according to a two-tailed Spearman’s correlation analysis.
6.4.5 The pattern of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) compared with symptomatology and clinical assessments (Table 6.6, Figure 6.20, 6.21)

It was next investigated whether cell soma volume changes of parvalbumin-positive pallidal neurons in the GPI was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh).

Figure 6.20 shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI) with Total Functional Capacity, Mini-Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. In general terms, no significant correlation was found between the mean cell soma volume of parvalbumin-positive pallidal neurons and all variables examined.

Figure 6.21 shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI), with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale motor and chorea scores for 5 HD cases. In general terms, no significant correlation was found between the mean cell soma volume of parvalbumin-positive pallidal neurons and all variables examined.

6.4.6 Overall summary of cell soma volume changes of parvalbumin-positive pallidal neurons in the globus pallidus internal segment compared with all variables (Table 6.6)

A summary of the comparisons between the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPI for all cases and all variables discussed in sections 6.4.3, 6.4.4 and 6.4.5 is highlighted in Table 6.6.
Figure 6.20 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the HD globus pallidus internal segment (GPi), with measures of Total Functional Capacity, cognition and mood

This figure shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPi), with Total Functional Capacity, Mini Mental State Examination score, Mood Hospital Anxiety scale, Mood Hospital Depression Scale, Outward Irritability score (irritability expressed towards others), and Inward Irritability score (irritability directed towards oneself) for 5 HD cases. Each square symbol indicates an HD case.

**A:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Total Functional Capacity in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.30$, $P = 0.68$).

**B:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and Mini-Mental State Examination scores in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.30$, $P = 0.68$).

**C:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Hospital Anxiety scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.50$, $P = 0.45$).

**D:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Hospital Depression scale in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.05$, $P = 0.90$).

**E:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the outward irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.50$, $P = 0.45$).

**F:** There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the Inward Irritability score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.00$, $P = 0.80$).
Figure 4.20 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the HD globus pallidus internal segment (GPI), with measures of Total Functional Capacity, cognition and mood.

A. HD (n=5)
- $r = -0.30$
- $P = 0.68$

B. HD (n=5)
- $r = -0.30$
- $P = 0.68$

C. HD (n=5)
- $r = -0.50$
- $P = 0.45$

D. HD (n=5)
- $r = -0.05$
- $P = 0.90$

E. HD (n=5)
- $r = 0.50$
- $P = 0.45$

F. HD (n=5)
- $r = 0.00$
- $P = 0.80$
Figure 6.21 Comparison between the mean cell soma volume of parvalbumin-positive pallidal neurons in the HD globus pallidus internal segment (GPI) with clinical measures of motor impairment and chorea.

This figure shows the correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons in the globus pallidus internal segment (GPI), with the Quantitative Neurological Exam motor impairment and chorea scores, in addition to the Unified Huntington’s Disease Rating Scale (UHDRS) motor and chorea scores for 5 HD cases. Each square symbol indicates an HD case.

A: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and QNE motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.30, P = 0.68$).

B: There was no significant correlation between mean cell soma volume of parvalbumin-positive pallidal neurons and the QNE chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.40, P = 0.52$).

C: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the UHDRS motor impairment score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = 0.30, P = 0.68$).

D: There was no significant correlation between the mean cell soma volume of parvalbumin-positive pallidal neurons and the UHDRS chorea score in HD cases according to a two-tailed Spearman’s correlation analysis ($r = -0.56, P = 0.30$).
Table 6.6 Summary of comparisons between the mean cell soma volume of parvalbumin-positive pallidal neurons in the GPi and all variables examined for all HD and control cases with the information available

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample size (n)</th>
<th>Relationship</th>
<th>( r ) value (GPi pallidal neuron soma volume)</th>
<th>Two-tailed ( P ) value (GPi pallidal neuron soma volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD CAG repeat length</td>
<td>- 8</td>
<td>Direct</td>
<td>0.25</td>
<td>0.55</td>
</tr>
<tr>
<td>PM delay (hours)</td>
<td>8 8</td>
<td>Inverse</td>
<td>-0.07</td>
<td>-0.23 0.85 0.57</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>6 7</td>
<td>Direct</td>
<td>0.09</td>
<td>-0.64 0.92 0.14</td>
</tr>
<tr>
<td>Age of death (years)</td>
<td>8 8</td>
<td>Inverse</td>
<td>0.26</td>
<td>-0.30 0.54 0.45</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>- 8</td>
<td>Inverse</td>
<td>-0.20</td>
<td>0.60</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>- 8</td>
<td>Inverse</td>
<td>-0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>Overall GPI volume (mm(^3))</td>
<td>8 8</td>
<td>Inverse</td>
<td>Direct -0.38</td>
<td>0.38 0.36 0.36</td>
</tr>
<tr>
<td>Total number of Nissl-positive pallidal neurons</td>
<td>8 8</td>
<td>Inverse</td>
<td>Direct -0.74</td>
<td>0.26 *0.046 0.54</td>
</tr>
<tr>
<td>Density of Nissl-positive pallidal neurons</td>
<td>8 8</td>
<td>Inverse</td>
<td>Direct -0.12</td>
<td>0.79 0.79</td>
</tr>
<tr>
<td>Total Functional Capacity</td>
<td>- 5</td>
<td>Inverse</td>
<td>-0.30</td>
<td>0.68</td>
</tr>
<tr>
<td>Mini-Mental State Examination score</td>
<td>- 5</td>
<td>Inverse</td>
<td>-0.30</td>
<td>0.68</td>
</tr>
<tr>
<td>Mood Hospital Anxiety Scale</td>
<td>- 5</td>
<td>Inverse</td>
<td>-0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>Mood Hospital Depression Scale</td>
<td>- 5</td>
<td>Inverse</td>
<td>-0.05</td>
<td>0.90</td>
</tr>
<tr>
<td>SNAITH Outward Irritability Score</td>
<td>- 5</td>
<td>Direct</td>
<td>0.50</td>
<td>0.45</td>
</tr>
<tr>
<td>SNAITH Inward Irritability Score</td>
<td>- 5</td>
<td>none</td>
<td>0.00</td>
<td>0.80</td>
</tr>
<tr>
<td>QNE Motor Impairment score</td>
<td>- 5</td>
<td>Direct</td>
<td>0.30</td>
<td>0.68</td>
</tr>
<tr>
<td>QNE Chorea Score</td>
<td>- 5</td>
<td>Inverse</td>
<td>-0.40</td>
<td>0.52</td>
</tr>
<tr>
<td>UHDRS Motor Assessment Score</td>
<td>- 5</td>
<td>Direct</td>
<td>0.30</td>
<td>0.68</td>
</tr>
<tr>
<td>UHDRS Chorea Assessment Score</td>
<td>- 5</td>
<td>Inverse</td>
<td>-0.56</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*denotes a significant correlation according to a two-tailed Spearman’s correlation analysis: *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \). Correlations with regression values close to +1.0 and -1.0 are highlighted in red.
6.5 The density of pallidal neurons in the globus pallidus internal segment in Huntington’s disease

In order to study pallidal neuron density in the globus pallidus internal segment in detail, the stereological cell counting data obtained using the Optical Fractionator (the total number of Nissl-positive pallidal neurons, see 6.3.1) was divided by the GPi volume estimate data obtained using the Cavalieri Estimator (GPi volume in mm$^3$, see 6.2.1) in order to obtain a density estimate of pallidal neurons per mm$^3$. The pallidal neuron density measures were obtained for 8 Huntington’s disease (HD) and 8 control cases.

6.5.1 The density of pallidal neurons in the globus pallidus internal segment in all HD and control cases (Table 6.7, Figure 6.22)

After assessment of pallidal neuron density in the GPi, the mean cell density of Nissl-positive pallidal neurons was increased by 28%, when all 8 HD cases are grouped together and compared with 8 controls (Figure 6.22). The mean cell density ± SEM of 8 controls was 441 ± 40 cells/mm$^3$ compared to the mean cell density ± SEM of all 8 HD cases which was 566 ± 50 cells/mm$^3$ (Table 6.7). Despite the considerable increase, the mean changes in cell density in HD compared to control were not significant according to the two-sided Mann-Whitney test, with the $P$-value being $> 0.05$ ($P = 0.06$). It is likely that high levels of variability within the control and HD cohorts are contributing to a lack of significance, as highlighted by the large standard deviations amongst control (SD = 127 cells) and HD (SD = 147 cells) cohorts (Table 6.7).

Table 6.7 Variation in mean cell density of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPi) between control and neuropathological grades in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HD (all grades combined)</th>
<th>HD grade 0-1</th>
<th>HD grade 2-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell density of Nissl-positive pallidal neurons (cells/mm$^3$)</td>
<td>441</td>
<td>566</td>
<td>564</td>
<td>568</td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
<td>127</td>
<td>141</td>
<td>213</td>
<td>109</td>
</tr>
<tr>
<td>Standard error of the mean (SEM)</td>
<td>45</td>
<td>50</td>
<td>123</td>
<td>49</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Percentage reduction (%) as compared to control</td>
<td>-</td>
<td>28% increase</td>
<td>28% increased</td>
<td>29% increased</td>
</tr>
</tbody>
</table>

*denotes significant difference compared with control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$
Figure 6.22 Mean cell density of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) in all HD cases compared to control cases in the human brain

The graph shows the variation in mean cell density of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) in 8 HD and 8 control brains, determined from data obtained using the Optical Fractionator (total number of cells) divided by the data obtained using the Cavalieri Estimator (overall volume in mm$^3$) for each case. Each blue diamond indicates the cell density of Nissl-positive pallidal neurons in the GPI (cells/mm$^3$) for each control case, and each red diamond indicates the cell density of Nissl-positive pallidal neurons in the GPI (cells/mm$^3$) for each HD case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. **The graph shows a 28% increase in mean cell density of Nissl-positive pallidal neurons in the GPI in Huntington's disease**, although this was not significant based on a 2-tailed $P$-value from the Mann-Whitney test ($P = 0.06$). See Table 6.7 for detailed results.
6.5.2 The pattern of pallidal neuron density changes in the globus pallidus internal segment in relation to striatal neuropathological grade (Table 6.7, Figure 6.23)

In order to investigate the relationship between pallidal neuron density in the GPi and striatal neuropathology, the HD case results were grouped according to striatal neuropathological grade and compared to the control group. After assessment of the density of pallidal neurons in the GPi, the data shows a 28% increase in the mean density of Nissl-positive pallidal neurons in the GPi in grade 0-1 HD, when compared with 8 controls (Figure 6.23). The mean ± SEM of 8 controls was 441 ± 45 cells/mm\(^3\), compared to the mean ± SEM of the grade 0-1 cases which was 564 ± 123 cells/mm\(^3\) (Table 6.7). However, the mean changes in cell density of pallidal neurons in HD grade 0-1 compared to control were not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted \(P\)-value of > 0.05 (\(P = 0.341\)). It was also important to highlight the extreme variability amongst the grade 0-1 HD cases, with an SD of 213 cells/mm\(^3\), which is considerably higher than the SD of the control cohort. Analysis of the grade 2-3 group showed a 29% increase in the density of pallidal neurons compared to 8 controls (Figure 6.23), highlighted by the mean ± SEM of 568 ± 49 cells/mm\(^3\) (Table 6.7). This increase in pallidal neuron density in HD grade 2-3 compared to control was not significant according to the Kruskal-Wallis test, with a Dunn’s multiple comparisons post-test, giving a multiplicity adjusted \(P\)-value of > 0.05 (\(P = 0.187\)).

In summary, it was observed that pallidal neuron density did not clearly correlate with striatal neuropathological grade, as the increase in density in HD observed did not increase progressively with more advanced striatal pathology. Although the increase in pallidal neuron density was not statistically significant at grade 0-1 or grade 2-3 compared to control, this could be due to the large variation within each cohort.
The graph shows the variation in mean cell density of Nissl-positive pallidal neurons in the globus pallidus internal segment (GPI) in 8 control brains compared with three HD brains of neuropathological grade 0-1, and 5 HD brains of neuropathological grade 2-3, determined from data obtained using the Optical Fractionator (total number of cells) divided by the data obtained using the Cavalieri Estimator (overall volume in mm$^3$) for each case. Each blue diamond indicates the cell density of Nissl-positive pallidal neurons in the GPI (cells/mm$^3$) for each control case, each red diamond indicates the cell density of Nissl-positive pallidal neurons in the GPI (cells/mm$^3$) for each HD grade 0-1 case, and each green diamond indicates the cell density of Nissl-positive pallidal neurons in the GPI (cells/mm$^3$) for each HD grade 2-3 case. Individual case numbers are shown beside each data point. The mean ± SEM for each data set is highlighted with a solid line accompanied with error bars. The graph shows a 28% increase in the mean cell density of Nissl-positive pallidal neurons in the GPI in HD grade 0-1, compared to control, though this was not statistically significant ($P = 0.341$). By HD grade 2-3, a 29% increase in mean cell density of Nissl-positive pallidal neurons in the GPI was found compared to control, but was not statistically significant ($P = 0.187$) based on a multiplicity adjusted $P$-value from a Kruskal-Wallis test combined with Dunn’s multiple comparisons post-test. See Table 6.7 for detailed results.
6.6 Discussion

This chapter provides a discussion of the results presented in this thesis on the degeneration of the globus pallidus internal segment (GPI) in Huntington’s disease (HD), using histology, immunohistochemistry, and unbiased design-based stereological techniques. Three different stereological probes were used to measure GPI volume, pallidal neuron loss, and pallidal soma volume. These results show that there was a significant reduction in overall GPI volume (40%), accompanied with a minor loss of pallidal neurons (19%), and minor reduction in pallidal cell soma volume (21%), when all HD cases were grouped together \(N = 8\) and compared with control cases \(N = 8\) (Figures 6.1, 6.8, 6.15). It is important to note that these changes in GPI volume, pallidal neuron number and soma volume occurred at a far lesser degree in comparison to the lateral counterpart of the GPI, the external segment (Chapter 4, GPe). However, a notable point of difference can be seen in the density of remaining pallidal neurons in the GPI, which was found in this study to increase by 28% when all HD cases were compared with control.

