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Interneuron Cell Loss in the Human Cerebral Cortex in Huntington’s Disease

Eric H. W. Kim

ABSTRACT

Huntington’s disease (HD) is characterised by variable symptoms (choreiform movements, cognitive, mood and neuropsychological change) and variable neuropathology in the basal ganglia and the cerebral cortex. Our recent studies have shown that the phenotypic variability in HD is associated with the variable pattern of pyramidal cell loss in the cerebral cortex (Thu et al., 2010). We are now extending these studies to the cortical GABAergic interneurons to determine whether the symptom variation in HD is also associated with the variable pattern of interneuron cell loss in the cerebral cortex. The GABAergic interneurons in the cortex are inhibitory neurons that act locally to modulate the activity of pyramidal neurons, hence are critical determinants of cortical output. To undertake this study, unbiased stereological cell counting methods were used to quantify three major types of GABAergic interneurons immunoreactive for calbindin-D28k, calretinin, and parvalbumin in the primary motor and anterior cingulate cortices in 13 HD and 14 matched control cases of the post-mortem human brain. The HD cases were categorised into 3 dominant symptom groups (“motor”, “mood”, or “mixed” groups). Detailed data on the symptomatology of HD cases was collected from family members and clinical records as previously described (Tippett et al., 2007; Thu, et al., 2010). The results demonstrated a heterogeneous loss of interneurons across the two cortical regions in HD cases compared to control cases. Most interestingly, the major findings of the present study showed a significant association between the pattern of cortical interneuron cell loss and the variable symptomatology in HD. The results showed that in the anterior cingulate cortex, there was a major significant interneuron loss in all three interneuronal populations (71% loss of calbindin-D28k⁺, 60% loss of calretinin⁺, 80% loss of parvalbumin⁺ interneurons) in HD cases with major “mood” disorder, but no significant interneuron loss was observed in the cingulate cortex in the HD cases with major “motor” symptoms. By contrast, in the primary motor cortex, there was a selective loss of calbindin-D28k⁺ interneurons (57% loss) in HD cases with major “motor” symptoms, but no significant interneuron loss was observed in the motor cortex in cases with a dominant mood phenotype. These findings show that there is a major heterogeneous pattern of interneuron loss in the cerebral cortex which correlates with symptom variation in HD, which suggests that cortical neurodegeneration is a key component in understanding the neural basis of clinical heterogeneity in HD.
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CHAPTER 1. GENERAL INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder characterised by progressive involuntary choreiform movements, cognitive and psychiatric symptoms (Nance, 1998; Walker, 2007). The disease was previously documented by others (Waters, 1842; Lund, 1860; Lyon, 1863), but only received widespread recognition after a comprehensive description in the paper “On Chorea” by George Huntington (1872). The discovery of the HD gene and its mutation on chromosome 4 in 1993 (Huntington's Disease Collaborative Research Group, 1993) lead to more reliable measures for diagnosis of HD (Kremer et al., 1994) and offered new strategies for therapeutic interventions. Huntington’s disease belongs to a family of polyglutamine diseases with expanded CAG trinucleotide repeats in the mutant gene (Everett & Wood, 2004). The protein encoded by the HD gene, huntingtin, is a large protein with an expanded polyglutamine tract near the N-terminus (~350 kDa) that is highly conserved and expressed ubiquitously throughout the body (Strong et al., 1993). In the brain, it is predominantly found in neurons (Landwehrmeyer et al., 1995; Ferrante et al., 1997). The exact pathogenic mechanism by which mutant huntingtin causes degeneration and dysfunction of neurons is not entirely known; however, abnormal depositions of huntingtin fragments in the nuclei and cytoplasm of neurons and the formation of protein aggregates have been postulated to initiate a pathogenic cascade leading to neuronal death (DiFiglia et al., 1997; Gutekunst et al., 1999; Imarisio et al., 2008).

The major neuronal degeneration in HD occurs in the striatum of the basal ganglia and the cerebral cortex (Vonsattel & DiFiglia, 1998; Vonsattel et al., 2008). An early account of neuropathological abnormalities was described by Meynert (1877) and proposed that chorea may be explained by lesions in the corpus striatum. Indeed, the most characteristic neuropathological features are neuronal loss and gliosis in the neostriatum (caudate-putamen). But the pathology is not only restricted to the neostriatum. The cerebral cortex and several subcortical structures are also affected. The progression of striatal degeneration
follows a characteristic course, while the sequence of extrastriatal degeneration, especially the cerebral cortex, and the differential vulnerability of neurons and their interactions are yet to be fully established.

Despite the characteristic triad of clinical symptoms (motor, mood, cognitive) in HD, there is considerable variation in symptoms at clinical onset and throughout the disease course. This can be observed by remarkable symptom differences in monozygotic twins with the same genetic mutation and environmental factors (Georgiou et al., 1999; Anca et al., 2004; Friedman et al., 2005; Gomez-Esteban et al., 2007). Although the number of CAG repeats in the HD gene has been correlated with the age of symptom onset in HD (Andrew et al., 1993; Duyao et al., 1993; Wexler et al., 2004), there is no clear association between the CAG repeat length and the symptom variation. Consequently, there is much interest in whether there are any underlying pathological differences in HD brains which may account for symptom heterogeneity. Characteristic phenotypic manifestation is likely to represent neuronal degeneration and dysfunction in the brain regions responsible for specific functional roles. This is of particular interest in our lab, and neuronal loss in a number of regions across the brain has been investigated in extensively characterised HD cases with specific symptom profiles (Thu, 2006; Tippett, et al., 2007; Nana, 2009; Thu, et al., 2010).

For example, recent evidence suggests that the variation in clinical symptoms exhibited by each HD case is strongly associated with the variable pattern of neurodegeneration in two major compartments of the striatum and also in the different functional regions of the cerebral cortex. In the striatum, there was a differential pattern of degeneration in the two compartments, namely the striosomes and the matrix which are involved in the limbic and motor functions, respectively (Tippett, et al., 2007). Some cases showed a selective loss of striatal neurons and neurochemical markers such as GABA_A receptor loss in the striosomes, whereas others showed selective changes in the matrix compartment. This differential compartmental pattern of striatal degeneration has been shown to correlate with the variable symptomatology in HD cases, where cases with major mood symptoms showed a profound degeneration in the striosomes (Tippett, et al., 2007).
In the cerebral cortex, the phenotypic variability in HD has also been associated with the variable neuronal degeneration. For example, alterations in the circuitry of primary motor cortex and anterior cingulate cortex may contribute respectively to impairments of certain motor and mood functions in HD. The detailed pathological studies across 8 regions of the cerebral cortex in our laboratory by Dr. D. Thu and Dr. A. Nana showed that there was a major cell loss in all neuronal types (using NeuN marker) and pyramidal cell loss (using SMI32 marker) (Thu, 2006; Nana, 2009; Thu, et al., 2010). In particular, HD patients with severe motor impairment showed a major cell loss in the primary motor cortex (BA 4), while HD patients with major mood component did not show significant cellular changes in the motor cortex. Conversely, there was a significant cell loss in the anterior cingulate cortex (BA 24) in cases with predominant mood disorder, while no change was observed in the HD cases with severe motor symptoms in the cingulate cortex (Thu, et al., 2010). In addition, functional MRI studies have also suggested that cortical changes play an important role in the development of heterogeneous clinical symptoms in HD (Sax et al., 1996; Rosas et al., 2002; Montoya et al., 2006; Rosas et al., 2008; Paulsen, 2009; Nopoulos et al., 2010).

These studies suggest that the underlying cortical degeneration is associated with the heterogeneous pattern of symptoms in HD and have facilitated further investigation in addressing whether there is a differential or selective cell loss of the different cortical neuronal types, i.e., GABAergic interneurons, and its relation to the symptom profile in HD.

Therefore, the overall aim of this study is to provide an insight into the pathological outcomes of GABAergic interneurons in the cerebral cortex of the human brain in HD and thus to provide a better understanding of the cortical pathogenesis in HD. In addition, it is hoped that this study will provide the basis for evaluating the validity of animal based models of HD and above all, that this research will provide a better understanding on the anatomical-functional relationships of the human brain, which will aid in research on other neurodegenerative diseases of the central nervous system. The literature review below outlines the neuronal architecture and the pathways of the cerebral cortex with specific emphasis on the involvement of GABAergic interneurons in HD and the potential link to the pattern of symptoms in HD.
CHAPTER 2. LITERATURE REVIEW

2.1. Cerebral Cortex

2.1.1. Introduction to the Cerebral Cortex

The cerebral cortex is a sheet of brain tissue that forms the outermost layer of the cerebrum. This highly complex structure develops from the dorsal pallial sector of the telencephalic hemispheres (Nieuwenhuys et al., 2008). All mammals possess a neocortex, which is differentiated into a six-layered structure. The term neocortex refers to its phylogenetically most recent appearance, in comparison to the older allocortex which can be divided into the palaeocortical olfactory cortex and the more ancient archicortical hippocampal cortex (Filimonoff, 1947). The relative thickness of the different regions and layers of the cerebral cortex does not vary substantially between different species, and averages between 1.5 to 4.5 mm (Brodmann, 2006). The surface area, however, is markedly increased in the human, which is reflected by its distinct convoluted shape (Zilles et al., 1988; Toro et al., 2008; Rakic, 2009).

2.1.2. Anatomical Organisation

The human cerebral cortex is divided into five major lobes: frontal, parietal, occipital, temporal, and limbic (Figure 2.1). The five lobes are anatomically defined by prominent sulci (or fissures) that demarcate gyri (or convolutions) of the cortex (Nieuwenhuys, et al., 2008). On the lateral surface, the deep central sulcus (fissure of Rolando) separates the frontal and parietal lobes. Basolaterally, the lateral sulcus (or Sylvian fissure) separates the frontal and parietal lobes from the temporal lobe. The border between the parietal and occipital lobes is indicated by the parieto-occipital sulcus present on the medial aspect of the hemisphere. The border of the occipital and temporal lobes is indicated by the preoccipital notch at the ventral surface. The limbic lobe is used to describe the gyri located at the inner edge (or limbus) of the hemisphere (Broca, 1878).
The frontal lobe is highly developed and comprises approximately one third of the entire hemispheric surface. The precentral gyrus and the three frontal gyri occupy the lateral surface of the frontal lobe; its basal or ventral (orbital) surface consists of the orbital gyri and the gyrus rectus. On the medial surface, the frontal lobe is separated from the limbic lobe by the cingulate sulcus, and from the parietal lobe by an arbitrary “extended” vertical line connecting the central sulcus with the cingulate sulcus. The parietal lobe can be subdivided into the postcentral gyrus and the superior and inferior parietal lobules. The latter includes the supramarginal and the angular gyri. The occipital lobe is located in the rearmost portion of the brain. On its medial aspect, the calcarine sulcus separates the cuneus from the occipital part of the medial occipitotemporal gyrus. The temporal lobe can be subdivided into superior, middle and inferior gyri on the lateral surface. The ventral and medial aspects of the temporal lobe are dominated by the occipitotemporal gyrus and by structures belonging to the inferior portion of the limbic lobe. The limbic lobe comprises of the medial mantle of the hemisphere and consists of the cingulate gyrus, which dorsally surrounds the corpus callosum, and the parahippocampal and hippocampal gyri, which are located medially on the ventral side of the temporal lobe.

![Figure 2.1 Brain lobes of the human cerebral cortex](image)

**Figure 2.1 Brain lobes of the human cerebral cortex**
The anatomically defined five major lobes of the cerebral cortex from the (A) lateral view, and the (B) medial view of the human brain.

In addition, distinctive brain functions have been shown to be localised on specific structures within each lobe of the brain. Prominent functional domains that are investigated in this study, namely the primary motor cortex of the frontal lobe and the anterior cingulate cortex of the limbic lobe are described under Sections 2.1.5 and 2.1.6.
2.1.3. Laminar Organisation of the Neocortex based on Cytoarchitecture

The systematic study of the arrangement of neurons in the brain is known as cytoarchitectonics (Brodmann, 2006; von Economo & Koskinas, 2008). Korbinian Brodmann began his classic studies in 1903 and provided exact localisation of what he later called area 4 and his concept of a six-layered fundamental tectogenetic cortical type during fetal development. The neocortex is also designated as the isocortex or homogenetic cortex. The isocortical areas that retained a six-layered pattern are termed homotypical homogenetic cortex whereas heterotypical homogenetic cortex is used to describe areas in which the six layers are no longer evident in the adult brain, such as the agranular frontal motor cortex and the granular primary visual cortex. Cortical areas that do not show a six-layered pattern during fetal development are called the heterogenetic cortex. By 1909, Brodmann recognised 44 sharply delineated cortical areas and provided a surface map of their locations. In 1925, von Economo and Koskinas further subdivided areas of Brodmann into smaller units and identified 107 functionally distinct fields.

The cortical layers are made up of a set of elements which include the two principal neuronal types (pyramidal neurons and local-circuit granule neurons), glia (astrocytes, oligodendrocytes and microglia), nerve fibres (extrinsic and intrinsic connections), and blood vessels. Beginning at the outer pia mater to the inner white matter, the six neocortical layers based on cytoarchitectural distinction are described as follows (Zilles, 2004; Brodmann, 2006; von Economo & Koskinas, 2008) (Figure 2.2):

(I) **Molecular layer** - this is the most superficial layer that contains very few cell bodies and made up of tangentially oriented dendritic processes of the cells. The axons travel through or form connections in this layer.

(II) **External granular layer** - is composed of small, densely packed granule cells. The name of this layer can be misleading because many of its constituent somata also belong to the small pyramidal neurons.
(III) **External pyramidal layer** - is a thick layer in which medium sized pyramidal somata prevail. Typical cortical pyramidal cells have basal and oblique dendrites and direct their apical dendrites toward the pial surface.

(IV) **Internal granular layer** - consists of small, densely packed granule cells. Thalamocortical axons predominantly terminate in this layer on the dendrites of both pyramidal and local-circuit neurons.

(V) **Internal pyramidal layer** - consists mainly of medium to large sized pyramidal neurons.

(VI) **Multiform layer** - or polymorphic layer, is comprised of a heterogeneous population of cells that borders with the underlying white matter.

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**Figure 2.2 Cytoarchitectonic structure of the human neocortical areas**

Cytoarchitecture of several human neocortical areas, designated with Brodmann's six cortical layers (1909). Figure adapted from Brodmann (1909, 2006) and Nieuwenhuys and colleagues (2008).
2.1.4. Columnar Organisation of the Neocortex

Neurons in the neocortex are not only distributed in horizontal layers but are also composed of radially oriented, column-like modules. A cortical column or module, is a functional domain composed of interconnected neurons arranged in a vertically oriented dimension through the thickness of the cortex (Jones, 2000; DeFelipe et al., 2002; Raghanti et al., 2010; Rockland, 2010). The word “column” was first used by von Economo (1925) to describe cords of cells oriented vertically to the pial surface in the human auditory cortex. The concept of the column as the fundamental processing unit was introduced by Mountcastle, under the term minicolumn - a unit made of ~110 neurons arranged in a narrow chain extending radially across layers II-VI with a transverse diameter of about 40-50 μm (Mountcastle, 1957; Powell & Mountcastle, 1959; Mountcastle, 2003). Similarly, Lorente de Nó (1949) observed intrinsic trans-laminar connections made up of chains of 80-100 neurons in discrete cylindrical patches in lamina IV of the rodent cortex, which he postulated to be the basic “elementary unit” of cortical operation. The ontogenetic radial cell columns during the cortical formation are thought to be the precursors of adult minicolumns (Rakic, 1972, 1995, 2009). Other experiments and network models concluded that there are about \(2 \times 10^8\) minicolumns in humans in the order of 28-60 μm in diameter separated from adjacent minicolumns by cell sparse zones (Schlaug et al., 1995; Buldyrev et al., 2000; Tsunoda et al., 2001; Buxhoeveden & Casanova, 2002; Johansson & Lansner, 2007), except in the primate visual cortex which contains larger columns and approximately 2.5 times the number of neurons (Peters & Rockland, 1994).

Cortical columns (or sometimes referred to as macro- or hyper-columns) are formed by 50-100 minicolumns connected together by common afferents and short range horizontal projections (Mountcastle, 1997; Horton & Adams, 2005). Hence, columns can vary between 300-800 μm in transverse diameter. The prominent examples include the rodent somatosensory (barrel) cortex which possess a topographical representation of the vibrissal follicles (Harris & Woolsey, 1979; Lubke et al., 2000; Land & Erickson, 2005; Schubert et al., 2007) and the ocular dominance columns in the primary visual cortex of the cat, monkey and human (Hubel & Wiesel, 1959, 1968; Hubel & Freeman, 1977; Adams et al., 2007).
2.1.5. Primary Motor Cortex and the Motor Systems

2.1.5.1. Anatomical and Functional Organisation of the Motor Cortex

The term motor cortex describes regions of the cortex involved in the planning, control, and execution of voluntary movements. The pioneering studies on the mapping of the motor cortex were that of Fritsch and Hitzig (1870) and Ferrier (1873) who demonstrated movement of the limbs by applying electrical stimulation to the precentral cortex in small mammals. In addition, Penfield and colleagues (Penfield & Boldrey, 1937; Penfield & Rasmussen, 1950) provided a spatially organised disproportionate somatotopic map of the body of the precentral cortex as depicted by Penfield’s homunculus and similar to what was proposed by Woolsey’s simiusculus (1952). The notion of the structural architectonic division of two motor areas (area 4 and 6 of Brodmann) of the frontal lobe was suggested by Campbell (1905) and Brodmann (1909). Subsequently, Fulton (1935) proposed a functional division of the motor cortex into a primary motor area and a premotor (associative) area, which correspond to Brodmann areas 4 and 6, respectively. However, this classical division has become more intricate and modern parcellation studies have identified multiple nonprimary motor fields that can influence direct and indirect motor output and control. The anatomical and functional properties of the prominent motor areas are outlined as follows:

The primary motor cortex, or M1, is located on the precentral gyrus in the caudal portion of the frontal lobe, anterior to the central sulcus, and extends over the medial surface of the hemisphere (Figure 2.3). It corresponds to area 4 of Brodmann, which is characterised by large cortical thickness with poor lamination, the absence of layer IV and the presence of the giant pyramidal cells of Betz in layer V (Brodmann, 1909; Marin-Padilla, 1970; Sherwood et al., 2003). Lassek (1940) counted approximately 34,000 Betz cells in area 4 of the human brain. The detailed description of area 4 in Brodmann’s original paper (1903) describes a wedge-shaped field that is broader on the superior portion of the precentral gyrus and a narrow basolateral side which covers only the rostral wall of the central sulcus. The giant pyramidal cells send long axons down the spinal cord and synapse onto α-motor neurons that connect to the muscles. M1 works in association with premotor areas, the basal ganglia, the thalamus and the cerebellum (Matelli et al., 2004; Geyer & Zilles, 2005; Chouinard & Paus, 2006).
In humans, area 4 can be further subdivided into the anterior and posterior regions on the basis of cytoarchitectonic data, and denoted as area 4a and 4p, respectively (Geyer et al., 1996). Area 4a contains larger and more densely populated layer III pyramidal cells whereas 4p has smaller, less tightly aggregated pyramidal cells. Functionally, area 4a is more dependent on the feedback from other systems, e.g., the activity can be elicited by imaginary finger movement (Ehrsson et al., 2003), listening to speech without actual movements (Wilson et al., 2004), and the activation is associated with complex sensorimotor interaction (Terumitsu et al., 2009); whereas area 4p activation can be modulated by attention without any sensory feedback (Binkofski et al., 2002). Kinesthetic motor illusion elicited by vibro-tactile stimuli activated both areas (Naito et al., 2005). Hence, there is a differential mode of action in the two subregions of the primary motor cortex where area 4a has been shown to be more important for the execution of motion involving more complex sensorimotor interaction while area 4p is more important for the initiation of motion. In addition to direct motor control, the motor cortex has been shown to be active in the areas that relate to words associated with a particular action (De Lafuente & Romo, 2004).

**Figure 2.3 Motor areas 4 and 6 of the human brain**

Identification of the motor cortices from the (A) lateral view, and the (B) medial view of the human brain. The Brodmann cytoarchitectural motor areas 4 and 6 are indicated on the (C) lateral view, and the (D) medial view of the brain. Figure adapted from Brodmann (1909, 2006).
The premotor area, or area 6 of Brodmann lies immediately anterior to the primary motor cortex (Figure 2.3). The portions of area 6 situated on the lateral surface of the hemisphere corresponds to the premotor cortex (PM), which can be subdivided into a dorsal (PMd) and a ventral zone (PMv). Both of these regions can be further subdivided into the rostral and caudal parts. The medial portion of area 6 is occupied by two functional areas, the rostral presupplementary motor area (pre-SMA) and the caudal supplementary motor area (SMA proper), or M2, that forms a subregion of the paracentral lobule. In addition to the premotor area 6, other nonprimary motor fields include Broca’s speech area located on the caudal end of the inferior frontal gyrus and the cingulate motor areas (CMA) on the medial surface which include rostral and caudal cingulate motor areas, CMAr and CMAc (also denoted M3 and M4, respectively), buried in the cingulate sulcus. The CMAr forms a subfield of Brodmann area 24 (areas 24c and 24c’) and CMAc (area 23c) borders on the SMA. The nonprimary motor fields also comprises the frontal eye field (FEF) and the supplementary eye field (SEF) which, as their names imply, are both involved in oculomotor function (Luppino & Rizzolatti, 2000; Schieber, 2001; Chouinard & Paus, 2006).

2.1.5.2. The Afferent and Efferent Circuitry of the Primary Motor Cortex

The primary motor cortex receives afferents from both cortical and subcortical sources. M1 maintains extensive reciprocal connections with the primary somatosensory cortex, or S1 (areas 1, 2, 3b), and the somatosensory association cortex (area 5) in the parietal lobe (Rizzolatti et al., 1997). The cortical afferents also originate from the premotor and other nonprimary motor areas. Somatotopically arranged subcortical afferents to the M1 and premotor areas arise principally from the rostral and dorsal dentate and anterior interposed nuclei of the cerebellum, via the posterior division of the ventral lateral thalamic nucleus (VLp). Striatopallidal projections are relayed by the anterior division of the ventral lateral thalamic nucleus (VLa) and the ventral anterior nucleus (VA) (Asanuma et al., 1983a, 1983b; Hirai & Jones, 1989).