6.6.1 The pattern of volumetric changes in the globus pallidus internal segment in Huntington’s disease using the Cavalieri Estimator

The results of stereological volume quantification using the Cavalieri Estimator shows that there was a significant 40% reduction in the mean volume of the GPI in HD, when all 8 HD cases are grouped together and compared with 8 controls \(***P = 0.0006\) (Figure 6.1). This finding is consistent with findings in other post-mortem studies (Lange et al., 1976, de la Monte et al., 1988, Mann et al., 1993) although none of these studies used design-based stereological techniques. Out of these post-mortem studies mentioned, Lange et al reported a 50% decline in the fresh volume of the GPI which reinforces the findings of this study (Lange et al., 1976). One study used stereological techniques to delineate the external and internal pallidum segments separately (although detailed delineation with immunohistochemical techniques were not carried out), and used the Cavalieri Estimator protocol to obtain a volume estimate (Halliday et al., 1998). This study reported a 21% reduction in mean GPI volume based on analysis of 7 HD cases. It can be noted that although Halliday et al reported a smaller degree of GPI atrophy compared to this study, it is most likely due to methodological variation, as Halliday et al did not use immunohistochemical-marker based delineation of boundaries which was used in this study,
and also prepared 3-5mm based thick sections for overall volume determination (compared to 70 µm thick sections used in this study). What is important to note is that Halliday et al reported a smaller degree of GPi atrophy (21%) in HD compared to the GPe (42%), which is generally consistent with our detailed results.

Region-of-interest based MRI studies have also reported a loss of volume in the globus pallidus of HD patients (Aylward et al., 1997, Rosas et al., 2003, Fennema-Notestine et al., 2004). However, delineating the GPi boundaries and obtaining a separate measurement of volume for this region using ROI-based MRI has not been currently achieved. One such study using ROI-based combined with voxel based morphometry (VBM), reported a 56% loss in mean volume of the globus pallidus, demonstrating that improvements in MRI can illustrate pallidal atrophy in a non-invasive manner (Douaud et al., 2006). ROI-based MRI combined with VBM is operator dependent and time-consuming, and although the globus pallidus was observed and quantified using this method, there is currently no way to manually distinguish the GPi from the GPe, thereby restricting the amount of data available using this technique. This reinforces the importance of detailed post-mortem tissue studies of regional volume, particularly small regions such as the GPi, which is currently not able to be accurately isolated using in vivo imaging methods.

The major reduction in GPi volume, unlike the GPe, is more likely to be due to neuropil loss, resulting from striatal fibres and terminals, and to a lesser extent the loss of neurons (Reiner et al., 1988, Albin et al., 1990, Storey and Beal, 1993). This is supported by the findings in this study, with only a minor 19% reduction in pallidal neurons found overall in the HD cohort studied. Therefore, the loss of striatal projections into the GPi must be a key contributor to the dramatic 40% loss of volume. It has been thought that the loss of white matter traversing the GP might be a contributor to the reduction in overall GPi volume in HD. Many bundles of myelinated fibres traverse the GP in fresh preparations, accounting for a paler colour than the neighbouring striatum (Nieuwenhuys et al., 2008). And as highlighted by Douaud et al, the atypical striatal and pallidal increase in fractional isotropy (FA), as determined using diffusive tensor imaging (DTI), was concurrent to a decrease of the dispersion of the fibre orientation, unambiguously characterising a preferential loss of connections along specific radiating directions from these structures, while others are comparatively spared (Douaud et al., 2009). Further DTI analysis of striatal and pallidal white matter tracts by the same group, revealed that striato-pallidal projections are most affected compared to other subcortical projections. Mean
diffusivity (MD), a value which increases in pathological tissue, was observed to be higher in the pallidum, and was confirmed by other recent in vivo DTI imaging studies of HD symptomatic patients (Rosas et al., 2006, Seppi et al., 2006). This increase in MD was either attributable to the loss of projections from striatal spiny neurons (Douaud et al., 2009), or to neuronal shrinkage or depletion within the pallidum itself (Lange et al., 1976). Since this study has found a minimal decline in neuronal depletion within the GPi, it is more likely that the loss of striato-pallidal projections are accounting for the volumetric decline.

6.6.2 The pattern of volumetric changes in the globus pallidus internal segment correlated with striatal neuropathological grade

The results of stereological volume quantification using the Cavalieri Estimator, shows that there is a 29% reduction in the mean volume of the GPi in grade 0-1 HD, when compared with 8 controls. However, a clear statistical association was not evident ($P > 0.05$) and this is likely to be due to the high levels of variability amongst the grade 0-1 cohort. Analysis of the grade 2-3 group showed a 46% reduction in mean GPi volume compared to 8 controls ($**P = 0.003$) (Figure 6.2).

The progressive volume reduction of the GPi with advancing HD grade could mirror the pace of striato-GPi projection loss in HD. While many striatal projection neurons collateralize on to more than one target, striatal projection neurons can be subdivided into four major populations based on their primary projection target. One of the four includes those projecting mainly to the GPi, which are rich in substance-P but poor in enkephalin, and have cell bodies located in the striatal matrix compartment (Mai et al., 1986, Reiner et al., 1988, Graybiel, 1990, Deng et al., 2004). The boundaries of the GPi for volumetric measurements in this study were outlined with substance-P immunoreactivity (see Chapter 3, Materials and Methods). Within the GPi, substance-P immunoreactivity is localised in terminals outlining the dendritic tree of pallidal neurons (Allen et al., 2009). In a study by Deng et al, quantification of substance-P immunolabelled terminals within the GPi, in a sample of HD cases ranging from grade 0-4, highlighted that the loss of striatal projections to the GPi proceeded far more gradually than the loss of enkephalin immunolabelled striatal terminals to the GPe (Deng et al., 2004). By HD grade 1, only 29.1% of substance-P positive fibres in the GPi were lost compared to control (in the GPe, 66.4% of enkephalin was lost at the same stage) (Deng et al., 2004). By HD grade 3,
46.9% of substance-P positive fibres in the GPi were lost compared to control (in the GPe, 76.7% of enkephalin positive fibres were lost at the same stage).

These results have been confirmed by a previous study from our group (Allen et al., 2009), that this substance-P immunoreactivity, a characteristic peptide marker of striato-pallidal terminals, described as “woolly fibres” in earlier studies (Haber and Elde, 1981), is progressively reduced in HD grades from 0-4 in the GPi. Density analysis of these substance-P terminals in the GPi showed an average 8% reduction at grades 0-1, 11% for grade 2, and 43% for grades 3-4 compared to control (Allen et al., 2009). This grade-wise reduction in substance-P positive striatal projections is much more subtle compared to the 23% reduction at grades 0-1, 33% for grade 2, and 59% for grades 3-4 as seen with enkephalin in the GPe (Allen et al., 2009).

These immunohistochemical findings support the notion that the striatal neurons, which preferentially project to the GPi (containing substance-P positive efferent terminals) are in fact less vulnerable in HD than other striatal projection neuron types, in particular, the enkephalin positive projection neurons into the GPe. As mentioned in Chapter 4, the overall volume of the GPe, as delineated using enkephalin immunoreactivity, was shown to decline by 45% at grade 0-1, and by 60% at grade 2-3 compared to controls. These changes were shown to mirror the greater decline in enkephalin immunoreactivity with greater striatal neuropathological grade, which reinforced the notion that the enkephalin-containing indirect pathway projecting from the striatum to the GPe is involved earlier and more predominantly than the substance-P/dynorphin-containing direct pathway to the GPi (Reiner et al., 1988, Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009). Therefore, it is likely that the pattern of GPi atrophy and the relationship with striatal neuropathology mirrors the progressive grade-wise degeneration of striatal projections immunoreactive for substance-P to the GPi. Furthermore, the resistance of striato-GPi projections to HD degeneration, compared to the greater vulnerability of striato-GPe projections, results in a lesser degree of subsequent grade-wise GPi atrophy compared to its lateral counterpart.
6.6.3 The relationship between the pattern of volumetric changes in the globus pallidus internal segment with CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, and disease duration

This study also investigated the differences in CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset and disease duration, which may also be contributors to the dramatic volumetric reduction of the GPi. In general terms, no significant correlation was found between GPe total volume and all variables considered for all control and HD cases.

Striatal pathology and volume loss has shown to be closely associated with the number of CAG repeats in HD (Penney et al., 1997, Rosas et al., 2001, Vonsattel et al., 2008). However, as shown by the lack of statistical association between CAG repeat length and GPi volume, it appears that the reduction in GPi volume does not follow the same association according to Spearman’s correlation analysis ($P > 0.05$). The analysis of post-mortem delay showed no overall correlation with GPi volume for both HD and control cases, thereby suggesting that the differences in time interval between death and tissue processing did not influence the volume reduction of the 8 HD cases in this study.

It has been long known that the overall brain weight of HD patients is decreased compared to control (Vonsattel et al., 1985, de la Monte et al., 1988, Vonsattel et al., 2008). Therefore, it was important to evaluate the relationship between brain weight and GPi volume. There was no significant correlation between the total volume of the GPi and the post-mortem brain weight in control or HD cases ($P > 0.5$). This suggests that unlike the GPe (Chapter 4), the extent in which the GPi degenerates in volume does not coincide with extent of overall brain weight loss. Halliday et al analysed the proportional volume (i.e. the regional volume expressed as a percentage of the total cerebrum volume in HD cases compared with control), and found that with a significant 42%, 33% and 30% reduction in proportional atrophy of the caudate, putamen and GPe respectively, the proportional volume of the GPi was not selectively reduced in HD, highlighting that the GPi does not undergo greater proportional atrophy in HD than the cerebrum as a whole, and therefore is not a major contributor to brain weight reduction (Halliday et al., 1998).

Quantitative analysis of several post-mortem and neuroimaging studies analysed by Raz et al, (Raz et al., 1995), have suggested that there are age-related declines in the volume of the
striatum. However, there is no data available on the aging of the globus pallidus. Rosa’s et al demonstrated a correlation between striatal volume and normal aging, although there was no information provided about neighbouring structures (Rosas et al., 2001). The correlation analysis of age of death with GPi volume showed no overall relationship for both control and Huntington’s cases ($P > 0.05$), suggesting that age of death does not relate to volume decline. There was also no correlation between GPi volume and age of disease onset, or disease duration ($P > 0.05$) (Figure 4.4). The lack of association between GP volume decline and both disease onset and duration has also been noted in a longitudinal MRI study (Aylward et al., 1997), reinforcing that the GPi, when isolated from the rest of the pallidum, also has no association.

6.6.4 The pattern of volumetric changes in the globus pallidus internal segment compared with pallidal neuron number, average pallidal soma volume, and pallidal neuron density

This study investigated whether the total volume of the GPi, was correlated with the other stereological variables measured, including total pallidal neuron number, average pallidal soma volume, and pallidal neuron density in both control and HD cases. **In general terms, the overall GPi volume decreased with pallidal neuron number for HD cases only**, which was statistically significant (**$P = 0.005$**). A recent study by Guo et al found a trend for correlation between the overall volume of the putamen and the number of DARPP32 positive spiny neurons in HD post-mortem tissue using design based stereology. Their results suggested that loss of DARPP32 positive spiny neurons contributes, at least in part, to the atrophy of the putamen (Guo et al., 2012). Since the GPi is an output structure of the putamen, it is possible that the minor loss of pallidal neurons found in the GPi in this study, could also be a contributor to part of the GPi atrophy, as inferred by Guo et al with reference to the putamen.

No significant correlation was found between GPi total volume and average pallidal soma volume for both control and HD cases ($P > 0.05$), thereby suggesting that the cell soma volume of pallidal neurons does not majorly contribute to the overall volume of the GPi, based on a lack of statistical association. The density of pallidal neurons in control cases were found to increase with lower GPi volumes ($P = 0.046$). However this relationship was not seen in the HD cohort ($P > 0.05$), suggesting that the disease alters the relationship between the density of pallidal neurons and GPi volume.
6.6.5 The relationship between the volumetric changes in the globus pallidus internal segment with symptomatology and clinical assessments

It was also investigated whether the total volume of the GPi was correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. The symptom information of the HD cases presented in this study were carefully examined independently by two neuropsychologists (Dr L. Tippett and V. Hogg), with motor examinations carried out by a neurologist (Dr R. Roxburgh). Their research have previously contributed to other HD neuropathological studies from our lab on the striatum and cerebral cortex, published in *Brain* (Tippett et al., 2007, Thu et al., 2010).

In general terms, no significant correlation was found between GPi total volume and Total Functional Capacity, Mini-Mental State Examination score, Mood Hospital Anxiety scale and Mood Hospital Depression Scale ($P > 0.05$). **However, the overall GPi volume increased with both of the SNAITH outward ($r = 0.9$, $P = 0.08$) and inward ($r = 0.95$, $P = 0.07$) irritability scores**, although these correlations were not statistically significant despite a highly positive regression value, indicative of a strong trend. In HD, irritability has been reported in over 50% of patients (Paulsen et al., 2001, Nimmagadda et al., 2011). Recent studies have also suggested that psychiatric symptoms such as irritability may be the first manifestation of HD in up to 79% of patients (Morris and Scourfield, 1996), with irritability also being reported in the prediagnostic phase (Walker, 2007, Klöppel et al., 2010).