The primary motor cortex contributes substantially to the corticospinal and corticobulbar tracts. The efferent fibres that originate from layer V pyramidal cells of area 4 terminate onto α-motorneurons, interneurons and then to the γ-motorneurons of the spinal cord via
interneurons (corticospinal tract), and thereby controls the movement of both the proximal and distal muscle groups of the limbs (He et al., 1993). These direct efferent fibres of area 4 constitute about 30% to the pyramidal tract (Dum & Strick, 1991). The axons of area 4 also terminate in the brainstem motor nuclei where the tract directly innervates the motor nuclei for cranial nerves V, VII, XI and XII, and indirectly innervates nuclei for nerves III, IV, and VI via interneurons (corticonuclear tract), as well as to the pontine nuclei (corticopontine tract), both of which are also commonly referred to as the corticobulbar tract. In addition, it has been shown that the fibre motor system originates from many nonprimary motor areas, including primary and secondary somatosensory cortices (He et al., 1995), the caudal PMv and PMd (Dum & Strick, 1991; Picard & Strick, 2001), the SMA proper (Picard & Strick, 1996) and the CMAr and CMAc (Luppino et al., 1991; Morecraft & Van Hoesen, 1992; Paus et al., 1993; Paus, 2001), thus allowing parallel processing of motor information rather than a hierarchical relationship between M1 and the premotor and other nonprimary areas (Leyton & Sherrington, 1917; Denny-Brown & Botterell, 1948; Penfield & Rasmussen, 1950).

2.1.6. Anterior Cingulate Cortex and the Limbic System

2.1.6.1. Anatomical Organisation and Cytology of the Cingulate Cortex

The cingulate gyrus (CG) occupies a large part of the limbic lobe that forms a cingulum or a belt that encircles the corpus callosum (Figure 2.4). On the ventral side, the gyrus is separated from the corpus callosum by the callosal sulcus and from the superior frontal gyrus on the dorsal border by the cingulate sulcus. This structure is traditionally divided along a rostro-caudal axis into a broad anterior agranular region (Brodmann areas 25, 33, 24, 32) and a posterior granular region (Brodmann areas 23, 31, 26, 29, 30). In addition, a ventro-dorsal distinction divides the old periallocortex adjacent to the corpus callosum (area 33) to the proisocortex (areas 24, 25) and the paralimbic cortex (area 32) on the basis of stepwise laminar differentiation (Vogt et al., 1995; Paus, 2001). Cytoarchitectonically, the anterior cingulate cortex (ACC) is distinct from much of the neocortex in lacking layer IV, and having a prominent layer V. The ACC is similar in these laminar specialisations to the adjacent motor areas of the neocortex (i.e., primary motor cortex).
In humans, the CG is divided into fundamental subregions into: the anterior cingulate cortex (ACC), which can be further separated into the perigenual anterior cingulate cortex (pACC) and the midcingulate cortex (MCC); the posterior cingulate cortex (PCC); and the retrosplenial cortex (RSC) (Vogt, et al., 1995; Bush et al., 2000; Vogt et al., 2004; Palomero-Gallagher et al., 2008). The pACC forms the rostral part of the CG that abuts the genu of the corpus callosum and includes areas 25, 33, 24 and 32 of Brodmann. The MCC forms the middle one-third of the CG that include caudal parts of areas 33, 24 and 32, designated as areas 33’, 24’ and 32’. The PCC corresponds to areas 23 and 31 and RSC corresponds to areas 29 and 30. The RSC extends around the splenium of the corpus callosum and bounded by the ventral parahippocampal regions.

**Figure 2.4 Cingulate gyrus of the human brain**

Identification of the cingulate cortices from the (A) medial view of the human brain showing the anterior agranular and posterior granular regions. The Brodmann cytoarchitectural cingulate areas of the anterior region (areas 25, 33, 24, 32) are indicated on the (B) medial view of the brain. Figure adapted from Brodmann (1909, 2006), Paus and colleagues (2001), and Vogt and colleagues (1995, 2003, 2004).
2.1.6.2. The Function and Circuitry of the Anterior Cingulate Cortex

Early theories viewed the entire cingulate gyrus as a band of ventral forebrain that is involved in olfactory and emotional processing, and termed this the grand lobe limbique (Broca, 1878). In 1937, Papez proposed a mechanism of emotion based on a medial circuit involving the cingulate cortex and other medial structures such as the hippocampus, postcommissural fornix, mammillary body, and the anterior thalamic nucleus (Papez, 1937, 1995). Early electrical stimulation studies in the anterior region elicited changes in blood pressure, heart rate and respiration, as well as vocalisations and facial expressions (Smith, 1945; Kaada, 1951; Lewin & Whitty, 1960; Talairach et al., 1973). In 1952, MacLean linked the medial circuit of Papez with the lateral and subcortical structures involved in emotional expression to first coin the term limbic system (Yakovlev, 1948; MacLean, 1949, 1993). These data, together with modern studies suggest that the cingulate cortex (CC) is a specialised region devoted to the regulation of emotion, motor and cognitive function. The three key roles of the CC can be functionally divided into a rostro-caudal axis: the rostral-most portion that includes the ACC mainly involved in affect, emotion and autonomic control; MCC that regulates motor control; and the caudal regions, PCC and RSR which are involved with visuo-spatial processing and memory functions, respectively. In addition, a broad ventro-dorsal axis divides the ventral limbic tier (areas 24a, 24b, 25) involved in emotion and autonomic functions and the dorsal paralimbic tier (areas 24c and 32) involved in motor and cognitive functions (Koski & Paus, 2000; Paus, 2001).

The areas 25, 33, 24a and 24b, i.e., the rostral and ventral portion of ACC, has extensive connections with the amygdala, ventral striatum, periaqueductal grey, anterior insular cortex, autonomic brainstem motor nuclei and the hypothalamus. These direct relationships indicate that this region engages in the assessment of salience of emotion, regulation of autonomic and endocrine control, assessments of attentional and motivational content and maternal-infant interactions. Hence this region is also termed the “affect” division (Barbas & De Olmos, 1990; Bandler et al., 1991; Kunishio & Haber, 1994; Posner, 1995; Fisk & Wyss, 2000; Critchley, 2005). Another source of input arises from the mediodorsal and the anterior thalamic nuclei as well as the midline nuclei, which are reflected in the regulation of cortical arousal and nociception (Gabriel et al., 1989; Barbas et al., 1991; Talbot et al., 1991; Sikes & Vogt, 1992; Donahue et al., 2001; Bush et al., 2002). Furthermore, arousal-
related changes are modulated via the brainstem monoamine nuclei, such as extensive
dopaminergic fibres from the ventral tegmental area. Also, noradrenaline input from the
locus coeruleus mainly targets deep layers of the ACC that provides a complementary
source of neuromodulation along with serotonin fibres (Aston-Jones et al., 2000; Paus,
2001).

The “motor” and “cognitive” divisions include area 32 of the dorsal ACC and caudal areas
24’ and 32’ of the MCC. The dorsal tier of the cingulate motor areas (areas 32 and 32’) is
found in the banks of the cingulate sulcus (Dum & Strick, 1991; Shima et al., 1991). These
regions provide connections with cortical regions that are involved in vocalisation.
Vocalisation regions also include area 24 and the rostral part of area 25 and therefore, it is
thought to be modulated in conjunction with visceromotor and emotional control, e.g.,
vocalisation evoked from this region in nonhuman primates has affective content (Aitken,
1981; Frisztak & Neafsey, 1991; Devinsky et al., 1995). In addition, there are strong inputs
from the auditory association cortex, suggesting auditory-vocal interactions (Grasby et al.,
1993; Barbas et al., 1999). The cingulate motor areas also project to the more distant
prefrontal regions, particularly the dorsolateral prefrontal cortex (PFC), in addition to the
spinal cord and red nucleus (Dum & Strick, 1991; Morecraft & Van Hoesen, 1992; Bates &
Goldman-Rakic, 1993; Morecraft & Van Hoesen, 1993; Picard & Strick, 1996). These
fields, especially in the dorsal MCC (area 32’) contain prominent large layer V pyramidal
neurons that play an important role in the execution of voluntary movements and cognitive
control. Anatomically, this area is reciprocally connected with the primary motor cortex,
premotor cortex and supplementary motor area, and has skeletomotor and premotor
recently, the contributions of ACC in cognitive control, namely in error detection, conflict
monitoring, task anticipation, response selection and problem solving has been further
refined in functional imaging studies (Pardo et al., 1990; Frith et al., 1991; Carter et al.,
1998; Shima & Tanji, 1998; Carter et al., 2000a; Gehring & Knight, 2000; Luu et al., 2000;
MacDonald et al., 2000; Procyk et al., 2000; Paus, 2001; Simpson et al., 2001a; Simpson et
al., 2001b; Critchley et al., 2005).
2.2. Interneurons

2.2.1. The Discovery of Cortical Neurons

Ehrenberg (1833, 1836), an early microscopist, appears to have been aware of cells in the cortex of man and animals, referring to them as “granules” or “globules”. Clearer descriptions of “ganglion bodies” were given by Valentin (1836) and Remak (1841). The broad recognition of the variety of neuronal types emerged with the application of the reazione nera (black reaction) method of Camillo Golgi (1873), a silver stain that bears his name (Pannese, 1996). By 1883, Golgi was first to suggest that, in general, there are two morphologically and physiologically different types of neurons: motor neurons (type I) had a long main axon that left the grey matter while sensory neurons (type II) had short axons that did not leave the grey matter. There were several excellent studies of the cortex using the Golgi method, by Golgi himself (1884, 1894), by Retzius (1893), by von Kolliker (1852) and particularly by Ramón y Cajal (1891, 1954) - reviewed in (Jones, 1984a). The studies of Santiago Ramón y Cajal (1909-1911) represent the first detailed description of the cortical neurons systematically, layer by layer, in human and small mammals. His basic classification is into pyramidal cells and short-axon cells. In particular, he found a great variety of morphological cell types with short axons which were based on their pattern of axonal and dendritic branching in each layer. Since then, the term short-axon cell has commonly been used synonymously with the term interneuron.

2.2.2. Neuronal Types in the Cerebral Cortex

The neurons of the cerebral cortex can be divided into two major classes: pyramidal neurons and nonpyramidal neurons (Peters & Jones, 1984; McBain & Fisahn, 2001; DeFelipe, 2002; Markram et al., 2004; Batista-Brito & Fishell, 2009).

The pyramidal neurons represent the projection neurons that constitute the largest population of cortical cells (approximately 70-85% of the total cell population) and are located mainly in layers III, V, and VI. These cells form asymmetric spiny synapses and are known to use glutamate (GLU) as their excitatory neurotransmitter. Projection neurons typically have conical or pyramidal shaped cell bodies and possess a single dominant apical
dendrite that radiates out from the cell body to extend vertically toward the pial surface. This radially oriented apical dendrite forms a terminal tuft in layers I and II, and basal dendrites radiate out from the base of the soma and with an axon descending vertically toward the white matter. All of the dendrites of typical pyramidal neurons bear spines, although these spines may be absent from the proximal portions of the dendrites, and cover the more distal portions (Peters & Jones, 1984; DeFelipe & Farinas, 1992).

The nonpyramidal neurons are elaborately endowed with names that can be used interchangeably (with some exceptions) as aspiny, smooth spiny, inhibitory, local-circuit, GABAergic, short-axon, or interneurons. They represent 15-30% of the total cell population found in all cortical layers that form symmetric synapses, and use γ-aminobutyric acid (GABA) as their inhibitory neurotransmitter. Cortical GABAergic interneurons in human are generated in the neuroepithelium of the subcortical ganglionic eminence in the ventral forebrain and migrate tangentially to their destinations in the neocortex; and also in the ventricular/subventricular zones of the lateral ventricular wall in the dorsal forebrain which follow the same radial route as the glutamatergic neurons (Marin & Rubenstein, 2001; Anderson et al., 2002; Letinic et al., 2002; Butt et al., 2005; Wonders & Anderson, 2005; Huang et al., 2007; Jones, 2009; Petanjek et al., 2009). The nonpyramidal neurons are further subdivided into two large groups: spiny (or stellate cells); and aspiny or sparsely spiny nonpyramidal cells. Spiny stellate cells constitute the typical excitatory granule cells that are abundant in cortical layer IV. Aspiny or sparsely spiny nonpyramidal cells are more commonly referred to as “interneurons” and show a great diversity in morphological, biochemical and physiological features (Fairén et al., 1984).