The outwardly directed irritability scale is a 9 point questionnaire to assess whether a person will attack, or fear they will attack another person physically or verbally. The inward directed irritability assessment is also a 9 point scale, although this assessment is based on the patient’s self-report on the degree to which the patient felt angry with themselves and gave vent to this irritation by cursing, name calling, self-assault to the extent of causing pain and injury, or thoughts of self-harm (Snaith et al., 1978, Snaith and Taylor, 1985). Although the GPi has strong interactions with the motor cortices, this region also has an association with the limbic system (Nambu, 2007). The significance of this association with regard to irritability and GPi neurodegeneration will be discussed in Chapter 7. Since GPi volume was shown to be highly correlated with irritability in HD, this is reinforcing the importance of the GPi in limbic system and mood related pathways.
As the GPi is a final output station of the basal ganglia through which body movements are controlled, it was essential to look at the relationships between GPi volume and motor symptoms. In general terms, there was no significant correlation between GPi volume and both Quantitative Neurological Exam (QNE) and Unified Huntington’s Disease Rating Scale (UHDRS) scores of motor impairment or chorea (Figure 6.7). This was an unexpected result, as the volume of its lateral counterpart, the GPe, was shown to inversely correlate with HD motor impairment (Chapter 4). Thus, it can be inferred that unlike the putamen atrophy which has been shown to correlate with motor impairment (Guo et al., 2012), and unlike its lateral counterpart reflecting this same trend, GPi atrophy does not correlate with motor impairment or chorea.

6.6.6 The pattern of pallidal neuron loss in the globus pallidus internal segment in Huntington’s disease using the Optical Fractionator

The results of stereological pallidal neuron quantification, using the Optical Fractionator, shows that there is a minor 19% reduction in the mean total pallidal neuron number in the GPi in HD, when all 8 HD cases are grouped together and compared with 8 controls ($P = 0.19$) (Figure 6.8). It was also interesting to note that there was considerable variation in the total number of pallidal neurons in the GPi within the HD cohort, with a large spread of data points supported by a large range and SD, thus highlighting the variation in the extent of pallidal neuron loss within the group. This group variability is the rationale behind striatal neuropathological grade comparisons within the HD cohort (see section 6.6.7).

There is conflicting evidence as to whether or not pallidal neuron loss plays a role in the considerable volume reduction of the GPi in HD, as most studies suggest that GPi atrophy is apparently chiefly due to the loss of neuropil and hence striatal fibres and terminals, and to a lesser extent the loss of neurons (Reiner et al., 1988, Albin et al., 1992, Storey and Beal, 1993, Vonsattel et al., 2008). However, these studies have concentrated on striatopallidal afferents rather than pallidal neuron degeneration. Only a few investigators, including Lange et al and Roos et al, concluded that neuronal depletion occurs in the GPi in HD and therefore GPi atrophy should be attributed not only to striato-pallidal fibre loss, but also pallidal neuron loss (Lange et al., 1976, Roos, 1986).

Lange et al found a 43% decrease in absolute number of pallidal neurons in the GPi based on a non-stereological morphometric study carried out on 6 HD post-mortem brains of unreported
grades (Lange et al., 1976). Based on this data, Lange et al suggested that pallidal neuronal loss was also due to a cell autonomous degenerative process, rather than solely the consequence of striatal degeneration (Lange et al., 1976). However, in comparison to this, a more recent non-stereological histometrical study by Wakai et al concluded that no neuronal depletion was recognised in the GPi in HD based on a total pallidal neuron count of 5 coronal sections at 5 GP levels of 6 HD cases, despite considerable GPi atrophy (Wakai et al., 1993). Both studies have reported an increase in the density of pallidal neurons in the GPi, however Lange et al concluded it was erroneous to state that increase in pallidal neuron density means that neurons are relatively preserved while GPi tissue bulk decreases, simply because pallidal neurons were lost according to their study (Lange et al., 1976). Whereas, in comparison, Wakai et al concluded that a lack of neuron loss, combined with an increase in pallidal neuron density, means that a decrease in neuropil is responsible for GPi atrophy (Wakai et al., 1993). This study, which used design-based stereology, a technique which was neither employed by Lange et al or Wakai et al, highlights that there was a 19% minor loss of pallidal neurons in the GPi, which supports Lange et al’s claims of cell loss being present. However, the extent of pallidal neuron loss in HD was minor in the GPi despite a 40% decline in volume, and therefore supports Wakai et al’s hypothesis of striato-pallidal fibre loss being the main contributor to GPi atrophy. Further discussion for Wakai et al’s hypothesis will be highlighted in section 6.6.14 in the discussion of pallidal neuron density.

The minor loss of pallidal neurons expands on previous reports that the substance-P enriched projection neurons from the striatum to the GPi (direct pathway) are more resistant to HD pathogenesis until mid-late neuropathological grades, compared to striatal-GPe enkephalinergic and striatal-nigral projections which are more severely affected in the early and middle grades of HD (Graveland et al., 1985, Reiner et al., 1988, Glass et al., 2000, Deng et al., 2004). This also reflects on previous findings in this study (Chapter 4), where pallidal neurons in the GPe that receive input from striato-GPe enkephalinergic projection neurons were reduced by a striking 59% in HD. This highlights the greater vulnerability of the striato-GPe pathway which is well linked with progressive loss of enkephalin fibres from presymptomatic HD to grade 4; whereas there is no noteworthy loss if substance-P from the GPi at presymptomatic HD or HD grade 0 (Hedreen and Folstein, 1995, Deng et al., 2004, Allen et al., 2009).
6.6.7 The relationship between pallidal neuron loss in the globus pallidus internal segment with striatal neuropathological grade

After grouping the results of stereological pallidal neuron quantification using the *Optical Fractionator* based on striatal neuropathological grade, the data present showed that **there is a negligible 2% reduction in the mean total number of pallidal neurons in the GPi in grade 0-1 HD**, when compared with 8 controls (Figure 6.9). However, the mean changes in total cell number in HD grade 0-1 were not significant due to high case variability and a limited sample size ($P > 0.05$). **Analysis of the grade 2-3 group showed a 29% reduction in the mean total number of pallidal neurons** compared to 8 controls (Figure 6.9), although this was not statistically significant ($P = 0.12$). Therefore, the total number of pallidal neurons in the GPi decreased with increased HD striatal neuropathological grade. However, with a small cohort of HD cases in the grade 0-1 group, combined with high cohort variability, the reduction observed is minor. This reduction was greater beyond grade 2, suggesting that pallidal neuron loss occurs in the GPi at more advanced striatal neuropathological grades.

The minimal loss of pallidal neurons in the GPi at grade 1, with subsequent loss at higher grades (> grade 2), could be a by-product of the more gradual loss of substance-P striatal-GPi afferents compared to the earlier, more extensive loss of enkephalin positive striatal-GPe afferents. As mentioned in Chapter 4, at the same grades of striatal neuropathology, a 37% loss of pallidal neurons was observed in the GPe at grade 0-1, and a 72% loss at grade 2-3, highlighting the greater vulnerability of GPe pallidal neurons which receive input predominantly from striatal-GPe enkephalinergeric afferents. Several studies have investigated neuropeptides and additional markers of striatal neurons and their terminals to determine which projection pathways are more susceptible to the disease, with the conclusion that striatal-GPi projections are relatively preserved at early HD grades, with loss occurring at late grades.

By HD grade 1, the loss of striatal terminals in the GPi is minimal, with substance-P immunolabelled fibres being 70-80% of control abundance (Sapp et al., 1995, Deng et al., 2004). This has been reinforced by the minimal loss of additional markers of striatal neurons and their terminals including complete preservation of D1 dopamine receptors in the GPi at grade 1, and greater preservation of cannabinoid receptors in the GPi compared to the GPe (Glass et al., 2000, Allen et al., 2009). In contrast, enkephalin immunolabelled fibres are reduced to about 35% of control abundance in the GPe in grade 1 (Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009).
combined with a 90% loss of D2 dopamine and A2a adenosine receptors (localised to enkephalin positive neurons) 75% loss of striatal cannabinoid receptors, 50% loss of striatal D1 dopamine receptors, and near complete depletion of D2 and A2a receptors from the GPe (Glass et al., 2000, Allen et al., 2009). These findings are consistent with relative preservation of the striatal-GPi projection at grade 1, concomitant with considerable loss of striatal-GPe projections. Thus, the 2% reduction in GPi pallidal neurons, as found in this study, reflects the minimal neurochemical changes occurring at grade 1 HD.

In grade 3 HD, immunolabelled striatal fibres in the GPe is at 20% of control abundance, but in the GPi are at 50% of control abundance (Deng et al., 2004). At this stage, the striatum and GPe are nearly devoid of cannabinoid, D2 dopamine, and A2a adenosine receptors, with 30% of striatal D1 receptors still remaining, and GPi cannabinoid receptor levels still exceed those in the GPe (Glass et al., 2000, Allen et al., 2009). These neurochemical findings are consistent with greater preservation of the striato-GPi projection than the striato-GPe at grade 3. However, significant loss of input to the GPi is present. Thus, the 29% reduction in pallidal neurons in the GPe at grades 2-3, as found in this study, reflects the greater preservation of striatal input on to GPi pallidal neurons, compared to the 72% loss of GPe pallidal neurons at the same stage of disease (Chapter 4), reflecting the greater loss of striatal-GPe receptor systems.

6.6.8 The pattern of pallidal neuron loss in the globus pallidus internal segment compared with symptomatology and clinical assessments

It was also examined whether GPi pallidal neuron numbers were correlated with clinical information relating to mood and motor symptoms obtained from 5 of the 8 HD cases studied. In general terms, no significant correlation was found between the total number of Nissl-positive pallidal neurons and all variables examined. However, there is a general trend as shown in Figure 6.13, panel C, which shows that the total number of pallidal neurons was greater in HD cases with a higher score for the Mood Hospital Anxiety Scale. This correlation was not statistically significant according to a two-tailed Spearman’s correlation ($P = 0.08$), although the highly positive regression value of $r = 0.9$ highlights a very strong trend. The hospital anxiety sub-scale composes 8 questions as part of the Hospital Anxiety and Depression scale, which is a reliable, self-assessment scale for detecting states of anxiety (Zigmond and Snaith, 1983). In a study of 52 patients with HD, 51.9% exhibited anxiety (Paulsen et al., 2001). Furthermore, a study which examined self-reported psychiatric symptoms in a large sample of 681 prediagnosed individuals
who have the gene expansion for HD showed elevations in both anxiety and phobic anxiety (Duff et al., 2007). However, the anxiety which is reported during the prediagnostic phase (i.e. before the presentation of motor symptoms) often merges with the diagnostic phase during which the motor symptoms predominate (Walker, 2007). Therefore, the fact that GPi pallidal neurons are the highest in number in the HD cases with the highest levels of anxiety, suggests that anxiety levels are high before the onset of cell loss in the GPi, and once the basal ganglia motor circuits begin to dysfunction and nuclei within the basal ganglia begin to degenerate (as indicated by an increase in cell loss in the GPi), psychiatric symptoms such as anxiety tend to merge with the motor symptoms and possibly decline with disease management.

As the GPi is a final output station of the basal ganglia through which body movements are controlled, it was essential to look at the relationships between GPi pallidal neuron number and motor symptoms. In general terms, there was no significant correlation between the total number of Nissl-positive pallidal neurons and both Quantitative Neurological Exam (QNE) and Unified Huntington’s Disease Rating Scale (UHDRS) scores of motor impairment or chorea (Figure 6.14). It is known that an increase in motor impairment score correlates with the loss of DARPP-32 positive striatal neurons (Guo et al., 2012). However, in this study, it was shown in Chapter 4 that GPe pallidal neuron loss did not correlate with motor impairment despite being an output nucleus of the striatum. Therefore, similarly to its lateral counterpart, it is possible that the number of pallidal neurons in the GPi simply do not relate to the motor index scores of the cases studied.

6.6.9 The pattern of pallidal neuron cell soma size changes in the globus pallidus internal segment in Huntington’s disease using the Isotropic Nucleator

The results of stereological pallidal soma volume quantification of parvalbumin positive pallidal neurons, using the Isotropic Nucleator, shows that there is a 21% reduction in the pallidal soma volume in the GPi in HD, when all 8 HD cases are grouped together and compared with 8 controls (Figure 6.15). This reduction however, was not significant, with the P-value being > 0.05 (P = 0.13), which is likely to be attributable to the variability amongst control cases.

Early studies from Vonsattel have reported that pallidal neurons are smaller compared to control in advanced grade HD cases, although this was not quantified (Vonsattel et al., 1985, Vonsattel et al., 2008). It is interesting to see that recently, a similar finding has been reported by Guo et al
using design-based stereology, where remaining DARPP-32 positive striatal neurons were found to be reduced in volume by 40%, as determined using the Isotropic Nucleator (Guo et al., 2012). Furthermore, as shown in Chapter 4 of this study, there was a 35% reduction in pallidal soma volume in the GPe, thereby highlighting that GPi pallidal neuron atrophy in HD could be mirroring its lateral counterparts, the GPe and striatum.

The atrophy of remaining pallidal neurons could be indicative of ongoing neuronal dysfunction which proceeds cell death in Huntington’s disease (Ross and Tabrizi, 2011). In many neurodegenerative diseases, cells which remain may show degenerative changes in terms of size, shape, and morphology. Although these changes are not specific, they might represent definite ongoing pathogenesis of neurons (Kanazawa, 2001, Graeber and Moran, 2002). In Parkinson’s disease, nearly 50% of remaining nigral neurons exhibit cellular shrinkage (Kanazawa, 2001), and in Amyotrophic lateral sclerosis, remaining spinal motor neurons shrink to ~70-80% in size (Kiernan and Hudson, 1993). It has been thought that dysfunctional “sick” neurons might be maintained at a lower level than control in terms of metabolism and function of neurons, providing a protracted survival for more than 3-10 years in an atrophic state (Kanazawa, 2001). In terms of HD, Guo et al hypothesised that the shrinkage of DARPP-32 positive striatal neurons may represent neurons at different stages of neuronal degeneration, which could be the same case for the shrinkage of pallidal neurons.