2.2.3. Classification of Cortical Interneurons

The classification of cortical interneurons into precise anatomical and functional subpopulations has been an ongoing challenge on the studies of interneuron function due to their great diversity. Since the pioneering work of Cajal, many authors examined and refined various forms of short-axon cells and provided concepts of the organisation of cortical connectivity, such as Lorente de Nó (1922, 1949), Szentágothai (1969), Valverde (1971), Lund (1973), Fairén (1984), Peters (1984), Hendry (1987), Kisvarday (1990), Jones
(1993), DeFelipe (1993), Kawaguchi (1997), and Somogyi (1998). Recently, a consortium of scientists convened at Petilla de Aragón, Navarre, Spain (birthplace of Cajal) to propose a standardised nomenclature of interneurons, which they designated as the Petilla terminology (Petilla Interneuron Nomenclature Group, 2008). Nonetheless, at present, cortical interneurons are typically classified according to their unique morphological, biochemical, and physiological features, and their synaptic connections (McBain & Fisahn, 2001; Letinic, et al., 2002; Markram, et al., 2004; Batista-Brito & Fishell, 2009; Jones, 2009).

2.2.3.1. Morphological Features

The cortical interneurons were first identified and classified based on the morphological features that were readily observed in Golgi-preparations. The main structural criteria of interneurons are the size and shape of the soma which typically vary in diameter from 10-30 μm, and the branching patterns of prominent axonal and dendritic fields. Accordingly, interneurons can be subdivided into three main types on the basis of the axonal and dendritic branching patterns: Group 1, cells with long vertically oriented axonal and dendritic fields; Group 2, cells with horizontal axon collaterals; and Group 3, cells with multiple axonal polarity. Thus, what follows is a brief description of the main types of morphologically heterogeneous populations of interneurons (Fairén, et al., 1984; DeFelipe, 2002; Nieuwenhuys, et al., 2008).

**Group 1: Cells with vertically oriented axonal collaterals**

- **Double bouquet cells** - are distinguished by long, tight vertically oriented arrays of axonal branches designated as “bundles” or “horse-tails”. Typical double bouquet cells have only been observed in layers II and III of the neocortex of cats and primates, but not in rodents (Lorente de Nó, 1922; Fairén, et al., 1984). These cells typically express calbindin-D28k (CB) and calretinin (CR) (see next Section 2.2.3.2).

- **Bipolar cells** - occur in layers II-VI and have single long vertically oriented dendrites that extend from both poles of the soma. The axons usually arise from one of the primary dendrites and form a narrow plexus that also spreads in the vertical orientation. These cells typically express calretinin (CR).
Martinotti cells - are multipolar, bitufted or bipolar neurons with smooth or sparsely spinous dendrites that occur in all cortical layers except layer I. Their distinguishing feature is a long ascending axon which reach layer I, where it forms a terminal arborisation. These cells typically express calbindin-D28k (CB).

Group 2: Cells with horizontal axonal collaterals

Horizontal cells - are bipolar neurons with horizontal orientation that are found in layers I and VI. Cajal referred to the special cells of layer I as Cajal’sche Zellen of Retzius (now known as Cajal-Retzius cells) (Marín-Padilla, 1998; Meyer et al., 1999). These cells typically express calretinin (CR). In addition, the deep zone of layer VI contains numerous small to medium sized horizontal cells.

Group 3: Cells with multiple axonal and dendritic fields

Stellate (or local plexus) cells - are found in all cortical layers. The dendrites branch out from the soma in all directions and their axonal arborisation forms a local plexus. The basket, chandelier, and neurogliaform cells represent the three distinct types of specialised stellate cells.

Basket cells - are among the largest nonpyramidal neurons and are found throughout layers II-VI. The basket cells are named due to the formation of weaves of preterminal axons that surround the cell bodies of pyramidal cells (“pericellular basket formation”). The three subtypes of basket cells include: large basket cells that are characterised by their large soma, long smooth or sparsely spinous dendrites, and horizontally branching myelinated axon collaterals; small basket cells characterised by small to medium sized soma with ascending dendritic tufts and numerous “curvy” preterminal axonal branches; and net basket cells that occupy an intermediate position between large and small basket cells. These cells typically express parvalbumin (PV).

Chandelier cells - were first described by Szentágothai and Arbib (1974). Chandelier cells are multipolar or bitufted cells which are distinguished by their preterminal axonal branches that give rise to a large number (up to 300) of short, vertically oriented rows of specialised terminals that resemble candlesticks (“axon terminal cartridges”).
Chandelier cells occur in layers II-V, but are most common in layer II-III. These cells typically express parvalbumin (PV).

- **Neurogliaform (or spiderweb) cells** - have small, round soma and short multipolar dendrites. Their axons arborise profusely around the soma, forming a dense thread that interweaves with the thin dendrites. Neurogliaform cells are particularly concentrated in layer IV of sensory cortices that receive thalamic afferents and synapse mainly with spiny stellate cells. These cells typically express calbindin-D28k (CB).

Nevertheless, it should be noted that there are many other inhibitory interneurons in the neocortex that are difficult to classify and sometimes referred to as *common type cells or remaining inhibitory interneurons* (DeFelipe, 2002; Nieuwenhuys, et al., 2008).

### 2.2.3.2. Biochemical Distinction Based on Calcium-Binding Proteins

The inhibitory function of cortical interneurons has been suggested by the presence of axon terminals with flattened vesicles and the formation of symmetrical synapses onto their postsynaptic targets; and also by early immuncytochemical studies using the GABA synthesising enzyme, GAD (glutamic acid decarboxylase) (Chattopadhyaya et al., 2007). In addition, these inhibitory GABAergic interneurons have been found to express a number of different novel molecular markers and are characterised into biochemically definable subgroups with particular morphologies. The prominent members include neurotransmitters, calcium-binding proteins, neuropeptides, ionic channels, receptors, and connexins - reviewed in (Hof et al., 2000; McBain & Fisahn, 2001; Baraban & Tallent, 2004; Monyer & Markram, 2004; Petilla Interneuron Nomenclature Group, 2008; Batista-Brito & Fishell, 2009; Kubota et al., 2011). Among these molecular and neurochemical markers, one of the most valuable characterisations of interneuron subtypes has been based on the differential immunoreactivity of the **calcium-binding proteins**, such as calbindin-D28k, calretinin, and parvalbumin. These markers are used in the present study and provide excellent cytoarchitectonic staining, and visualise a Golgi-like cellular morphology (Celio, 1990; Rogers, 1992; Andressen et al., 1993; DeFelipe, 1993; Conde et al., 1994; Kubota et al., 1994; DeFelipe, 1997; Gonchar & Burkhalter, 1997; Kawaguchi & Kondo, 2002; Kubota, et al., 2011).
Calbindin-D28k

Calbindin-D28k (CB) stains subpopulations of nonpyramidal cells in the brain. The calbindin-D28k expressing (CB⁺) neurons are frequent in the upper layers II and III. CB⁺ interneurons represent less than 5% of the total neuronal population (Hornung & De Tribolet, 1995). In layers III, V, and VI, a small population of pyramidal cells are also weakly stained. Among the nonpyramidal neurons, the double bouquet, Martinotti, and neurogliaform morphological cell types typically contain CB (Figure 2.5).

Calretinin

The calretinin expressing (CR⁺) neurons are found throughout all cortical layers, but are more frequent in the supragranular layers. These cells make up approximately 10% of the total neuronal population (Hornung & De Tribolet, 1995). The morphology of CR⁺ interneurons in the rat is typical for bipolar and vertically oriented multipolar neurons. Therefore, CR⁺ interneurons present a second, non-overlapping cell population. The number of CR⁺ neurons increase in the monkey and human brains, where this protein is also expressed by a small number of pyramidal neurons. CR is typically expressed in Cajal-Retzius, bipolar, and double bouquet morphological nonpyramidal cell types (Figure 2.5).

Parvalbumin

The parvalbumin expressing (PV⁺) neurons make up approximately 6.5% of the total neuronal population in the cortex (Hornung & De Tribolet, 1995), and are present in all cortical layers, except layer I. These cells are found more frequently in layers III-V. The PV⁺ neurons belong to the basket and chandelier cells with cell bodies that are mostly multipolar and less often bitufted (Figure 2.5). PV⁺ neurons are involved in the generation of γ-frequency (30-80 Hz) oscillations associated with cognition and sensory processing (Muller et al., 2000; Engel & Singer, 2001; Bartos et al., 2007). In the hippocampus and cerebellum, PV deficiency leads to altered γ-band activity (Vreugdenhil et al., 2003; Cheron et al., 2005; Cheron et al., 2008). In addition, change in the inhibitory activity of PV neurons in the cortex increased susceptibility to epileptic seizures (DeFelipe, 1999; Schwaller et al., 2004).
Figure 2.5 Morphological and biochemical diversity of cortical GABAergic interneurons

The representative morphologically and biochemically diverse populations of inhibitory GABAergic interneurons in the cerebral cortex. The different morphological types are further subdivided into the three calcium-binding protein (calbindin-D28k, calretinin, parvalbumin) populations. Calbindin-D28k mainly labels **MC**, Martinotti, **DB**, double bouquet, and **NGF**, neurogliaform cells. Calretinin mainly labels **C-R**, Cajal-Retzius cells of layer I, **DB**, double bouquet, and **BP**, bipolar cells. These cells mainly form axo-dendritic contacts with its neighbouring **PC**, pyramidal cells. Parvalbumin mainly labels **BC**, basket, and **CC**, chandelier cells, which form axo-somatic and axo-axonic synapses with pyramidal cells, respectively.
2.2.3.3. Electrophysiological Features

A further criterion widely utilised for interneuron classification is by their electrophysiological properties. Cortical neurons can be divided into five main categories according to their distinct firing characteristics in response to intracellular current injection: The pyramidal neurons almost exclusively exhibit regular spiking (RS), intrinsically bursting (IB), fast repetitive bursting or chattering (FRB) characteristics. The other two electrophysiological classes, the low threshold spike (LTS), and fast spiking (FS) cells constitute interneurons with aspiny or sparsely spiny dendritic trees (Kawaguchi & Kubota, 1997; Klausberger et al., 2003; Contreras, 2004; Toledo-Rodriguez et al., 2004).