6.6.10 The pattern of pallidal soma volume changes in the globus pallidus internal segment compared with striatal neuropathological grade

The results revealed a **17% reduction in the mean soma volume of pallidal neurons in the GPi in grade 0-1 HD** ($P > 0.99$) (Figure 6.17). **Analysis of the grade 2-3 group showed a 24% reduction in the mean pallidal soma volume** (Figure 6.17), This decline in pallidal soma volume in HD grade 2-3 was not significant ($P = 0.157$), which is likely to be attributable to the variation in the control cohort. Early studies from Vonsattel et al have reported that pallidal neurons are smaller compared to control in HD grade 3, and even more so in grade 4, although this was not quantified (Vonsattel et al., 1985, Vonsattel et al., 2008). This present study is the first quantitative evidence of pallidal cell soma reduction which is greater with advancing striatal neuropathological grade.
6.6.11 The density of pallidal neurons in the globus pallidus internal segment in Huntington’s disease

After assessment of pallidal neuron density in the GPi, the **mean cell density of Nissl-positive pallidal neurons was increased by 28%**, when all 8 HD cases are grouped together and compared with 8 controls (Figure 6.22). Despite the considerable increase, the mean changes were not statistically significant ($P = 0.06$). It is likely that high levels of variability within the control and HD cohorts are contributing to a lack of significance.

According to Lange et al (Lange et al., 1976), despite a dramatic loss of pallidal neurons in the GPi being found in HD, pallidal neuronal density was up to 27% higher in HD cases compared to controls. Although stereology was not used by Lange et al, this finding was reinforced by Vonsattel et al, who stated that neurons in the GP were more densely packed by grade 4 HD, suggesting that neurons are relatively preserved while tissue bulk decreases (Vonsattel et al., 1985). A more recent study by Wakai et al, using a non-stereological method, concluded that there was no significant loss of pallidal neurons in the GPi in HD, however a significant increase in neuronal cell density was found, leading to the suggestion that pallidal neuron loss does not contribute to advancing GP atrophy (Wakai et al., 1993).

Wakai et al also found the density of cells to be higher in the HD GPe compared to the GPi, which formed the conclusion that the more severely the striatum is affected, the more densely the neurons are packed into the GP receiving the striatal fibres, although no relationship between these two variables were tested (Wakai et al., 1993). In this current stereological study, the density of pallidal neurons in the HD GPe, as highlighted in Chapter 4, was marginally reduced by 11%, and combined with the striking cell loss in the GPe, it is clear that the premise which Wakai suggested – that is – the more severely the striatum is affected, the higher the density of pallidal neurons, cannot be valid. It is clear by the findings in this study (as highlighted in Chapter 4), that the loss of pallidal neurons, with minimal changes in remaining cell density, means that bulk tissue atrophy is occurring in parallel with cell loss in the GPe (because density is derived from cell number and tissue volume). However in the case of the GPi, the increase in pallidal neuron density, which has been shown, is a by-product of the lack of pallidal neuron loss in the GPi, combined with greater tissue atrophy. Therefore, because of this pallidal neuron density increase, it is likely that GPi atrophy is mainly due to neuropil loss, resulting from striatal fibres and terminals, and to a lesser extent the loss of neurons (Vonsattel et al., 2008).
Whereas, GPe atrophy must have a cell loss contribution, as the density of pallidal neurons within that region showed minimal change.

6.6.12 The pattern of pallidal neuron density changes in the globus pallidus internal segment did not correlate with striatal neuropathological grade

It was also investigated if pallidal neuron density in the GPe is related to striatal pathology. After assessment of the density of pallidal neurons in the GPi, the data shows a 28% increase in the mean density of Nissl-positive pallidal neurons in the GPi in grade 0-1 HD, when compared with 8 controls (Figure 6.23). However, the mean changes in cell density of pallidal neurons in HD grade 0-1 were not significant ($P = 0.341$). It was also important to highlight the extreme variability amongst the grade 0-1 HD cases. Analysis of the grade 2-3 group showed a 29% increase in the density of pallidal neurons compared to 8 controls (Figure 6.23). This increase in pallidal neuron density in HD grade 2-3 was not significant ($P = 0.187$). Therefore, pallidal neuron density did not clearly correlate with striatal neuropathological grade, as the increase in density in HD observed did not increase progressively with greater striatal pathology.

It was interesting to observe that the density of pallidal neurons in the GPi did not change with increased striatal neuropathology, which is consistent with a non-stereological study that found a lack of correlation between striatal grades and GPi cell density (Wakai et al., 1993). It could possibly be due to the fact that in early stages of the disease (i.e. grade 0-1), there is minimal loss of pallidal neurons occurring as mentioned previously in this chapter, which means, the ratio of the number of cells to unit volume of tissue is high, as tissue atrophy is occurring at a faster rate than neuronal loss at HD grade 0-1. However, the greater extent of pallidal neuron loss by grades 2-3, would gradually lower the number of cells to each unit volume of tissue, i.e. GPi atrophy and cell loss are occurring in unison, leaving no change in density as a result and thereby accounting for the lack of change in GPi density with HD grade.
6.7 Conclusion

The major findings of this present study includes a **striking 40% reduction in overall GPi volume**, accompanied with a **minor 19% loss of pallidal neurons**, a **21% decline in pallidal soma volume**, and a **28% increase in pallidal neuron density** in *post-mortem* Huntington’s disease cases. Although GPi atrophy was major and paralleled advancing striatal neuropathological grade, the lesser extent of grade-wise GPi atrophy compared to its lateral counterpart, the GPe, showcases differential vulnerability in terms of tissue atrophy. It was also shown, for the first time, a link between GPi atrophy and irritability in HD, reinforcing the role of the GPi in limbic system and mood related pathways. In addition, the minor loss of pallidal neurons also coincided with striatal pathology. However, the resistance of striato-GPi projections to HD degeneration results in a lesser degree of subsequent grade-wise GPi atrophy and cell loss, compared to its lateral counterpart. Furthermore, an interesting relationship was found between cell number and anxiety, suggesting that anxiety proceeds GPi cell loss in HD. Surviving pallidal neurons were smaller in volume, suggesting neuronal dysfunction. In addition, the overall density of pallidal neurons in the GPi is increased in HD, thereby reinforcing that GPi atrophy is mainly a by-product of striato-pallidal projection loss, and to a lesser degree pallidal neuron loss – suggestive of a different pattern of degeneration of the GPi compared to the GPe in HD.
CHAPTER 7: GENERAL DISCUSSION

7.1 Introduction

The results of this study provide the first comprehensive and detailed stereology-based research on the neurodegeneration of the human globus pallidus in Huntington’s disease (HD). This study focused on three functionally diverse regions within the basal ganglia: the globus pallidus externus (GPe), the globus pallidus internus (GPi) and the ventral pallidum (VP). The GPe, a major output of the striatum, projects widely to other basal ganglia nuclei. However, one of the most important pathways through which the GPe connects to other nuclei is the indirect pathway, where it plays a major role in motor activities. The GPi is a final output station of both the direct and indirect pathways of the basal ganglia, through which body movements are controlled. The VP, in contrast to its dorsal GPe and GPi motor nuclei, is a primary output of the limbic ventral striatum (nucleus accumbens), mediating reward and motivation functions, in addition to aiding translation to movement. The overall goal of this study was to investigate changes in the GP complex in HD. Particular attention was focused on the overall volumetric changes, the extent of pallidal neuron loss, and pallidal neuron volume changes in the GPe, GPi and VP and to compare these findings with: (1) striatal neuropathological grade, (2) CAG repeat length, post-mortem delay, brain weight, age of death, age of disease onset, disease duration; and (3) symptom heterogeneity and clinical assessments.

To carry out this study, histology, immunohistochemistry, and unbiased design-based stereological techniques were employed. The results in Chapters 4 and 6 investigated overall volume, total number of pallidal neurons, average pallidal cell soma volume, and pallidal neuron density changes in HD human brains compared to control brains, using stereology in the GPe and GPi respectively. The results in Chapter 5 investigated the overall volume, total number of pallidal neurons, and pallidal neuron density changes in the VP of HD human brains compared to control brains, using stereology. The stereological data described in each Chapter were then grouped and compared according to striatal neuropathological grade, a grade independently determined by a neuropathologist. Furthermore, the stereological data from each HD case were also correlated with clinical symptom heterogeneity, determined by retrospective analysis of
clinical symptom data collected from patients, family members and clinical records carefully examined by neuropsychologists and neurologists. The overall results and key findings are now discussed in this chapter in comparison to previous studies involving the GP in HD.

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of CAG trinucleotide repeats in the *HD* gene on chromosome 4, which encodes a mutant protein called huntingtin (MacDonald et al., 1993). The most pronounced and well characterised neuropathology in HD occurs within the striatum of the basal ganglia, in which there is gross atrophy. This is principally due to the well documented loss of medium spiny GABAergic projection neurons in the striatum, which follows a dorsal-ventral gradient (Vonsattel et al., 1985, Vonsattel et al., 2008). Efferent fibres from the striatum converge towards the globus pallidus (GP), where they constitute a massive fibre system that passes through the GPe (via the *indirect* pathway) and GPi (via both the *indirect* and *direct* pathways).

Despite the extensive literature examining the implications of HD on the striatum, very little exists with regard to the impact of striatal cell loss on the main neurons that receive striatal input, pallidal neurons. Currently, there is a disagreement in the literature about the extent of pallidal neuron loss in the GP in HD. In 1976, Lange et al reported a quantitative loss of pallidal neurons in the GPe and GPi in HD (Lange et al., 1976). In contrast, a more recent study of the GPe and GPi in HD by Wakai et al reported no such loss of pallidal neurons (Wakai et al., 1993). However, the study by Lange et al was carried out in the 1970’s before design-based stereological techniques were used, utilised a small sample size with limited clinical information, did not use immunohistochemical markers to delineate the regions, and was conducted prior to the establishment of the striatal neuropathological grading criteria. Furthermore, the more recent study by Wakai et al also used non-stereological techniques, a small predetermined and biased selection of sections, and a small sample size. Thus, there is disagreement in the literature about the extent of pallidal neuron loss in the GPe and GPi in HD, and there is a lack of detailed stereological knowledge with regard to the relationship between GPe and GPi neurodegeneration with striatal neuropathological grade and symptom heterogeneity. In addition, no current evidence exists in the literature with regard to the fate of the main output structure of the ventral (limbic) striatum in HD, the VP (Heimer and Wilson, 1975, Haber et al., 1990b).

This present study has extended the basal ganglia pathology studies to the external, internal, and ventral portions of the pallidum to investigate total regional volume, total number of pallidal
neurons, pallidal neuron soma volume and pallidal neuron density in the globus pallidus external
segment (GPe), globus pallidus internal segment (GPI), and ventral pallidum (VP) of the human
brain in 8 HD brains compared to 8 control brains for our specific cohort of neurologically
defined brains from the Neurological Foundation of New Zealand Human Brain Bank in the
Centre for Brain Research, The University of Auckland. This project forms an important part of
a larger, systematic study on the pathological changes in various cell populations across the
human brain in HD, which is being carried out by our laboratory at the Centre for Brain
Research, The University of Auckland.

The results reveal a variable extent of pallidal regional atrophy, variable degrees of pallidal
neuron loss, variable degrees of pallidal neuronal atrophy, and differential patterns of pallidal
neuron density changes in the three functionally diverse pallidal regions. In addition, the
association between the patterns of regional atrophy, cell loss, cell atrophy, and cell density with
the HD striatal neuropathological grade was also investigated to determine the pathological
relationship between the pallidum and the striatum. Furthermore, with a subset of HD cases, it
was shown that there were various relationships between the stereological data obtained with
both clinical data and symptom heterogeneity. These findings greatly highlight the importance
of pallidal dysfunction in HD, thereby contributing to the understanding of overall basal ganglia
pathogenesis and dysfunction in HD.
7.2 Methodological Considerations

**Human brain tissue**

There were limiting factors on the number of cases which were available to be used in this study. The basal ganglia in the New Zealand Neurological Foundation Human Brain Bank is extensively utilised in studies in New Zealand and overseas on HD research. Due to the availability of human basal ganglia tissue, and the requirements of whole basal ganglia blocks to be used for stereology, a total of 8 HD and 8 control cases were analysed in this study. The HD cases were further subdivided according to striatal neuropathological grade. The grades of the 8 HD cases ranged from 1 to 3. There were three grade 0-1 cases, one grade 2 case, and four grade 3 cases. For the purpose of this investigation, the only grade 2 case was pooled with the grade 3 cases, with analyses conducted with and without this case to ensure it did not skew the data. It can be noticed that particularly in the Grade 0-1 group in each results chapter, there was considerable individual variation between cases. Furthermore, clinical correlations were conducted with a sample size of 5 cases in the HD cohort, as only 5 of the 8 cases had the symptom and clinical information available. It is clear that a greater sample size would increase the validity and statistical power of this study, and the addition of more cases into the grade 0-1 group would probably reduce the extreme variability between cases, thereby increasing statistical power. Because it was not always possible for patients on the donor programme to have full quantitative assessments of symptoms, it was not always possible to obtain complete data sets of symptom scores for all patients, particularly if the duration of HD symptoms prior to death was short.