The LTS cells are characterised by the generation of a burst of 3-4 Na⁺ spikes which rides on an underlying low threshold calcium current ($I_t$). The burst is only elicited from a hyperpolarised membrane potential and never sustains a repetitive discharge. The LTS cells are found in layers II-VI and correspond mainly to bipolar, double bouquet, and Martinotti cells, which correspond to nonpyramidal neurons that express calbindin-D28k (CB) and calretinin (CR) (Kawaguchi & Kubota, 1997; Kubota, et al., 2011). These vertically oriented cells may also exhibit certain RS electrophysiological characteristics. The RS cells generate regular trains of action potentials with sustained repetitive firing in response to injected current. The spike lasts ~1 ms followed by various degrees of afterhyperpolarisations (AHP) and afterdepolarisations (ADP) (Nunez et al., 1993; Nowak et al., 2003). The RS phenotype is also typically expressed by layer IV spiny stellate cells which represent the only excitatory nonpyramidal neurons and by some large basket cells (Kawaguchi & Kubota, 1997). The FS cells were first described by Mountcastle (1969) and were named due to the presence of fast spikes from extracellular recordings in the rat barrel cortex (Simons, 1978). Intracellularly, FS cells show a rapid repolarisation and short lasting AHP that lasts 0.4-0.6 ms. In response to current injection, FS cells can generate regular trains of action potentials at very high frequencies (~800 Hz) with very little accommodation (Contreras, 2004). In addition, FS cells have the capacity to generate fast membrane potential oscillations (20-80 Hz) that may lead to single spikes at those frequencies. The FS cells are found in layers II-VI and mainly correspond with two morphological nonpyramidal classes - basket and chandelier cells that express parvalbumin (PV) (Cauli et al., 1997; Kawaguchi & Kondo, 2002).
2.2.3.4. Synaptic Connectivity

Interneurons are also distinguishable at the synaptic level, by their input and output specificity (Somogyi, et al., 1998; Gupta et al., 2000; Buzsaki & Draguhn, 2004; Silberberg et al., 2005; Somogyi & Klausberger, 2005; Molnar et al., 2008). The interneurons that have been studied in detail have been shown to form synapses with both pyramidal and nonpyramidal cells. Furthermore, interneurons are not only connected by chemical synapses (unidirectional connections), but also via electrical synapses through gap junctions (bidirectional connections). The GABA<sub>A</sub> receptors that mediate the outputs of interneurons also differ across subtypes (Ali & Thomson, 2008). Based on the observation that different portions of a pyramidal cell are innervated by different types of interneurons, the following main types of interneurons can be recognised:

- **Axo-dendritic cells:** Cells forming synapses only (or mostly) with the distal dendrites of pyramidal neurons, i.e., bipolar, double bouquet, and neurogliaform cells. These cells typically express calbindin-D28k (CB) and calretinin (CR).

- **Axo-somatic cells:** Cells forming synapses with the pyramidal somata exerting a powerful inhibitory action, i.e., basket cells (Pouille & Scanziani, 2001; Freund, 2003). Basket cells form major recipients of thalamic and commissural inputs. These cells typically express parvalbumin (PV).

- **Axo-axonic cells:** Cells forming synapses with the pyramidal cell axon initial segments, i.e., chandelier cells. Most interneurons form symmetrical synapses with both pyramidal cells and other interneurons, with the exception of chandelier cells, which only form axo-axonic synapses with the pyramidal neurons. These synapses are formed by axons that produce a dense plexus in the vicinity of the parent soma. From this axonal plexus, numerous (up to 300) vertically oriented arrays of terminals arise that contact the initial axonal segments of pyramidal cells. These cells typically express parvalbumin (PV).
2.3. Calcium-Binding Proteins

2.3.1. Intracellular Calcium Homeostasis and the EF-hand Family
Calcium (Ca$^{2+}$) ions play a pivotal role in intracellular transmission of signals. In neurons, Ca$^{2+}$ is involved in an array of key functions, including membrane excitability, exocytosis, axonal transport, apoptosis, transcription, and plasticity (Berridge et al., 2003; Rusakov, 2006; Clapham, 2007). Therefore, the concentration of Ca$^{2+}$ ions is tightly regulated due to its ability to affect a wide range of physiological processes. The speed and efficacy of Ca$^{2+}$ is achieved by maintaining a steep gradient between the intracellular ($\sim 10^{-7}$ M) and extracellular ($\sim 10^{-4}$ M) concentrations (Kirichok et al., 2004; Strehler & Treiman, 2004). The Ca$^{2+}$ that enters the cytoplasm during multiple cellular functions does not remain free. Instead, the cells have developed ways to sequester Ca$^{2+}$ by chelation, compartmentalisation or extrusion. In particular, the influx of Ca$^{2+}$ into the cell becomes bound to Ca$^{2+}$ chelators - the calcium-binding proteins - which contain a calcium-binding structural motif termed the “EF-hand” domain. There are more than 240 different calcium-binding proteins encoded by the human genome (International Human Genome Sequencing Consortium, 2001) and over 600 in all different species (Carafoli et al., 2001).

The EF-hand proteins belong to a homologous family that binds Ca$^{2+}$ using a characteristic helix-loop-helix structural motif (29 amino acids), and are composed of two orthogonal $\alpha$-helices bridged by highly conserved short chelation loop region ($\sim 12$ amino acid residues) (Capozzi et al., 2006; Grabarek, 2006; Gifford et al., 2007). Unlike helices and sheets, the turn-loop structure provides flexibility and readily supplies ligands to provide an interaction with free Ca$^{2+}$. The Ca$^{2+}$ ions are coordinated within the loop in a pentagonal bipyramidal configuration. The six residues within the loop involved in the binding are in positions 1 (+X), 3 (+Y), 5 (+Z), 7 (-Y), 9 (-X) and 12 (-Z) (also referred to as the “canonical EF loop”). The Ca$^{2+}$ ions are bound by negatively charged amino acid residues (glutamic and aspartic acid) which make electrostatic interaction with the positively charged Ca$^{2+}$. The EF-hand motif frequently occurs in pairs to bind two Ca$^{2+}$ ions (Biekofsky & Feeney, 1998). Therefore, the pair contains 4 helix bundles with the amphipathic regions facing together to form a hydrophobic core.
Functionally, EF-hand proteins can be divided into two general classes: the Ca$^{2+}$ sensors and the Ca$^{2+}$ buffers. The Ca$^{2+}$ sensors (or effectors) such as calmodulin, S100, troponin C and recoverin undergo a conformational change after binding Ca$^{2+}$ to trigger a biochemical response (Cheung, 1980; Shaw et al., 1990; Tanaka et al., 1995; Donato, 2001; Hoeflich & Ikura, 2002; Burgoyne, 2007). The Ca$^{2+}$ buffers such as calbindin, calretinin, and parvalbumin are a subset of the EF-hand protein family that modulate the signal both spatially and temporally by binding to free Ca$^{2+}$ or remove them from the cytoplasm to modulate cytosolic calcium transients (Schwaller et al., 2002; Schwaller, 2009).

2.3.2. Structural and Functional Role of Calcium-Binding Proteins (Ca$^{2+}$ buffers)

The function of the Ca$^{2+}$ buffers (CB, CR, PV) in the nervous system is not fully understood, although these proteins have been known to act as buffers to modulate cytosolic calcium transients, thus play an important role in neurodegeneration. These proteins also provide valuable markers of neuronal subpopulations for anatomical and developmental studies (Baimbridge et al., 1992; Heizmann & Braun, 1992; Schwaller, et al., 2002; Wojda et al., 2008; Schwaller, 2009). Brief accounts of the three Ca$^{2+}$ buffer calcium-binding proteins used in this study (CB, CR, PV) are described as follows:

2.3.2.1. Calbindin

Calbindin (CB) was first discovered in the cytosolic fractions of chicken intestine and kidney with high affinity for Ca$^{2+}$ (Oberholtzer et al., 1988). Calbindin was initially known as the “vitamin D-dependent calcium-binding protein” as their expression was induced with vitamin D and its metabolites such as calcitriol, especially in vitamin D-deficient animals (Christakos et al., 1979; Christakos et al., 2007). In the brain, CB is independent from vitamin D-derived synthesis. In human, CB is encoded by the CALB1 gene on chromosome 8p11 (Parmentier et al., 1991) and exist in two forms with a molecular weight of 9 kDa and 28 kDa. Calbindin-D9k (CB-D9k) is found in the mammalian intestine and calbindin-D28k is found in the chicken intestine, kidney, and is also highly expressed in the brain. Functionally, both types of CB have been suggested to mediate the transport of Ca$^{2+}$ across intestinal enterocytes. There is no homology between the two types, apart from the
structural EF-hand domains. CB-D9k contains two EF-hand domain (one pair) whereas calbindin-D28k contains six EF-hands (three pairs) that forms an ellipsoid-type conformation to bind four Ca$^{2+}$ in loops EF1, EF3, EF4 and EF5 (Cedervall et al., 2005; Kojetin et al., 2006).

Calbindin-D28k (CB-D28k) in the brain acts as a Ca$^{2+}$ buffer to affect a number of neuronal processes such as modulation of Ca$^{2+}$ transients and neuronal firing (Schwaller, 2009). CB-D28k is colocalised with L-type Ca$^{2+}$ channels (Celio, 1990) and more recently, it has been suggested that the presence of CB-D28k reduces Ca$^{2+}$ influx through the voltage dependent L-type Ca$^{2+}$ channels and enhance the sensitivity of the channels to evoked Ca$^{2+}$ transients (Lee et al., 2006). CB-D28k has also been shown to specifically interact with the C-terminus of the L-type Ca$^{2+}$ channel $\alpha_{1c}$ subunit (Ca$\nu$1.2) (Christakos, et al., 2007). In addition, the neuroprotective role of CB-D28k has been implicated by its buffering role against increases in intracellular Ca$^{2+}$ which can lead to neuronal death in ischemic injury and neurological diseases such as Alzheimer’s and Huntington’s disease (Baimbridge, et al., 1992; Andressen, et al., 1993; Schwaller, et al., 2002). CB-D28k also modulates apoptosis by interacting with caspase 3 (Bellido et al., 2000; Christakos & Liu, 2004).

2.3.2.2. Calretinin

Calretinin (CR) is another vitamin D-dependent calcium-binding protein (29 kDa) first identified in the avian retina (Rogers, 1987). CR is encoded by the CALB2 gene located on chromosome 16q22.1 in human (Parmentier, et al., 1991). CR contains six EF-hand domains (three pairs) in which four motifs display a high affinity for Ca$^{2+}$ (Schwaller et al., 1997) and the other two exhibiting lower affinity binding (Stevens & Rogers, 1997). The closest homologue of CR is CB-D28k (58% homology) and therefore was also known as the “29 kDa calbindin”. However, CR and CB-D28k have also shown to possess different domain organisation (Palczewska et al., 2003) thus suggesting distinct functional roles in the brain (Schwaller, 2009). CR is abundantly expressed in neurons and is implicated as a modulator of neuronal excitability (Gall et al., 2003) and may also provide protection against calcium-induced glutamate excitotoxicity (Lukas & Jones, 1994; D'Orlando et al., 2002).
2.3.2.3. Parvalbumin

Parvalbumin (PV) is a calcium-binding protein with a low molecular weight of 9-12 kDa. In human, PV is encoded by the *PVALB* gene on chromosome 22q12-q13.1 (Berchtold, 1989; Zuhlke et al., 1989). The term “EF-hand” was first described and named after the E and F regions of the PV protein (Kretsinger & Nockolds, 1973; Nakayama & Kretsinger, 1994). PV contains less frequent three EF-hand motifs where the EF2/3 pair is involved in the binding of Ca$^{2+}$, and the unpaired EF1 domain serves to stabilise and maintain its structural integrity (Babini et al., 2005). PV exists in two isoforms (α and β). In mammals, only α-isoform is found which is preferentially expressed in muscles and neurons (Celio & Heizmann, 1981, 1982). The β-isoform (also called oncomodulin) is found in the mammalian-derived tumour cells (Gillen et al., 1987). In the brain, PV is found in a subpopulation of interneurons in the hippocampus, cerebellum and cortex to exert perisomatic inhibition of the principal neurons and modulate neuronal excitability (Freund & Buzsaki, 1996; Schwaller, et al., 2002; Gall et al., 2005; Kubota, et al., 2011). PV neurons are involved in the generation of γ-frequency (30-80 Hz) oscillations associated with cognition and sensory processing (Muller, et al., 2000; Engel & Singer, 2001; Bartos, et al., 2007).

2.4. Huntington's Disease

2.4.1. Introduction to Huntington's Disease

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder that is characterised by a triad of motor, mood and cognitive symptoms. The disease aetiology was successfully identified as an unstable expansion of CAG (cytosine-adenosine-guanosine) trinucleotide repeats on chromosome 4, which encodes a mutant cytoplasmic protein called *huntingtin* (Huntington's Disease Collaborative Research Group, 1993). Depending on the individual CAG expansion, disease onset generally occurs in mid-life, entailing an invariably lethal progression and death between 10 to 20 years from the time of symptomatic onset. The mutant huntingtin is ubiquitous in somatic tissues; however the pathology of HD is extensive in the brain. Characteristic neuropathological features include major degeneration in the corpus striatum as well as pronounced cortical atrophy (Vonsattel, et al., 2008). Within the corpus striatum, there is an increasing degeneration of medium
spiny projection neurons with increasing grade of the disease, resulting in near total degeneration at advanced stages of the disease. In addition, cortical degeneration in HD has been well documented in a number of reports (Cudkowicz & Kowall, 1990a; Hedreen et al., 1991; Sotrel et al., 1991; Sotrel et al., 1993; Heinsen et al., 1994; Jackson et al., 1995; Macdonald et al., 1997; Rajkowska et al., 1998; Gutekunst, et al., 1999; Sapp et al., 1999; Sieradzan & Mann, 2001; Macdonald & Halliday, 2002; Selemon et al., 2004; Thu, 2006; Nana, 2009; Thu, et al., 2010). However, despite the single gene aetiology of HD, there is considerable variation in the presentation of symptoms from one individual to the next. Consequently, there is much interest in whether there are any underlying pathological differences in HD brains which may account for the variance in symptoms. While there is currently no cure, timely assessment and symptom management are of key importance in a range of treatment strategies. Moreover, it is important to have a clear understanding of the neuropathology in the brain regions responsible for specific functional roles which may underpin the characteristic phenotypic manifestation in HD.

2.4.2. Genetic Features

The prevalence of clinically identified HD is highest among most European populations (5-7 per 100,000). In New Zealand, the prevalence of HD patients is 5.7 per 100,000 populations (Harper, 1992). The rate is much lower in Asian (Takano et al., 1998) and African populations (Wright et al., 1981). The gene for HD also known as “IT15” (for “Interesting Transcript”) is located on the short arm of chromosome 4, 4p16.3 (Huntington's Disease Collaborative Research Group, 1993). The genetic mutation responsible for HD is associated with the expansion of the CAG (cytosine-adenosine-guanosine) trinucleotide repeats that codes for glutamine within the N-terminal coding region of the HD gene, encoding a mutant ~350 kDa protein termed huntingtin with an expanded stretch of polyglutamine tracts (Figure 2.7). The CAG repeat expansion is common to a group of 9 neurological disorders including dentate-rubro-pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA) and several spinocerebellar ataxias (SCA; types 1, 2, 3, 6, 7, 17) (Everett & Wood, 2004). However, there is no clear homology between the aetiopathology of the gene products in these disorders. In the case of HD, the number of CAG repeat expansions is largely inversely proportional to the disease onset and proportional to symptom severity, rate of disease progression or with age of death (Snell et
al., 1993; Persichetti et al., 1995; Nance, 1997; Penney et al., 1997; Andresen et al., 2007; Langbehn et al., 2010). The number of CAG repeats accounts for approximately 44-60% of the variation in age of onset, indicating that there are other factors such as modifying genes and the environment playing a role in disease onset and progression (Snell, et al., 1993; Illarioshkin et al., 1994; Djousse et al., 2004; Wexler, et al., 2004). Another intriguing phenomenon is that the expression of the clinical phenotype is normally delayed to adulthood, even though the expression of the mutated gene is likely to occur early during development.

The inheritance pattern proceeds in a Mendelian fashion (autosomal dominance), with unstable, polymorphic expansion of CAG repeats that alter during meiosis (Myers et al., 1993). Trinucleotide CAG repeats exceeding 28 show instability on replication and lead to gene expansion (Rubinsztein & Carmichael, 2003), especially during spermatogenesis than oogenesis (MacDonald et al., 1993; Zuhlke et al., 1993; Trottier et al., 1994; Kremer et al., 1995; Ranen et al., 1995; Wheeler et al., 2007). The phenomenon of increase in HD gene length with each successive generation is termed genetic anticipation. However, contraction can also take place (in 23% of cases) (Djousse, et al., 2004; Chattopadhyay et al., 2005; Walker, 2007). Anticipation in HD is more pronounced when the disease is of paternal origin (Gusella et al., 1993; Nance, 1997). For example, in 90% of the patients who developed the disease at 10 years of age or younger, the gene was paternally transmitted (Vonsattel & DiFiglia, 1998; Vonsattel, et al., 2008). Individuals with a CAG repeat length of less than 36 will not develop HD, whereas a CAG repeat length over 36 are associated with an increased probability of developing the disease. Individuals with 36-39 CAG repeats show variable, incomplete penetrance with respect to the HD phenotype. When the repeat length reaches >39, the disease is considered to be fully penetrant. Most adult-onset HD patients have expansions ranging from 40 to 55 CAG repeats and expansions of more than 70 are associated with juvenile-onset HD patients. Rarely patients have expansions exceeding 100 (Duyao, et al., 1993; Huntington's Disease Collaborative Research Group, 1993; Vonsattel & DiFiglia, 1998; Vonsattel, et al., 2008).
2.4.3. Huntingtin and Pathogenesis of HD

2.4.3.1. Characteristics of Wild-Type Huntingtin

The wild-type huntingtin is a large protein (3144 amino acid residues) expressed mostly in the cytoplasm, dendrites and axon terminals of neurons in the brain (Trottier et al., 1995; Ferrante, et al., 1997). Huntingtin is associated with various intracellular organelles, including endoplasmic reticulum (ER), Golgi apparatus (DiFiglia et al., 1995; Hilditch-Maguire et al., 2000), and microtubules (Li et al., 2003). A small proportion is also found in the nucleus (Kegel et al., 2002). Structurally (Figure 2.6), the huntingtin protein contains a conserved nuclear export signal (NES) near its C-terminus as well as an N-terminal 17 amino acid sequence that act as a NES (Xia et al., 2003; Cornett et al., 2005; Imarisio, et al., 2008). Also, polyglutamine (polyQ) and polyproline (polyP) sequences are found near the N-terminus (Steffan et al., 2004) followed by downstream clusters of conserved HEAT (huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase A and the lipid kinase Tor) motifs that interact with proteins involved in intracellular transport and microtubule dynamics (Takano & Gusella, 2002; Kegel et al., 2005; Li et al., 2006; Atwal et al., 2007; Rockabrand et al., 2007). Another feature is the presence of post-translational modification sites such as ubiquitination, SUMOylation, palmitoylation, phosphorylation and various proteolytic cleavage consensus sites (Kalchman et al., 1996; Humbert et al., 2002; Huang et al., 2004; Steffan, et al., 2004; Warby et al., 2005; Yanai et al., 2006).

![Figure 2.6 Domain structure of the wild-type huntingtin protein](image)

Schematic diagram of the full length wild-type huntingtin protein with locations of main polypeptide sequence features, including the nuclear export sequence (NES), polyglutamine (polyQ) and polyproline (poly P) sequences, and clusters of HEAT motifs. Sites of post-translational modifications such as SUMOylation, ubiquitination, palmitoylation, phosphorylation and cleavage by proteases (caspase cleavage sites: 513, 552, 586; calpain cleavage sites: 469, 536) are also indicated. Figure adapted from Wellington and colleagues (2000), Kim and colleagues (2001), Gafni and Ellerby (2002), Cattaneo and colleagues (2005), Imarisio and colleagues (2008).
Huntingtin has no clear homology to the known proteins and its normal function is yet to remain fully understood. However, a considerable effort in understanding huntingtin structure and function has lead to suggested roles in development, protein trafficking, signalling pathways, anti-apoptotic role and transcriptional regulation. Huntingtin is essential for normal embryonic development, as knockout zebrafish and mouse embryos produce a wide variety of developmental defects and results in embryonic lethality (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995; White et al., 1997; Cattaneo et al., 2001; Lumsden et al., 2007). Also, increased expression of normal huntingtin protein improved neuronal survival and alleviated the effects of mutant protein (Cattaneo et al., 2005). The role of huntingtin in vesicle and protein trafficking are also proposed on the basis of its localisation to endosomal vesicles in axons and synaptic terminals and from its interaction with a number of proteins involved in intracellular trafficking such as α-adaptin, clathrin, dynamin, HIP1, HIP14, HAP1 (the first identified huntingtin interactor), HAP40, PACSIN1 and SH3GL3 (Gauthier et al., 2004; Huang, et al., 2004; Li & Li, 2004; Borrell-Pages et al., 2006; Yanai, et al., 2006; Imarisio, et al., 2008). Huntingtin also interacts with the SH3 domain of PSD-95 (postsynaptic density protein 95), a key regulator for exo- and endocytosis at synaptic terminals (Smith et al., 2005), and also mediates post-synaptic clustering of ionotrophic glutamate receptors such as NMDA (N-methyl-D-aspartate) and kainite receptors (Sun et al., 2001; Fan & Raymond, 2007). The anti-apoptotic role of huntingtin is suggested by proteolytic cleavage by various intracellular proteases, including caspases 1, 3, 6, 7, 8, and calpain and an aspartyl protease (Zeitlin, et al., 1995; Kim et al., 2001; Gafni & Ellerby, 2002; Lunkes et al., 2002; Wellington et al., 2002). The overexpression of huntingtin also protects against ischemic injury (Zhang et al., 2003) and excitotoxicity (Zeron et al., 2002; Leavitt et al., 2006). The pro-survival function is shown by inhibition of pro-caspase 9 processing (Rigamonti et al., 2001) and the HIP1-HIPP1 complex (Gervais et al., 2002) by wild-type huntingtin, as well as being a substrate for IGF1/Akt pathways (Humbert, et al., 2002; Rangone et al., 2004b). Huntingtin is involved in transcriptional regulation by interacting with various transcription factors and proteins such as BDNF (brain-derived neurotrophic factor) to act as a positive transcriptional regulator (Fusco et al., 2003; Zuccato et al., 2003), and also interacts with tryptophan (WW) domain-containing proteins implicated in non-receptor signalling and pre-mRNA splicing (Faber et al., 1998).
2.4.3.2. Mutant Huntingtin and Mechanisms of HD Pathogenesis

The mutant huntingtin shows a similar expression level and regional distribution to the wild-type huntingtin in the brain (Aronin et al., 1995), but a difference in huntingtin epitope localisation have been observed, e.g., abnormal accumulation of N-terminal fragment of mutant huntingtin in the nucleus and dystrophic neurites in HD brains (DiFiglia, et al., 1997). Through several mechanisms described below, mutant huntingtin may not only have a toxic gain of function but also provide a loss of function effect, which hinders the normal function of wild-type huntingtin. Several lines of evidence suggesting the role of mutant huntingtin and related pathogenic mechanisms are briefly outlined as follows (Figure 2.7):

**Huntingtin aggregation**

The pathological characteristic of HD is the formation of nuclear and cytoplasmic inclusions (Davies et al., 1997) that appear early in the disease (Weiss et al., 2008). The toxic mechanism of these inclusions leading to a differential loss of a specific subset of neurons, however, is still a topic of debate. There is some evidence to suggest that the inclusions are not pathogenic as neuronal dysfunction or loss was not observed in mouse with widespread huntingtin expression (Davies, et al., 1997; Slow et al., 2005) and cells with inclusions had prolonged survival (Arrasate et al., 2004). In the CAG140 knock-in and other mouse models of HD, inclusions appeared after the symptom onset (Wheeler et al., 2000; Menalled et al., 2002; Menalled et al., 2003; Slow et al., 2003; Nguyen et al., 2006). Also, a recombinant compound that promotes formation of inclusions reduced neuronal pathology (Bodner et al., 2006).