However, significant changes were observed in the pallidal regions even with the limited number of available cases. In addition, because detailed stereological studies require considerable amounts of tissue, it was not feasible to use a large sample size of cases to obtain a valid estimate of sampling precision, which is why pilot studies were used to increase sampling rates within each case, rather than increasing the number of cases to obtain a valid estimate of volume, cell number, or cell volume (West, 1999).
In this study, immunohistochemical markers were used to delineate the extent of each pallidal region for this stereological study (see Chapter 3, Materials and Methods). Clear delineation of each region of interest is a requirement for any stereological study (West, 1993). Unlike the clear separation between the GPe and GPi, based on differential neurochemical markers, the delineation of the ventral pallidum borders both rostrally and caudally in coronal sections was more difficult. This is because both substance-P and enkephalinergic positive profiles are found within the subcommissural region, where these peptidergic tubular profiles intermingle. The ventral pallidum was distinguished ventrally from the GPe in this study by a dense wedge of enkephalin positive terminals, extending directly under the temporal limb of the anterior commissure. Enkephalin immunoreactivity was selected because of the continuity seen between the GPi and VP based on substance-P immunoreactivity, thereby using substance-P to delineate between these regions caudally could not be consistently carried out across cases (Mai et al., 1986). However, using enkephalin alone to delineate the VP region leaves open the possibility of exclusion of some of the VP, as it is known that a strongly substance-P positive subcommissural area extends further medially than the enkephalin positive area. However, this could not be avoided, as enkephalin provided the more definitive and consistent delineation in human tissue for stereological purposes.
7.3 The Overall pattern of neurodegeneration in the globus pallidus and ventral pallidum in Huntington’s disease

The results of this study provide the first comprehensive and detailed characterisation, using stereology, to examine the overall volume, pallidal neuron number, and neuronal volume of three functionally diverse pallidal subregions within the basal ganglia: the globus pallidus externus (GPe), the globus pallidus internus (GPi) and the ventral pallidum (VP). This level of characterisation was conducted for 8 Huntington’s disease (HD) cases, and 8 neurologically normal control cases.

Currently, reviews and book chapters of the basal ganglia describe the normal human globus pallidus with limited detail. As far as cell number is concerned, the majority of reviews quote a single non-stereological study from 1975, reporting 600,000 pallidal neurons in the entire normal human globus pallidus (Thorner et al., 1975). In addition to characterising the neurodegeneration in HD using stereology, this study has also enhanced the overall understanding of the normal human globus pallidus in terms of its subdivisions, and gathered a detailed understanding of volumetric, pallidal neuron population, and pallidal soma size differences in each pallidal subdivision. Based on the pooled volumetric data obtained using the Cavalieri Estimator, the overall average volume of the normal control globus pallidus is 1,278 mm$^3$, with the GPe forming 65.4% (836 mm$^3$), GPi comprising 30.6% (390.9 mm$^3$) and VP occupying 4% (51.43 mm$^3$) of the total volume respectively. This is the first proportional volumetric breakdown of the three pallidal subregions reported. Furthermore, this is the first study to validate the total pallidal neuron figure quoted in 1975. Based on the pooled stereological cell counting data obtained using the Optical Fractionator, the total number of pallidal neurons in the entire normal human globus pallidus is 637,906 cells, with the total proportion of pallidal neurons within the GPe being 68.9% (439,564 cells), GPi comprising 26.1% (166,600 cells), and VP comprising 5% (31,742 cells) respectively. The results of cell quantification using stereology are generally consistent with Thorner and Lange et al (Thorner et al., 1975, Lange et al., 1976). Furthermore, this is the first study to report a measurement of pallidal neuronal soma volume in the normal control GPe and GPi. It was interesting to find that the average pallidal soma volume in the GPe (17,945 µm$^3$) is considerably smaller in comparison to the average pallidal soma in the GPi (27,081 µm$^3$). Thus, the stereological data obtained for each pallidal region in normal control
tissue could provide baseline data for future studies of the globus pallidus and ventral pallidum, particularly with regard to studying the impact of disease on these regions.

These data obtained from 8 normal control brains, served as a baseline to compare and contrast the degree of regional atrophy, cell loss and cell atrophy in the GPe (Chapter 4), VP (Chapter 5) and GPi (Chapter 6) of 8 HD cases, using detailed design-based stereology. The results in Chapter 4 detailed the extreme vulnerability of the GPe to the disease, highlighted by a significant striking reduction in overall GPe volume (54%), pallidal neuron number (59%), and pallidal cell soma volume (35%), when all HD cases were compared with control cases (Figures 4.1, 4.8, 4.15, Chapter 4). In comparison, the results presented in Chapter 6 suggest that the GPi is less vulnerable to the disease process, with a smaller but significant reduction in overall GPi volume (40%), accompanied with a lower loss of pallidal neuron number compared to the GPe (19%), and minor reduction in pallidal cell soma volume (21%), when all HD cases were compared with control cases (Figures 6.1, 6.8, 6.15, Chapter 6). However, a notable point of difference can be seen in the density of remaining pallidal neurons in the GPi, which was found to increase by 28% in HD (Figure 6.22, Chapter 6), suggesting that there is a large decrease in the GPi volume probably due to, in part, the major loss of basal ganglia efferent heavily myelinated axons known to traverse the GPi. Furthermore, this study reports the first evidence, as highlighted in Chapter 5, that the VP is affected in HD, with a reduction in overall VP volume (31%) and pallidal number (24%) when all HD cases were compared with control cases (Figures 5.1 and 5.8, Chapter 5).

It is clear that the substantial heterogeneity of neurodegeneration in each pallidal region could possibly be related to the severity of degeneration in the striatum of the basal ganglia, as well as symptom variation in HD. Therefore, these investigations were carried out next and the results are discussed below.
7.4 The relation of the pattern of neurodegeneration in the globus pallidus and ventral pallidum to striatal neuropathological grade

The most pronounced and well characterised neuropathology in HD occurs within the striatum of the basal ganglia, in which there is gross atrophy. This is principally due to the well documented loss of medium spiny GABAergic projection neurons in the striatum, which is one of the key measures used in assigning a pathological grade (Vonsattel et al., 1985, Vonsattel et al., 2008). What is important to note, is that there is a differential vulnerability among striatal projection neurons to the disease, with subsequent impact on the various output pathways of the striatum. Since the globus pallidus and ventral pallidum are output nuclei of the striatum, it was essential to look at the relationship between striatal neuropathological grade and the extent of pallidal degeneration.

In this study, it was found that amongst the pallidal nuclei studied, the extent of GPe atrophy as a whole, pallidal neuron loss, and individual cellular atrophy was strongly correlated with the striatal neuropathological grade; i.e., the greatest atrophy, cell loss, and soma atrophy coincides with advancing striatal neuropathological grade in the GPe compared to the GPi and VP (Figure 4.2, 4.9, 4.17, Chapter 4). These results are consistent with previous studies detailing the earlier and more predominant loss of the enkephalin-containing indirect pathway projecting from the striatum to the GPe, which has been shown to coincide with advancing striatal neuropathological grade (Reiner et al., 1988, Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009). Further support for the link between striatal degeneration and pallidal degeneration arises from the relationships between GPi atrophy, cell loss, and cell atrophy, with striatal neuropathological grade (Chapter 6). It was interesting to see that the extent of atrophy, cell loss, and cell atrophy in the GPi was not as large as the GPe when correlated with striatal neuropathological grade, although neurodegeneration did increase with advanced grade (Figure 6.2, 6.9, 6.17, Chapter 6). Therefore, these findings are consistent with the later and less prominent involvement of the substance-P-containing direct pathway projecting from the striatum to the GPi. Thus, it is likely that the differential pattern of GPe and GPi neurodegeneration associated with striatal neuropathology, mirrors the characteristic pattern of early striatal enkephalinergic projection loss to the GPe, and later striatal substance-P projection loss to the GPi. Furthermore, these data reinforce the resistance of striato-GPi projections to HD degeneration, suggested by the lesser extent of downstream GPi degeneration, compared to
the greater vulnerability of striato-GPe projections. **The functional significance of these findings in terms of disease symptomatology will be discussed in sections 7.5.**

In addition, for the first time, the relationship between striatal neuropathological grade and VP neurodegeneration was explored (Chapter 5). It was interesting to find that VP atrophy and cell loss seem to occur only when striatal neuropathology progresses beyond grade 2 in HD, suggesting that its extent of degeneration differs from its dorsal GPe and GPi counterparts (Figure 5.2, 5.9, Chapter 5).

As discussed in Chapter 5, it is likely that pronounced VP atrophy, and cell loss exclusively at striatal neuropathological grades greater than 2 mirrors the pace of ventral striatum/nucleus accumbens degeneration in HD. The VP receives its main input from the ventral striatum, a component of the limbic pathway which is relatively more spared in terms of HD pathology compared to the severely degenerated dorsal “motor” striatum (Bots and Bruyn, 1981, Vonsattel et al., 1985, Ferrante et al., 1986, de la Monte et al., 1988). This dorsal-ventral gradient of striatum pathology in HD could possibly account for the lesser extent of VP atrophy and cell loss compared to its dorsal counterparts, the GPe and GPi, which receive predominantly dorsal striatal input. Also mentioned in Chapter 5, based on the sparing of enkephalin immunoreactivity in the ventral ‘limbic’ striatum in all grades of HD, combined with a lack of neuronal death or fibrillary astrocytosis until advanced HD grades beyond grade 2 (Vonsattel et al., 1985, Ferrante et al., 1986), this could translate to the possible sparing of striato-VP projections until >grade 2, which is reflected in later VP atrophy, and thereby latter loss of pallidal neurons until grade 2. The functional implications of these findings, in terms of disease symptomatology will be discussed in section 7.5.

In summary, the neurodegenerative patterns of the GPe, GPi and VP closely parallel the pattern of striatal medium spiny neuron loss. The heavy degeneration of the GPe is closely connected with the major loss of enkephalinergic MSN loss in the striatum. The resistance of substance-P MSN neuron loss until advanced HD is reflected in a lesser degeneration of the GPi compared to its lateral counterpart. And finally, the neurodegeneration of the VP being spared until advanced grades of HD reflects the relative preservation of its ventral striatal input until advanced HD. In conclusion, the findings of the present study reinforce the differential vulnerability of striatal-GPe, striatal-GPi and striatal-VP connections, as highlighted by the close link between striatal and pallidal degeneration.
7.5 Implications of pallidal neurodegeneration on cellular dysfunction of the basal ganglia circuitry and symptom profiles in HD

One of the prevailing questions in the field of HD research is how changes in terms of neuropathology relate to the development of symptomatic features. Clinically, HD is characterised by cognitive impairments, motor dysfunctions, and psychiatric changes, with the latter often preceding the other symptoms (Lawrence et al., 1996, Paulsen et al., 2001, Petrasch-Parwez et al., 2012). Therefore, in addition to the strong implications of pallidal degeneration on the basal ganglia motor circuitry in HD, the association of the pallidum with the limbic system might also explain some of the psychiatric and cognitive symptoms in HD (Petrasch-Parwez et al., 2012). This portion of the discussion will focus on integrating the results of GPe, GPi, and VP atrophy, cell loss, and cell atrophy in HD with dysfunction of various motor and limbic pathways within the basal ganglia. In addition, clear hypotheses will be raised to relate the pattern of basal ganglia dysfunction with the heterogeneity of mood and motor symptoms. A summary of the discussed pathways is highlighted in Figures 7.1, 7.2, and 7.3.

The implication of GPe degeneration on the basal ganglia circuitry – relevance to HD symptoms (Figure 7.1, 7.2)

Based on the results of GPe neurodegeneration in HD (Chapter 4), the earlier and more predominant loss of the enkephalin-containing indirect pathway, projecting from the striatum to the GPe, has been shown to coincide with advancing striatal neuropathological grade (Reiner et al., 1988, Sapp et al., 1995, Deng et al., 2004, Allen et al., 2009). The indirect pathway is a multisynaptic pathway which firstly involves the transmission of corticostriatal information to the striatum, then via complex processing connects the GPe with the STN which projects to the output nuclei, the GPi and SNr (Albin et al., 1989, DeLong, 1990, Flaherty and Graybiel, 1994). In the normal brain, cortical activation (primarily from the primary motor, premotor, and supplementary motor cortices) of the striatal inhibitory GABAergic and enkephalin-containing medium spiny neurons (MSNs) reduces the output of GABAergic GPe activity, resulting in disinhibition of glutamatergic STN neurons, which causes an increase in the glutamatergic activation of the GPi and SNr. The activation of the inhibitory output of the GPi and SNr centres reduces the thalamic activation of cortical neurons in the normal brain (Albin et al., 1989,
DeLong, 1990, Smith et al., 1998a, Bolam et al., 2000). This is because pallidal neurons in the GPe are inhibited by GABA released from striato-pallidal terminals and in turn, provide ‘tonic’ inhibition to the thalamus. This is reinforced by previous studies showing that application of GABA_A and GABA_B receptor agonists into the GPe inhibits pallidal activity (Galvan et al., 2005). Thus, the effect of activating the basal ganglia indirect circuit on cortical motor activity is inhibitory, which is the opposite of the direct circuit and will be discussed later in this Chapter. In HD, the loss of the enkephalinergic indirect pathway to the GPe, with relative sparing of the direct pathway, is thought to result in imbalance of indirect and direct pallidal circuits, favouring unopposed glutamatergic release by the VA/VL motor thalamus onto the motor cortex by actions of the direct pathway. This could possibly account for hyperactive chorea movements seen commonly in HD (Crossman, 1987, Albin et al., 1989, Hedreen and Folstein, 1995). Studies in primates and other animal models show that chorea is induced when GABA_A receptor antagonists are injected into the GPe, supporting the idea that loss of projections from the striatum to the GPe are involved in the expression of chorea (Crossman et al., 1988, Matsumura et al., 1995). This is because injection of such antagonists into the GPe increases neuronal firing, thereby leading to chorea in primates and small animal models, due to the over activity of GPe pallidal neurons (Crossman et al., 1988, Matsumura et al., 1995, Chen et al., 2004, Kita et al., 2004).

However, what is interesting to note, and almost contradictory, is that bilateral lesions confined specifically to the GPe in humans, cause akinetic-rigid syndromes similar to parkinsonian symptoms (Bhatia and Marsden, 1994, Bucher et al., 1996, Münchau et al., 2000). Bhatia and Marsden concluded that lesions of the GPe might cause parkinsonism due to disinhibition of the STN and the GPi with subsequent over inhibition of the VA/VL nucleus of the thalamus and hence the motor cortex (Bhatia and Marsden, 1994). Therefore, based on the involvement of the indirect pathway in HD, the dysfunction of this pathway can only result in hyperactive chorea movements if the GPe pallidal neurons are still present and functional. Based on this pathway, reducing GABAergic striatal inhibition on the GPe would subsequently result in aberrant GABA release from pallidal neurons within the GPe. GABA release from functional pallidal neurons in the GPe is required to inhibit glutamatergic STN activity. The reduction in STN activity would reduce GABA release from the GPi onto the thalamus and thereby lead to excess excitation of glutamatergic neurons projecting from the thalamus to the motor cortex.
As shown in Chapter 4, a 37% loss of pallidal neurons was found in the GPe at HD grades 0-1 (Figure 4.9, Chapter 4). This means that 63% of the pallidal neuron population remains in early HD. In addition major atrophy of remaining pallidal neurons was found (Figure 4.15, Chapter 4), which could be indicative of ongoing neuronal dysfunction including aberrant neurotransmitter release (Ross and Tabrizi, 2011). Taken together, it can be hypothesised that in early stages of HD (i.e. at striatal neuropathological grades less than 2), the loss of striatal inhibition on to pallidal neurons in the GPe is likely to lead to dysfunctional activity of pallidal neurons (reinforced by cell soma morphology changes), thereby leading to aberrant GABA release by GPe pallidal neurons, resulting in a loss of inhibition on the motor thalamus and motor cortex by the indirect pathway, and thereby leading to chorea. This is highlighted in Figure 7.1.
In the *indirect pathway*, the excitatory corticostriatal projection terminates onto striatal medium spiny neurons that contain GABA/ENK. The striatal output first passes to the inhibitory GPe and then to GPi via the excitatory STN whereby disinhibition of the subthalamic neurons reduces thalamic activation of the cortex. The disruption of the excitatory glutamatergic projection onto the striatum results in dysfunction of the striatal output pathways in HD which ultimately leads to the development of motor dysfunction.

In HD, hyperkinesia and chorea are caused by the initial preferential damage in the *indirect* GABA/ENK striato-pallidal pathway (indicated by a large red X) that project from the striatum to the GPe. The loss of striatal neurons that give rise to the *indirect* pathway reduces the inhibitory action of the GPe upon the STN, which is reinforced by early atrophy of pallidal neurons and atrophy of the GPe region itself (indicated by thunderbolt symbols). The STN then becomes hypofunctional and causes reduction of the inhibitory action of the GPi upon the thalamus. This subsequent disinhibition of the thalamus leads to the overactivation of the motor cortex which results in hyperkinetic movements.

The continuous and dotted lines indicate excitatory and inhibitory pathways, respectively. ENK, enkephalin; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral thalamic nuclei.
Figure 7.1 The impact of degeneration of the globus pallidus external segment (GPe) on the \textit{indirect} motor circuitry of the basal ganglia at striatal neuropathological grade 1

\textbf{Grade 1 HD – Motor dysfunction}

Atrophy of GPe due to loss of striatal input

Increase GABA release from atrophic dysfunctional GPe pallidal neurons leads to excess glutamate release from thalamo-cortical neurons, resulting in hyperkinetic movements
In principle, based on the functions of the indirect pathway, a dramatic loss of GPe pallidal neurons would result in disinhibition of the subthalamic nucleus and thereby contribute to akinesia and possible rigidity (Reiner et al., 2011). Although most patients display hyperkinetic movements such as chorea early in the disease (which as explained above, could be linked to GPe dysfunction), these movements are often progressively replaced by a more hypokinetic (akinetically-rigid) syndrome in which bradykinesia, rigidity, and dystonia predominate. Thus, it was interesting to find in this study that atrophy of the GPe was found to correlate with the severity of motor impairment in HD and not chorea (Figure 4.7, Chapter 4). This result was paralleled by findings from Guo et al (Guo et al., 2012), who reported that the overall reduction in putamen volume is correlated with the severity of motor impairment and not chorea. This suggests that GPe atrophy, like striatal atrophy is more related to the pathogenesis of motor dysfunction, as opposed to chorea, reinforcing the hypothesis that chorea may be a manifestation of cellular dysfunction (such as early morphological changes to pallidal neurons as mentioned above) within the GPe, not degeneration itself (Ross and Tabrizi, 2011, Guo et al., 2012). Taken together, these findings suggest that pallidal neuron dysfunction of GPe neurons leads to the early symptoms of chorea based on indirect pathway dysfunction. However, chronic pallidal neuron loss is linked to the “symptom shift” towards akinetic-rigid syndrome later in HD, which is reinforced by the correlation with the severity of motor impairment. The impact of severe neurodegeneration of the GPe on advanced grades of HD in terms of the indirect pathway is highlighted in Figure 7.2.

Thus, based on the existing models of the indirect pathway and its dysfunction in HD, the question has previously been explored as whether or not GPe lesions might prove an effective treatment for motor disturbances in HD (Reiner, 2004). In principle, GPe lesions would only benefit the earlier stages of HD (i.e. pathology stages of grade 0-1, before sufficient pallidal neuron loss), during which a hyperactive and dysfunctional GPe (in response to the loss of input from inhibitory striatal-GPe neurons) leads to choreic symptoms (Reiner, 2004). Since the disease in many cases progresses to hypokinesia (Penney et al., 1997), and GPe neurodegeneration was shown to coincide with the severity of motor impairment and striatal neuropathology in this study, an irreversible GPe lesion would not be advisable, because as shown by the data presented here, neurodegeneration of the GPe is more extensive than previously thought, and would likely be a contributor to the later akinesia/bradykinesia symptoms. In conclusion, if GPe lesions were to be a potential HD therapy for chorea alleviation, they would need to be limited to patients who are unlikely to progress to
akinesia/bradykinesia (i.e. those with a lower CAG repeat number) (Penney et al., 1997, Reiner, 2004). Alternatively, direct GPe depolarisation blockage via implanted electrodes or drug infusion (e.g., GABA$_A$ agonist) would be needed to provide a reversible inactivation of the GPe during early HD (Reiner, 2004). Animal studies have shown a potential therapeutic approach for HD hyperkinetic symptoms consisting of GPe inhibitory stimulation to indirectly reduce thalamocortical hyperactivity. For example, bilateral GPe deep brain stimulation has been shown to improve cognitive and motor symptoms in a transgenic rat model of HD (Temel et al., 2006). This finding prompted an investigation into a potential therapeutic approach for HD hyperkinetic symptoms in humans using GPe bilateral high-frequency deep brain inhibitory stimulation in 5 HD subjects. The results showed an overall decrease in neuronal activity, and modulated cerebral connectivity within the basal ganglia-thalamo-cortical circuitry (Ligot et al., 2011). However, neurologic confirmation within the framework of this clinical trial is needed to confirm the actual value of this therapeutic strategy.

**The implication of GPi degeneration on the basal ganglia circuitry – relevance to HD symptoms (Figure 7.2)**

Based on the results of GPi neurodegeneration in HD (Chapter 6), it is clear that there is a lesser extent of neurodegeneration in the GPi compared to the GPe (Chapter 4). This is consistent with the later and less prominent involvement of the substance-P-containing direct pathway projecting from the striatum to the GPi, suggestive of a resistance to early HD pathogenesis until mid-advanced neuropathological grades, compared to the striatal-GPe enkephalinergic pathway (Graveland et al., 1985, Reiner et al., 1988, Hedreen and Folstein, 1995, Glass et al., 2000, Deng et al., 2004).

In the direct pathway, cortico-striatal information is transmitted to the striatum, and next, either via the GPi or SNr to the ventral nuclear complex of the thalamus (VA/VL). The VA/VL complex then projects to the premotor, supplementary motor, and motor cortex (Albin et al., 1989, Smith et al., 1998a, Wilson, 2004). In the normal brain, cortical activation exerts an excitatory influence on GABAergic striatal MSNs. The increased activity of these GABAergic elements leads to a decreased activity of GPi and substantia nigra output neurons. Because these neurons are also GABAergic and inhibitory, their inhibition results in an increase in activity of the thalamocortical neurons on which they impinge. Thus, excitation of striatal neurons by
cortical efferents ultimately disinhibits (and hence activates) thalamocortical neurons. In other words, the direct circuit mediates positive feedback of thalamocortical interactions (Alexander and Crutcher, 1990, Gerfen and Wilson, 1996, Smith et al., 1998a). In mid to advanced HD, particularly grade 3 and higher in terms of striatal pathology, the later loss of substance-P striatal-GPi projections of the direct pathway, is thought to contribute to the rigid-akinetic HD. This is because the loss of substance-P GABAergic MSNs, increases the output of GPi pallidal neuron activity, thereby reducing the excitatory effect of glutamatergic thalamic neurons, reducing glutamatergic cortical output, thereby potentially contributing to the bradykinesia that develops late in HD, while the near complete loss of this projection is likely to explain akinesia in terminal grade 4 HD (Albin et al., 1989, Albin et al., 1990).

Indeed, lesions to the GPi lead to a variety of movement disorders in small animal and primate models, including slowing of movements and dyskinesia (Horak and Anderson, 1984, Crossman, 1987, Mink and Thach, 1991). In studies involving humans, GPi lesions have also been associated with both akinesia (Molinuevo et al., 2003), and dystonia (Bhatia and Marsden, 1994, Bucher et al., 1996, Münchau et al., 2000). Thus, based on the findings in Chapter 6 and the collective literature described, due to the lesser degree of cell loss found in the GPi, which is only apparent at striatal neuropathological grades greater than 2, reinforced by the increase in density of remaining pallidal neurons, it is clear that the increase in aberrant GABAergic GPi pallidal neuron activity in response to the loss of striatal inhibitory input on the direct pathway, might be a potential contributor to the akinetic-rigid and dystonic symptoms in more advanced stages of HD. The impact of GPi degeneration on the direct pathway is highlighted in Figure 7.2.
Figure 7.2 The impact of degeneration of the globus pallidus external segment (GPe) and internal segment (GPI) on the indirect and direct motor circuitry of the basal ganglia at striatal neuropathological grade 3

In the direct pathway, the cortical excitatory fibres terminate on the medium spiny striatal projection neurons that contain GABA/SP which project to the GPi. These result in inhibition of the GPi and disinhibition of the VA/VL thalamic output to the cerebral cortex. Thus, the result of cortical activation in the direct pathway is opposite to that of the indirect circuit, reinforcement rather than reduction of cortical activity.

In advanced HD, the subsequent later loss of the direct GABA/SP striato-pallidal pathway that projects from the striatum to the GPi (indicated by the thunderbolt symbol), results in the dysfunction of atrophic pallidal neurons and causes increased inhibition of the thalamus which decreases the activation of the motor cortex and causes rigidity (hypoactivity).

Furthermore, severe neurodegeneration of the GPe in HD (indicated by a skull), leads to disinhibition of the STN and further exaggerates the disinhibition of the VA/VL thalamic complex.

The continuous and dotted lines indicate excitatory and inhibitory pathways, respectively. ENK, enkephalin; SP, substance-P; GPe, globus pallidus external segment; GPI, globus pallidus internal segment; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral thalamic nuclei.
Figure 7.2 The impact of degeneration of the globus pallidus external (GPe) and internal segments (GPi) on the *indirect* and *direct* motor circuitry of the basal ganglia at striatal neuropathological grade 3

**Grade 3 HD – Motor dysfunction**

Atrophy of GPi due to loss of striatal input:

- Increase GABA release from atrophic dysfunctional pallidal neurons leading to dysfunction of the direct pathway, contributing to akinesia and rigidity

Complete ablation of GPe leads to disinhibition of STN and contributes to an akinetic-rigid syndrome
Furthermore, another interesting finding was the relationship between neurodegeneration in the GPi with symptoms of mood, including irritability (Figure 6.6, Chapter 6) and anxiety (Figure 6.13, Chapter 6). Although the GPi has strong interactions with the motor cortices and controls somato-motor behaviours, the GPi can also be subdivided into the motor (ventral two-thirds), associative (dorsal one-third), and limbic (medial tip) territories (Nambu, 2007). The GPi also projects to the lateral habenular (LHb), which is a small nucleus located in the epithalamus, and is associated with the limbic system (Lecourtier and Kelly, 2007, Hong and Hikosaka, 2008). A recent study of reward-based tasks in primates found that the activity of LHb-projecting neurons, located on the dorsal and ventral borders of the GPi, were modulated by expected reward outcomes, suggesting that the GPi may initiate reward related signals via an extrabasal ganglia pathway from the striatum/GPi/LHb/dopaminergic reward system/striatum connections (Hong and Hikosaka, 2008). This same signal may also be used to control mood and social behaviours via the projections from the LHb to the raphe nuclei (Strecker and Rosengren, 1989). Therefore, since GPi neurodegeneration was shown to be highly correlated with irritability and anxiety in HD, this is reinforcing the importance of the GPi in limbic system and mood related pathways. The contribution of GPi degeneration to dysfunction of the limbic reward pathways is highlighted in Figure 7.3.