On the other hand, the inclusions have been suggested to be toxic and involved in the pathogenesis of HD. The important steps of the aggregate toxicity hypothesis may involve proteolysis, nuclear translocation, and aggregation. The mutant huntingtin possess a higher likelihood of proteolytic cleavage than its wild-type counterpart (Goldberg et al., 1996; Saudou et al., 1998; Wellington et al., 2000). The smaller cleaved fragments are suggested to be more toxic (Gong et al., 2008) and the toxicity is also associated with nuclear translocation (Martindale et al., 1998; Peters et al., 1999; Atwal, et al., 2007). The accumulation of prolonged production of mutant huntingtin causes the neuron to lose its
ability to degrade huntingtin fragments, principally via the ubiquitin-proteasome system or autophagic vacuolisation (Sherman & Goldberg, 2001; Nagata et al., 2004; Rangone et al., 2004a; Rubinsztein, 2006). Thus, aggregated mutant huntingtin may obstruct normal protein-protein interactions that are involved in cellular pathways such as excitotoxicity, transcription, apoptosis, energy metabolism, protein trafficking and axonal transport.

**Excitotoxicity**

The neuronal degeneration due to the overactivity of glutamate neurotransmission is a process termed excitotoxicity (Martin et al., 1998; Doble, 1999; Sattler & Tymianski, 2000; Arundine & Tymianski, 2003). The striatum is susceptible to excitotoxic insult as this structure receives massive glutamatergic input from the cortex. The pathogenic mechanisms of excitotoxicity in HD emerged from the observation that striatal injections of glutamate agonists such as quinolinic acid (QA) that act on NMDA receptors can reproduce certain clinical and pathological features of HD (Beal et al., 1991; Ferrante et al., 1993; Fan & Raymond, 2007). In addition, HD mouse models showed enhanced excitotoxicity (Hodgson et al., 1999), increased sensitivity to NMDA receptor activation (Zeron, et al., 2002; Starling et al., 2005), and increased release of glutamate and its agonist from the cortex leading to excitotoxic striatal degeneration (Cepeda et al., 2003; Stack et al., 2007). Mutant huntingtin can impede the normal function of wild-type protein by associating with PSD-95 to alter interactions with NMDA receptors and promote sensitisation (Sun, et al., 2001), and interact with endocytic protein HIP1 (huntingtin-interacting protein 1) which is involved in the intracellular trafficking of AMPA receptors to potentiate glutamate-mediated excitotoxicity (Metzler et al., 2007).

**Apoptosis and caspases**

The role of apoptosis and caspases (cysteine proteases) has been proposed for neuronal loss in HD (Mattsson, 2000). For example, cultured striatal neurons from YAC transgenic mice exhibited cell death via apoptotic mechanisms (Zeron et al., 2004). The mutant huntingtin is a substrate for caspases 1 and 3 and calpain, all of which generates smaller N-terminal fragments that translocates into the nucleus to form aggregates (Kim, et al., 2001; Gafni & Ellerby, 2002; Sun et al., 2002). Also as the disease progresses, caspases 8 and 9 are
activated which in turn release cytochrome c that triggers a cascade of apoptotic mechanisms (Sanchez et al., 1999; Gervais, et al., 2002).

**Mitochondrial dysfunction and altered energy metabolism**

Mitochondrial dysfunction leading to oxidative stress and changes in energy metabolism has been implicated in an array of neurodegenerative disorders, including HD (Lin & Beal, 2006; Mochel et al., 2007; Trushina & McMurray, 2007; Turner et al., 2007). Studies have shown defects in complexes II, III, and IV of the mitochondrial respiratory chain in HD (Gu et al., 1996; Browne et al., 1997), and increases in the transglutaminase activity that may impair mitochondrial function (Karpuj et al., 1999; Lesort et al., 1999; Battaglia et al., 2007). The mitochondrial dysfunction hypothesis is further supported indirectly by the observation that administration of various mitochondrial inhibitors such as irreversible inhibitor 3-nitropropionic acid (3-NP), which produced clinical and pathological characteristics of HD (Browne & Beal, 2006; McLin et al., 2006; Browne, 2008). The mutant huntingtin involvement in energy impairment has been evidenced by direct interaction of mutant protein with mitochondrial membranes, altered mitochondrial trafficking (Orr et al., 2008; Reddy et al., 2009; Li et al., 2010), reduced ATP production (Seong et al., 2005), and disruption of calcium homeostasis thus leading to indirect excitotoxicity (Panov et al., 2002; Rockabrand, et al., 2007). The mutant protein also suppresses the expression of PGC-1α, a transcriptional co-activator involved in mitochondrial biogenesis and respiration (Puigserver & Spiegelman, 2003; Cui et al., 2006; Weydt et al., 2006).

**Transcriptional deregulation**

The polyglutamine (polyQ) tract in the mutant huntingtin interacts with a number of transcriptional regulators leading to deregulation in the transcriptional activity in HD - reviewed in (Sugars & Rubinsztein, 2003; Landles & Bates, 2004; Thomas, 2006; Cha, 2007). Some of the prominent members include: TATA binding protein (TBP); transcription factor II F (TFIIF); TBP-associated factor (TAFII130); specific protein-1 (Sp1); cAMP response element (CRE) binding protein (CREB); and CREB binding protein (CBP) - all of which directly interact within the expanded polyQ tail. These proteins are involved in the
basal transcriptional machinery and thus form critical components of cell survival 
(Shimohata et al., 2000; Dunah et al., 2002; Li et al., 2002; Luthi-Carter et al., 2002;
Schaffar et al., 2004; Jiang et al., 2006). Other nuclear proteins, such as pro-apoptotic 
transcription factor, p53, interact via SH3 domain sequences in the distal part of the polyQ 
tract (Steffan et al., 2000; Bae et al., 2005). Conversely, mutant huntingtin can also lose 
interactions with a number of cellular proteins including a transcription factor complex 
REST/NRSF (repressor-element-1 transcription factor/neuron-restrictive silencer factor) 
that binds to NRSE (neuron-restrictive silencer element) in the cytoplasm. This leads to 
nuclear translocation where it binds to NRSE domain and endorses remodelling of the 
chromatin via histone deacetylation. In turn, there is decreased production of BDNF (brain-
derived neurotrophic factor), an important survival factor for neurons as the NRSE is 
located in the promoter of the BDNF gene (Zuccato, et al., 2003; Strand et al., 2007).

Axonal transport and synaptic changes

The abnormal expansion of polyQ in the mutant huntingtin leads to disruption in 
intracellular dynamic processes such as axonal transport, vesicle trafficking of the Golgi 
apparatus, and synaptic exo- and endocytic processes in HD (Smith, et al., 2005; Borrell-
Pages, et al., 2006). The mutant protein may inhibit the interaction with wild-type 
huntingtin/HAP1/p150 complex that plays a role in anterograde and retrograde axonal 
transport (Gunawardena & Goldstein, 2005; McGuire et al., 2006; Morfini et al., 2009). 
This in turn can impair association between microtubule and its related motor protein 
kinesin and dynein (Reed et al., 2006; Caviston et al., 2007), and also reduce BDNF 
transport (Gauthier, et al., 2004). In addition, the mutant huntingtin impairs vesicle secretion 
and trafficking from the Golgi apparatus (Strehlow et al., 2007), such as alteration of the 
interaction with huntingtin-interacting protein 14 (HIP14), a protein involved in Golgi-
related trafficking of neuronal proteins, the BDNF protein (del Toro et al., 2006), and 
huntingtin protein itself (Yanai, et al., 2006). Moreover, mutant huntingtin can disrupt 
synaptic neurotransmission (Li, et al., 2003; Smith, et al., 2005) by inhibiting the functions 
of various synaptic proteins including SNARE (soluble N-thylmaleimide-sensitive factor 
attachment protein receptor) protein synaptobrevin-2, G-protein rabphilin 3A (Morton et al.,
2001), PACSIN1/syndapin complex (Modregger et al., 2002), and complexin II (Morton & 
Edwardson, 2001).
The HD allele with more than 36 CAG repeats in the IT15 gene results in the altered expression of mutant huntingtin (htt) protein with expanded polyglutamine. The mutant htt protein may lead to neuronal cell death via various proposed cellular mechanisms. Each process can be directly influenced by the mutant htt protein and therefore contribute to neuronal dysfunction and death independently from all other processes. In addition, each process can modulate the activity of the other processes which may enhance neuronal dysfunction by multiple pathways.

Figure 2.7 Mechanisms of pathogenesis in Huntington’s disease
2.4.4. Clinical Features and Symptoms of HD

Huntington’s disease typically develops in mid-life although the disorder can manifest at any time between infancy and senescence. Generally symptoms advance rapidly after onset and the patients progressively develop a clinical triad of movement, cognitive and emotional disorders (Nance, 1998; Margolis & Ross, 2003; Walker, 2007). The disease duration is typically 10-20 years. In the prediagnostic phase, some individuals may show subtle changes of personality, difficulty in multitasking, increased forgetfulness, irritability and anxiety. In this early stage, family members note restlessness or fidgeting. Weight loss is also a common feature of the disease (van der Burg et al., 2009). Death usually results from aspiration pneumonia secondary to dysphagia, or from complications resulting from falls or chronic illness. Suicide is another cause of death. Although a cure for HD is still unavailable, the identification and assessment of presymptomatic gene carriers and in the early disease stages broadened the known spectrum of characteristic clinical symptoms. The intricate interaction of this particular genetic defect with innumerable environmental factors and modifier genes produces a wide range of different possible phenotypes among patients with the same disorder. For example, monozygotic twins with HD typically have an age of onset within several years of each other, but in some cases show quite different clinical phenotypes (Wexler et al., 1987; Georgiou, et al., 1999; Squitieri et al., 2003; Anca, et al., 2004; Walker, 2007).

2.4.4.1. Motor Dysfunction

Motor disabilities usually progress over a 10-15 year period. Involuntary, choreiform movements represent a common disturbance in motor function which is a classic feature that can be traced back to George Huntington’s original work in 1872 (Huntington, 1872, 2003). Although useful for diagnosis, chorea is a poor marker of disease severity. Most patients initially display hyperkinetic movements that are progressively replaced by a more hypokinetic (akineto-rigid) syndrome in which bradykinesia, rigidity and dystonia predominate. Other motor difficulties include incoordination, oculomotor deficits, akathesia, and particularly dyskinesia, progressing to failure of initiation and execution of voluntary movements. In addition, the patient’s ability to speak and swallow is also often affected thus leaving the patient susceptible to aspiration pneumonia (Young et al., 1986; Berardelli et al., 1999; Mahant et al., 2003; Walker, 2007).
2.4.4.2. Cognitive Deficits

The cognitive disorder begins insidiously with the loss of mental flexibility and progressive decline of intellectual processes that leads to profound dementia; another aspect of the clinical indicator originally depicted by George Huntington (Huntington, 1872, 2003). Paulsen (2001b) noted the appearance of subtle cognitive deficits years before the appearance of obvious motor signs. Early cognitive defects include impaired concentration, dysfunction of short-term memory (often sparing long-term memory) and impairment of executive functions, such as organising, planning, decision-making, or adapting alternatives, and delay in the acquisition of new motor skills (Snowden et al., 2002; Ho et al., 2003). As the disease progresses these deficits develop into a more widespread subcortical dementia, with the deterioration of verbal skills such as speech and comprehension, and difficulty in visuospatial functioning (Lawrence et al., 2000; Kirkwood et al., 2001; Montoya, et al., 2006).