The implication of VP degeneration on the basal ganglia circuitry – relevance to HD symptoms (Figure 7.3)

The VP is innervated by the ventral striatal region (also known as the nucleus accumbens), receiving substantial inputs from a variety of limbic and limbic-associated structures (Haber et al., 1985). The reward limbic pathway originates from various orbital and medial prefrontal cortical areas (OMPFC), and projects to the ventral striatum (Yeterian and Pandya, 1991, Eblen and Graybiel, 1995, Haber et al., 1995). The ventral striatal regions then project to the VP (Haber et al., 1990a, Hedreen and Delong, 1991, Parent et al., 1997). The VP then projects to the mediodorsal thalamic nucleus, which is then reciprocally interconnected with the OMPFC (Öngür and Price, 2000).

Based on the findings in Chapter 5, the pronounced VP neurodegeneration exclusively at striatal neuropathological grades greater than 2 reflects the pace of degeneration of the ventral striatum. It is widely known, that compared to the dorsal “motor” striatum, which projects to the GPe and
GPi and is severely degenerated in HD, the ventral “limbic” striatum (VS) is remarkably spared, with neurodegeneration only apparent beyond HD grade 2 (Bots and Bruyn, 1981, Vonsattel et al., 1985, Ferrante et al., 1986, de la Monte et al., 1988). Thus, it can be hypothesised that based on the preservation of MSNs which project to the VP (Vonsattel et al., 1985) and the preservation of enkephalin immunoreactivity in the VS in HD (Ferrante et al., 1986), this could translate to the possible sparing of VS-VP projections until >grade 2, which is reflected in later VP neurodegeneration in HD, compared to its motor pallidal (GPe and GPi) counterparts.

The link between VP neurodegeneration and symptom scores relating to cognition (Figure 5.6, Chapter 5) and motor impairment (Figure 5.7, Chapter 5), suggests that the reward limbic pathway, does not work in isolation, but its pathways interface with circuits that mediate cognitive function to affect motor planning (Haber and Knutson, 2010). Diffusion tensor imaging studies also support that such interactive networks exist in the human brain (Draganski et al., 2008), and that there are convergence zones that can link the reward with cognitive circuits to influence motor control circuits.

In studies of other movement disorders, such as Parkinson’s disease (PD), brain structures implicated in the development of cognitive decline includes the “limbic loop,” because bilateral impairment of this loop contributes to the appearance of amnestic dysfunctions and cognitive decline in PD and Alzheimer’s disease (Hyman et al., 1990, Braak et al., 2006). In HD, reversal learning (a measurement of cognition) deficits have been reported. Reversal learning implicates the “affective loop,” which constitutes the orbitofrontal cortex/anterior cingulate cortex/hippocampus/basolateral amygdala – ventral striatum – ventral pallidum – mediodorsal thalamus (structures similarly implicated in the limbic circuit). (Oscar-Berman and Zola-Morgan, 1980, Lawrence et al., 1998). Therefore, it can be possible that the volumetric decline in the VP in HD, as a component of the limbic pathway which shares common connectivity to the affective loop, could have an association with cognitive decline as highlighted by the correlation with MMSE (Figure 5.6, Chapter 5), which could relate to deficits in cognitive tasks such as reversal learning. **The impact of VP degeneration in advanced HD and its relevance to the cognitive decline is highlighted in Figure 7.3.**

The relationship between VP degeneration and motor impairment (Figure 5.7, Chapter 5) could indicate that the pattern of VP degeneration could share some commonalities to its dorsal motor counterpart, the GPe (Chapter 4). Notions of the VP as a striatal output for movement,
comparable to the GP, has originated from the view that it functioned as a motor expression site (Heimer et al., 1982). For example, based on a series of behavioural studies, Mogenson et al proposed that the nucleus accumbens projections to the VP translated limbic motivation signals into motor output (Mogenson and Yang, 1991). This account attributed the “limbic-motor integration” to accumbens-pallidal systems, and specifically identified ventral pallidal projections to the brainstem (i.e. pedunculopontine tegmentum) as a primary motor output for limbic motivation signals. Therefore, in addition to the predominant roles of the VP in reward and motivation, it must also be noted that the VP plays a role in transferring input from accumbens to brainstem motor-related targets (Mogenson and Yang, 1991, Smith et al., 2009). Thereby, it could be possible, that the relationship between VP atrophy and motor impairment is a reflection of processing and interaction of reward and motor circuits, possibly through a “motivation-to-movement” interface through the basal ganglia (Haber and Knutson, 2010). **The impact of VP degeneration in advanced HD and its relevance to the motor impairment, based on the dysfunction of VP projections to the brainstem, is highlighted in Figure 7.3.**

Thus, these findings show that although VP degeneration was found to be linked with limbic system related changes such as outward irritability (Figure 5.3, Chapter 5), which was discussed in section 5.5.8 Chapter 5, **its unexpected link with cognitive and motor impairment reinforces that the ventral limbic basal ganglia network, while at the heart of reward processing, interacts with cognitive and motor networks** (Percheron and Filion, 1991, Joel and Weiner, 1994, Haber et al., 2000, McFarland and Haber, 2001, Draganski et al., 2008, Haber and Knutson, 2010).

In conclusion, the findings of this present study provide a new and exciting perspective on the significance of the pattern of pallidal degeneration in HD, by demonstrating that symptom heterogeneity in HD is reflected by the variable pattern of degeneration of different pallidal regions in the basal ganglia. These novel findings support the view that the impact of variable degeneration of the **direct, indirect, and limbic** pathways of the basal ganglia may be an underlying principle of symptom heterogeneity in HD.
Figure 7.3 The impact of degeneration of the globus pallidus internal segment (GPI) and the ventral pallidum (VP) on circuits which integrate with the limbic pathway at striatal neuropathological grade 3

In advanced HD, a relationship was found between neurodegeneration of the GPI, and both anxiety and irritability. It could be possible that these relationships are signifying potential dysfunction of pallidal neurons projecting to the lateral habenular nucleus (indicated with an exclamation symbol), which is a structure heavily implicated in the reward pathway.

Furthermore, a relationship was found between neurodegeneration in the VP and cognitive impairment, which could implicate dysfunction of the “affective loop” involving the prefrontal cortex – ventral striatum – ventral pallidum – mediodorsal thalamic nucleus (as indicated with a thunderbolt between the VP and MD). In addition, a relationship was found between VP neurodegeneration and motor impairment, which indicates that VP-brainstem motor output may be compromised (as shown by a thunderbolt between the VP and brainstem).

The continuous and dotted lines indicate excitatory and inhibitory pathways, respectively. GPe, globus pallidus external segment; GPI, globus pallidus internal segment; MD, medial dorsal thalamic nucleus; L. Habenular nucleus, lateral habenular nucleus; Orbital and medial PFC, orbital and medial prefrontal cortex.
Figure 7.3 The impact of degeneration of the globus pallidus internal segment (GPI) and the ventral pallidum (VP) on circuits which integrate with the limbic pathway at striatal neuropathological grade 3

Grade 3 HD – Mood dysfunction

Atrophy of VP and minor cell loss:
- linked with dysfunction of the “affective” cognitive loop
- linked with dysfunction of “limbic-motor” integration systems through the brainstem

GPI neurodegeneration:
- linked with anxiety and irritability, possibly though connections with the lateral habenular nucleus and reward pathways
7.6 Possible mechanisms for pallidal neurodegeneration in HD

The mechanisms which provide the environment for a greater extent of neurodegeneration in the GPe compared to the GPi and VP is not understood. The exact mechanisms of neuronal cell death in HD are currently unclear in all aspects of HD research. The expanded CAG repeat of the HD gene is expected to interact with large numbers of other genes, as shown by the results of gene microarray studies, indicating large numbers of affected genes in studies on both post-mortem HD tissue (Hodges et al., 2006) and animal models of HD (Luthi-Carter et al., 2000). These interactions lead to a complex set of parameters that may involve transcriptional dysregulation, excitotoxicity, oxidative stress, changes in neurotransmitters, and breakdown of cellular and vesicular transport mechanisms which could be operative in neurons throughout the brain (Cha, 2000, Morton et al., 2001, Zuccato et al., 2001, DiProspero et al., 2004, Cattaneo et al., 2005, Rosas et al., 2008). However, regardless of what mechanisms are involved, the prevailing question remains, is the loss of pallidal neurons due to one of the above primary processes (cell autonomous mechanism)? Or is it consequential to striatal neuron dysfunction (a by-product of the loss of striatal input)?

As discussed in Chapter 4, the overall density of pallidal neurons in the GPe did not dramatically increase in HD as proposed by early studies (Lange et al., 1976, Wakai et al., 1993), challenging the current view that GPe atrophy is only a by-product of striato-pallidal projection loss. Thus, it is possible that there is involvement of cell-autonomous mechanisms in the GPe: that is, degeneration and neuronal death independent of mutant damage accumulated within other cell types that interact with pallidal neurons. It is, thereby, possible that the interaction of the HD gene on pallidal neuron gene expression in the GPe could be leading to pallidal neuron degeneration, possibly by the mechanisms of cell death as described above, which needs further investigation.

However, non-cell-autonomous mechanisms also need investigation, particularly with regard to GPi neurodegeneration. As highlighted in Chapter 6, the overall density of pallidal neurons in the GPi increased in HD, thereby, reinforcing that GPi atrophy is mainly a by-product of striato-pallidal projection loss, and to a lesser degree pallidal neuron loss – suggestive of a predominant role of non-cell-autonomous mechanisms with regard to pallidal neuron loss in the GPi. The HD mechanism of non-cell-autonomous cell death is based on pathological cell-cell interactions, at
least in small animal models as shown with cortical-striatal interactions. This has been demonstrated by selective expression of mutant huntingtin, with an expanded CAG repeat within specific neuronal cell types (Gu et al., 2005, Gu et al., 2007). In these rodent models, progressive motor deficits and cortico-striatal neuropathology was only observed when mutant huntingtin expression was activated in many neuronal (and glial) cell types, including striatal medium spiny neurons, cortical interneurons and cortical pyramidal neurons. However, deficits were not apparent when mutant huntingtin was restricted to either cortical neurons, or striatal medium spiny neurons alone (Gu et al., 2005, Gu et al., 2007). This suggests that cell-cell interactions, whether it is between cortico-striatal neurons, or neuronal-glia interactions, are required for HD pathogenesis in these models. Thus, this conclusion could also imply that striatal-pallidal pathological interactions are necessary for non-cell-autonomous death of pallidal neurons, especially in the GPi where striatal projections are lost later into the region, compared to striatal-GPe projections.

Furthermore, it is possible that non-neuronal cells which interact with pallidal neurons, such as glia, could be involved in non-cell-autonomous degeneration of pallidal neurons. Studies using primary cell culture showed that astrocytes derived from R6/2 transgenic mice models of HD have a reduced capacity to transport glutamate, and thus cannot protect mutant neurons from glutamate excitotoxicity (Shin et al., 2005). Rodent models of other neurodegenerative diseases, such as amyotrophic lateral sclerosis (Boillée et al., 2006), frontotemporal dementia (Higuchi et al., 2005), and another CAG repeat disorder, spinocerebellar ataxia (Custer et al., 2006), all provide in vivo genetic evidence that non-neuronal cells may contribute to neurodegeneration. These sources of evidence suggest that pathological cell-cell interactions might be a common cellular mechanism for neurodegenerative disorders, which could apply to pallidal neuron-glia interactions in HD. The possible ways for glia involvement in non-cell-autonomous degeneration of neurons could include: (1) toxicity within the affected neurons could trigger damaging responses from glia which are not directly damaged by this toxicity or by their own synthesis of the mutant huntingtin protein; (2) mutant huntingtin expression (or toxicity) in glia could perturb the normal glia response, amplifying initial damage to the vulnerable neurons; or (3) mutant huntingtin expression (or toxicity) within the glia could disturb normal glial function, therefore becoming a primary source of neurotoxicity, potentially independent of mutant effects within the neurons at risk (Lobsiger and Cleveland, 2007).
It could also be possible, that pallidal neurons within particular regions such as the GPi could generally be more resistant to cell death compared to neurons within the GPe. If it is assumed that cell death in the pallidum in HD is attributable to the selective and differential vulnerability of striatal projection neuron input, what makes striatal-GPi neurons more resistant than striato-GPe neurons to degeneration in HD is not known (Deng et al., 2004). Current research has focused on the role of glutamate subunit configuration, free radical defences, calcium sequestering, and anti-apoptotic mechanisms to HD pathogenesis which could account for the resistance (DiFiglia, 1990, Hedreen and Folstein, 1995, Huang et al., 1995, Chen et al., 1996, Medina et al., 1996, Calabresi et al., 1998, Chen et al., 1998, Figueredo-Cardenas et al., 1998, Hackam et al., 2000, Zeron et al., 2001, Gervais et al., 2002).

However, it must be emphasised that post-mortem human studies such as presented here, can only report on the end stage pathology and are therefore limited in providing evidence on pathogenesis during the course of neurodegeneration. No doubt, both cell-autonomous and non-cell-autonomous mechanisms play a role in neurodegeneration in HD, and our studies in the post-mortem human brain cannot make any definitive statement on the precise pathogenesis.
7.7 Future directions

Characterisation of the Thalamus in HD

The pattern of motor, behavioural and cognitive symptoms in HD implicates dysfunction of not only the basal ganglia, but the entire basal ganglia-thalamo-cortical circuits. As the GPe, GPi and VP all project to nuclei within the thalamus, it would be very interesting to investigate the role of neurodegeneration in the thalamus in Huntington’s disease using stereological-based techniques. Some stereological-based studies have found a 55% loss of neurons in the thalamic centromedial-parafascicular complex, and a 23.8% reduction in the number of neurons in the mediodorsal nucleus in post-mortem HD tissue (Heinsen et al., 1996, Heinsen et al., 1999). Furthermore, modern neuroimaging techniques combining MRI with voxel-based morphometry have found that the mediodorsal nucleus and the centromedian/parafascicular and ventrolateral nuclear complex displayed volume loss, in agreement with neuropathological studies (Kassubek et al., 2005). Thus, the further study of all thalamic nuclei in detail using design-based stereology, and linking changes to symptom heterogeneity, would thereby further outline the clear role of the thalamus in HD. Furthermore, the identification of specific cell types in the thalamus which are susceptible to cell death in HD has never been undertaken, and it would therefore be critical to use different immunohistochemical markers to delineate different cell types in the thalamus.

Morphological changes to pallidal neuron processes in HD

As highlighted in the findings presented above, in addition to pallidal neuronal loss, considerable soma atrophy was also found of remaining pallidal neurons (35% and 21% reduction in the GPe and GPi, respectively), which could be indicative of ongoing neuronal dysfunction which proceeds cell death in Huntington’s disease (Ross and Tabrizi, 2011). In many neurodegenerative diseases, cells which remain may show degenerative changes in terms of size, shape, and morphology. Although these changes are not specific, they might represent definite ongoing pathogenesis of neurons (Kanazawa, 2001, Graeber and Moran, 2002). It is postulated that a loss or thinning of dendrites are major contributors of neuronal degeneration (Klapstein et al., 2001). Thus, it would be of considerable interest to examine the density of pallidal dendrites using high resolution image analysis similar to the methodology of Deng and Sapp et al (Sapp et al., 1995, Deng et al., 2004). In addition, immuno-electron microscopy methods have been developed in our laboratory which could be used to detect, at an ultrastructural level, detailed pallidal neuron morphology changes in
terms of synaptic remodelling, as well as detailed GABA/glutamate receptor distribution along pallidal dendrites and axons. Detailed ultrastructural studies have been conducted in the human spinal cord by our laboratory (Waldvogel et al., 1990), and the basal ganglia of the primate (Waldvogel et al., 1998). Currently, no studies have been conducted on the detailed ultrastructural changes to pallidal neurons axons and dendrites at different striatal grades of HD using this method.

**Glutamate receptors in the globus pallidus in HD**

Although the globus pallidus is predominantly GABAergic in activity, neurons from the GPe, GPi and VP all receive excitatory glutamatergic input from the subthalamic nucleus (Kita, 2007, Nambu, 2007, Haber and Knutson, 2010). The GABAergic inhibitory system has been extensively characterised in the control and HD brain (Waldvogel et al., 1999, Thompson-Vest et al., 2003, Waldvogel et al., 2004, Allen et al., 2009). However, the glutamatergic system is yet to be fully characterised. The upregulation of GABA receptors in HD was a crucial finding, as it explained a possible compensatory coping mechanism for pallidal neurons to increase the sensitivity to reduced levels of GABA that occurs in HD due to the loss of MSN input (Allen et al., 2009). Thus, to better understand the pathophysiology of the basal ganglia circuitry in HD, an understanding of the excitatory system is also required, and therefore a characterisation of glutamate receptors in the globus pallidus may aid in understanding the heterogeneity of HD symptoms.

**Astrocytes, microglia and oligodendrocytes in the globus pallidus in HD**

As shown in this study, it was interesting to observe an increase in small, punctate Nissl-positive glial cells in each pallidal segment with advancing HD grade; these are likely to be a collection of astrocytes, oligodendrocytes and microglia (Figure 4.10, 5.10 and 6.10 Chapters 4, 5 and 6), which is consistent with other qualitative observations (Lange et al., 1976). Increased oligodendrocyte densities have been documented in the human caudate nucleus in HD, but limited information is available relating to the GP (Myers et al., 1991). Progressive reactive microgliosis is an established feature of HD in humans (Sapp et al., 2001), and a grade-dependent increase in microglia has been reported in the cortex and striatum (Sapp et al., 1999). Several studies have documented glial cell changes in HD and other neurodegenerative diseases (Shin et al., 2005, Lobsiger and Cleveland, 2007). Glutamate-mediated excitotoxicity is a prime example of neuroglia toxicity that has been proposed to be a significant component of HD (Shin et al., 2005).
Extracellular glutamate removal is performed by GLT-1 (glutamate transporter-1) and GLAST (glutamate aspartate transporter) receptors which are expressed on astrocytes and transported into the cytoplasm, where glutamate is then metabolised by glutamine synthase (Maragakis and Rothstein, 2004). In mouse models of HD, decreased levels of GLT-1 transporter and its activity are found. And furthermore, in mouse-neuron astrocyte co-cultures, mutant astrocytes increase neuronal vulnerability to excitotoxicity (Shin et al., 2005, Lobsiger and Cleveland, 2007). Thus, these studies, combined with the qualitative descriptions of increased gliosis in the HD globus pallidus as presented in this study, suggests that glial cells may play a role in HD pathogenesis. Therefore, quantitative characterisation of reactive gliosis in the HD globus pallidus, as well as astrocytes and oligodendrocytes would be a benefit to discerning the role of these cells in the GPe, GPi and VP in HD.

**Gene, protein and functional studies**

In order to further understand the functional significance of the patterns of neuronal degeneration in HD, it is important to gain an understanding of the molecular, protein, and functional basis of symptom heterogeneity in HD. Previous studies have shown that there are changes in gene expression in the striatum and cortex in HD (Luthi-Carter et al., 2000, Hodges et al., 2006). For example, after analysing messenger RNA (mRNA) profiles from a sample of human HD brains compared to control using microarray analysis, the greatest number and magnitude of differentially expressed mRNAs were detected in tissue homogenates of the caudate nucleus, highlighting that the molecular phenotype parallels neuropathology (Hodges et al., 2006). Furthermore, examination of mRNA levels in laser-capture microdissected neurons from grade 1 HD caudate nucleus confirmed that these mRNA changes were not simply due to the loss of neurons, because the changes in mRNA expression were independent of neuronal loss (Hodges et al., 2006). Thus, it could be possible that genome-wide profiling of pallidal neurons could be investigated using techniques such as microarrays and laser capture microscopy, where single pallidal neurons could be dissected out of the tissue for genetic and proteomic analysis, and thereby determine how HD affects the gene expression profile in different pallidal regions which play differential roles in the basal ganglia circuitry. Such investigations could extend on the findings of this study by revealing the regional pallidal gene expression profile at the cellular level which could help to explain the aberrant molecular processes underpinning the variable extent of pallidal regional degeneration and its relationship to the basal ganglia and symptom heterogeneity in HD. Furthermore, this study did not quantify the levels of the calcium-binding protein parvalbumin in pallidal neurons and whether
it changes in HD. According to recent studies, the unique cell type-specific distribution in different parts of the brain suggests that calcium-binding proteins have evolved as functionally distinct, physiologically relevant modulators of intracellular calcium transients, and are possibly involved in regulating calcium pools critical for synaptic plasticity (Schwaller et al., 2002). Thus, additional analyses including western blotting, ELISA (enzyme-linked immunosorbent assay), in situ hybridisation, real time PCR (polymerase chain reaction) and reverse transcriptase PCR techniques could be used to determine if there is variability in gene expression, or specific changes in the levels of protein concentrations, such as calcium-binding proteins including parvalbumin, in the HD globus pallidus.

Finally, electrophysiological studies in small rodent transgenic models of HD have shown a decrease in firing frequency of the majority of neurons in the globus pallidus in HD (Vlamings et al., 2012). Although not currently feasible on human tissue, further studies to elucidate the electrophysiological roles of pallidal neurons in HD would aid in understanding the significance of these neurons to basal ganglia function and dysfunction in disease states. Furthermore, these could extend to studies of overall neuronal activity at the cellular level, by looking at metabolic substrates such as cytochrome oxidase and other key players in the mitochondrial energy apparatus (Vlamings et al., 2012) during live cell imaging. The importance of electrophysiological studies with regard to pallidal neurons have been highlighted recently by Mallet et al (2008, 2012), who have defined an analogous division of labour in the GPe of 6-OHDA-lesioned Parkinsonian rats, showing the distinct temporal activities of two populations of GPe neurons in vivo. A first population of prototypic GABAergic GPe neurons fire antiphase to subthalamic (STN) neurons, whereas a second population (arkypallidal neurons) fire in-phase with STN neurons and only innervate the striatum (Mallet et al., 2008, Mallet et al., 2012). Thus, the study of electrophysiological properties of pallidal neurons in transgenic models of HD, similarly to what has been found in models of Parkinson’s disease, could contribute to a better understanding of the cellular dysfunction, particularly if functionally diverse cell types are found to be preferentially affected.

Therefore, these studies could contribute to the understanding of the molecular, protein and functional basis of the differential susceptibility of the GPe, GPi and VP to HD. This would enable a better understanding of the correlation between neurodegeneration, basal ganglia dysfunction and symptom heterogeneity in HD, as well as other neurodegenerative diseases including Parkinson’s and Alzheimer’s diseases which are also characterised by heterogeneous symptomatology.
7.8 Conclusion

This is the first comprehensive detailed research on the neurodegeneration of three functionally diverse pallidal regions of the basal ganglia in Huntington’s disease (HD). Our results suggest that the neurodegeneration of the globus pallidus externus (GPe), the globus pallidus internus (GPi) and the ventral pallidum (VP) have different impacts on the motor, limbic, and cognitive circuits, depending on the stage of striatal neurodegeneration, their main source of input. The general implication of the results is that the HD gene mutation produces variable degrees of pallidal degeneration, in terms of differential degrees of regional atrophy, cell loss, and cellular atrophy, which could contribute to variable dysfunction of basal ganglia circuits, and explain symptom heterogeneity. The relationship between striatal neuropathology and pallidal degeneration highlighted the differential vulnerability of various striatal projections into each pallidal region in HD. The relationship established between symptom scores and pallidal degeneration provides a novel perspective for understanding basal ganglia circuit dysfunction, and clinical heterogeneity involving the pallidum in HD.

The detailed pattern of neurodegeneration of the pallidal regions investigated in this thesis, have further implications for understanding the variability of disease progression in HD. Although these structures are known for their defined role in certain basal ganglia circuits, it is clear that their pattern of neurodegeneration with relation to symptom scores revealed unexpected additional roles. For example, in addition to the established role of the GPi in the direct motor circuit, GPi neurodegeneration in HD was shown to correlate with scores relating to irritability and anxiety, signifying an additional role in limbic and mood related pathways. In the case of the VP, its unexpected link with cognitive and motor impairment reinforces that the ventral limbic basal ganglia network interacts with cognitive and motor networks. Indeed, these results suggest that the degeneration of circuits implicating the basal ganglia in HD is not as simple as dysfunction of specific pathways relating to a certain symptom, but it is likely that there are multiple pathways of degeneration affecting different cell types in different brain regions, and these pathways intermingle. Finally, to fully define the impact of HD on the entire basal ganglia circuit, an investigation of the main structure of pallidal output, the thalamus, is required, and this will enhance our understanding of the circuits involved in HD, through relating pathological changes to clinical heterogeneity.
REFERENCES


Cavalieri B (1653) Geometria indivisibilibus continuorum nova quadam ratione promota: Duciis.


underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90:537-548.


Gerfen CR (1992) The neostriatal mosaic: multiple levels of compartmental organization in the basal ganglia. Annu Rev Neurosci 15:285-320.


3-D arrangements of particles by point processes with examples of application to
symptomatology and nursing care. Nurses must balance safety, quality-of-life issues, the
need for personal choice, and the meaning of human dignity when caring for patients
Kremer B, Almqvist E, Theillmann J, Spence N, Telenius H, Goldberg Y, Hayden M (1995) Sex-
dependent mechanisms for expansions and contractions of the CAG repeat on affected
Disord 11:136-142.
Kuo JS, Carpenter MB (1973) Organization of pallidothalamic projections in the rhesus monkey.
J Comp Neurol 151:201-235.
Langbehn DR, Hayden MR, Paulsen JS (2010) CAG-repeat length and the age of onset in
Huntington disease (HD): A review and validation study of statistical approaches. Am J
Lange H, Threrner G, Hopf A, Schröder KF (1976) Morphometric studies of the
Lavoie B, Parent A (1990) Immunohistochemical study of the serotonergic innervation of the
basal ganglia in the squirrel monkey. J Comp Neurol 299:1-16.
Lavoie B, Parent A (1994) Pedunculopontine nucleus in the squirrel monkey: projections to the
basal ganglia as revealed by anterograde tract-tracing methods. J Comp Neurol 344:210-
231.
Lavoie B, Smith Y, Parent A (1989) Dopaminergic innervation of the basal ganglia in the
squirrel monkey as revealed by tyrosine hydroxylase immunohistochemistry. J Comp
Neurol 289:36-52.
Lawrence AD, Sahakian BJ, Robbins TW (1998) Cognitive functions and corticostriatal circuits:
visuospatial cognition in Huntington's disease: implications for information processing in
O, Ross CA, Margolis RL (2003) A genome scan for modifiers of age at onset in
Ligot N, Krystkowiak P, Simonin C, Goldman S, Peigneux P, Naeman J, Monclus M, Lacroix F,
Devos D, Dujardin K, Delmaire C, Bardinet E, Delval A, Dellaux M, Defebvre L,
46.
homeostasis and mitochondrial dysfunction in striatal neurons of Huntington disease. J