2.4.4.3. Mood, Behavioural and Psychiatric Changes

Unlike cognition, behavioural and psychiatric symptoms arise with some frequency but do not show a stepwise progression with disease severity (Thompson et al., 2002). There is a wide range of associated mood and neuropsychiatric complications. HD patients are afflicted with depression, dysphoria, agitation, irritability, labile mood, apathy, and anxiety (Rosenblatt & Leroi, 2000; Paulsen et al., 2001a). Other authors additionally report a disproportionately high prevalence of obsessive-compulsive symptoms, sexual disorders, sleep disturbances, explosive behaviours, personality changes, psychotic symptoms, and suicidal tendencies (Cummings & Cunningham, 1992; Robins Wahlin et al., 2000; Anderson et al., 2001; Baliko et al., 2004).

2.4.5. Neuropathology

2.4.5.1. Degeneration of the Striatum in HD

The neuropathological hallmark of HD is the progressive degeneration of the striatum of the basal ganglia (Vonsattel & DiFiglia, 1998; Vonsattel, et al., 2008). Gross examinations of
HD brains and in vivo neuroimaging techniques reveal a striking bilateral atrophy of the striatum (caudate nucleus and putamen) and increased size of the lateral ventricles. With the progression of the disease, striatal degeneration has an ordered and topographic distribution - develops simultaneously in dorsolateral-ventral direction, and also follows a caudo-rostral gradient (Ferrante et al., 1987b; Ferrante, et al., 1997). The most affected neuronal populations in the striatum are the GABAergic medium-sized spiny projection neurons that make up ~90-95% of the striatal neuronal population. Striatal interneurons (~5-10%) form a morphologically and biochemically heterogeneous group of locally projecting cells, and are typically not affected or mildly affected at late stages of the disease (Ferrante et al., 1987a; Reiner et al., 1988; Albin et al., 1992; Richfield et al., 1995).

In addition, within the striosome-matrix organisation of the striatum (Holt et al., 1997), neuronal loss and gliosis seem to first appear in the striosomal compartment, indicating that the neurons in striosomes may be more vulnerable at an early stage of HD than those in the matrix (Hedreen & Folstein, 1995). However, this data conflicts with other studies which show a preferential loss of neurons and neurochemical markers in the matrix compartment (Ferrante, et al., 1987b; Seto-Ohshima et al., 1988; Faull et al., 1993). These findings of the heterogeneous pattern of compartmental striatal degeneration in HD is interesting, since the studies in the rodent and primate brains show that the striosome and matrix compartments have different patterns of connectivity. The striosome compartment is more involved with the limbic and emotional functions whereas the matrix compartment may be more implicated with sensory and motor functions (Flaherty & Graybiel, 1993; Eblen & Graybiel, 1995). Extending these observations, Tippett and colleagues (2007) have shown that the HD cases with a major mood disorder demonstrated a predominant loss in the striosome compartment. This suggests that the different patterns of cell death in the striatum could contribute significantly to the variability in the HD symptomatology.

2.4.5.2. Striatal Neuropathological Grading

The most widely adopted grading of post-mortem neuropathological severity was developed by Vonsattel (1985). This system consist of five grades (0-4) of severity based on both the macro- and microscopic findings in the striatum, such that **Grade 0** shows no discernible
cellular abnormalities despite obvious premortem clinical manifestations and positive family history; **Grade 1**, limited neuronal loss with astrogliosis which is more evident in the caudate tail region; **Grade 2**, caudate nucleus atrophy with a convex border between caudate and the lateral ventricle; **Grade 3**, atrophy of the caudate and putamen with marked neuronal loss; and **Grade 4** shows severe atrophy in the striatum with up to 95% neuronal loss with associated astrocytosis, and prominent shrinkage in the putamen and globus pallidus structures (Vonsattel & DiFiglia, 1998; Vonsattel, et al., 2008).

2.4.5.3. Degeneration of the Basal Ganglia and Other Brain Structures in HD

The pathology in the brain also occurs in other nonstriatal structures in HD. These regions include basal ganglia structures (globus pallidus (GP), substantia nigra (SN), subthalamic nucleus (STN)), thalamus, hippocampus, cerebellum, cerebral cortex and its underlying white matter (Vonsattel, et al., 2008). **Cortical involvement in HD is further discussed in Section 2.5.** Other studies have indicated a marked atrophy in the hypothalamus and dysfunction in neuroendocrine circuits in rodent models and patients with HD (Kassubek et al., 2004; Petersen & Bjorkqvist, 2006; Petersen et al., 2009).

2.4.5.4. Cortico-Basal Ganglia-Thalamo-Cortical Circuitry in HD

The major neurological alterations that occur in HD are changes in the anatomy, neurochemistry, and cellular morphology of the basal ganglia (BG). The BG are a major group of extra-pyramidal nuclei located in the base of the forebrain and are involved in modulating motor, mood and cognitive control. The BG integrates diverse inputs from the cerebral cortex and “channel” this information back to the cortex via the thalamus, and thus these collective circuits are called the **cortico-basal ganglia-thalamocortical** loop. The projections in the loop form several functionally segregated parallel and interconnected systems. Prominent among these are the **motor circuit**, which involves the motor and premotor cortices and the dorsal striatum, and the **limbic circuit**, involving the cingulate, orbital and medial prefrontal cortices, and the ventral striatum (Alexander et al., 1990; Parent & Hazrati, 1995; Holt, et al., 1997).
Motor circuit

The input of the motor circuit consists of the striatum, which receives glutamatergic inputs from the entire cortex and dopaminergic inputs from the substantia nigra compacta (SNC). The output nuclei consist of the GP, substantia nigra reticulata (SNr), and STN. The target nuclei of these output regions are the VA-VL (ventral anterior and ventral lateral) nuclei of the thalamus, which in turn make an excitatory action upon the motor regions of the cortex, especially to the primary motor cortex, premotor and supplementary motor areas. The intralaminar nuclei of the thalamus are also involved in the motor circuit and form reciprocal connections with the basal ganglia and the cortex (Sidibe et al., 2002). Two major efferent pathways, the indirect and direct pathways have apparently opposing effects upon the output nuclei and thalamic target nuclei (Penney & Young, 1983, 1986; Albin et al., 1989; DeLong, 1990; Flaherty & Graybiel, 1994; Takada et al., 1998; McFarland & Haber, 2002). In the indirect pathway, the excitatory corticostriatal projection terminates onto striatal medium spiny neurons that contain GABA and enkephalin (ENK) along with dopamine D2-class receptors. The output first passes to the external segment of the GP (GPe) and then to GPi via STN whereby disinhibition of the subthalamic neurons increases the subthalamic activation of the GPi. This increase in GPi activation reduces thalamic activation of the cortex, in a “negative feedback” scheme (Figure 2.8). In the direct pathway, the excitatory fibres of the corticostriatal projection terminate on the medium spiny striatal projection neurons that contain GABA and substance P (SP) along with dopamine D1-class receptors. These target the internal segment of the GP (GPI) and SNr, and return to cortex via thalamic output. 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 a “positive feedback” scheme.

The disruption of these striatal pathways in HD leads to the development of motor dysfunction in both hyperkinetic and dyskinetic movements. In HD, hyperkinesias are caused by the preferential damage in the “indirect” GABA/ENK striatopallidal fibres 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. 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 chorea (hyperactivity) (Crossman,
1987; Crossman et al., 1988; DeLong, 2000). In contrast, the loss of “direct” GABA/SP striatopallidal fibres that project from the striatum to the GPi following degeneration of the striatonigral projection neurons causes rigidity (hypoactivity) (Berardelli, et al., 1999). The early loss of “indirect” GABA/ENK striatal cells has been supported by post-mortem studies; a reduction in ENK staining in the GPe was a prominent feature in the early stages of the disease (grade 0-1), while substance P (SP) immunostaining in the GPi and SN were affected in the later stages (grades 2-4) in HD (Glass et al., 2000; Deng et al., 2004).

**Limbic circuit**

The ventral striatum is associated with the limbic system by virtue of its afferent connections from the allocortical and mesocortical areas (Alexander, et al., 1990). The ventral striatum comprises the nucleus accumbens, and the ventromedial and caudoventral parts of both the caudate nucleus and the putamen (Fudge & Haber, 2002). The ventral striatum together with the ventral pallidum form a loop system involved in the regulation of mood and emotion. The ventral striatum receive fibres from the medial (areas 14, 24, 25 and 32) and orbitofrontal (areas 11, 13 and 47/12) cortical regions, and in turn project to the ventral pallidum (Ferry et al., 2000; Ongur & Price, 2000; Chiba et al., 2001; Ongur et al., 2003). This information is relayed via the mediodorsal (MD) thalamic nucleus to the orbitofrontal and medial prefrontal cortical areas (OMPFC) (Haber et al., 1995; McFarland & Haber, 2002). In addition, the cingulate cortex in the medial region (areas 24, 25, 32) is reciprocally interconnected with the anterior nuclear complex and the lateral dorsal nucleus, which forms a part of the limbic system along with the amygdala, hippocampus, fornix, mammillary nuclei and the mamillothalamic tract (Figure 2.8).

In HD, since damage to the frontal cortex and the basal ganglia can lead to mood and behavioural symptoms, the emotional disturbances have been suggested to reflect disturbed functioning in the frontostriatal circuits (Cummings, 1993). In addition, symptoms of agitation, irritation and euphoria in HD are suggested to be secondary to the underactivity of the indirect pathways which arise from the ventromedial caudate and ventral striatum connecting the limbic circuit (Litvan et al., 1998; Joel, 2001). Hence, the dorsal striatopallidal system plays a prominent role in initiating motor activities while the ventral
The striatopallidum plays a role in regulating emotion and initiating movements in response to emotional or motivational stimuli.

**Figure 2.8 Schematic diagram of the cortico-basal ganglia-thalamo-cortical loop**

The two functional loops within the overall sequence of the cortico-basal ganglia-thalamo-cortical circuitry. The basal ganglia receive inputs from the cerebral cortex and “channel” the flow of information back to the cortex via the thalamus through these proposed (A) motor, and (B) limbic circuits. **VS/VP**, ventral striatum and ventral pallidum, **STN**, subthalamic nucleus, **SN**, substantia nigra, **VA/VL**, ventral anterior/ventral lateral thalamic nuclei, **Ant**, anterior thalamic nucleus, **MD**, mediodorsal thalamic nucleus. For other abbreviations, see text in Section 2.4.5.4. Figure adapted from DeLong (2000), Joel (2001), Nieuwenhuys and colleagues (2008).
2.5. Cortical Degeneration in HD

The most striking and well-characterised neuropathology is found in the striatum of the basal ganglia. However, significant pathology in the other brain regions, especially the cerebral cortex has been identified, and new studies continue to provide information about the major neurodegeneration of the cerebral cortex and its involvement in the pathogenesis of HD.

The earliest accounts of cortical neuropathological features were described by several authors including Bryun (1968), Forno and Jose (1973), Tellez-Nagel, Johnson and Terry (1974), Roizin, Stellar and Liu (1979), and Trifiletti and colleagues (1987). Evidence of global cortical atrophy has been observed by de la Monte and colleagues (1988). The authors demonstrated morphometric atrophic changes in the brain (30% of mean brain weight reduction) with 21-29% reduction in the cerebral cortex and 29-34% loss in the white matter. The findings of cortical atrophy was further supported by a reduction of total brain volume (19%) in all brain lobes except relative sparing of the medial temporal lobe (Halliday et al., 1998). Another study indicated a significant reduction in the frontal lobe volume (17%) and frontal white matter volume (28%) (Aylward et al., 1998). More recently, the advances in detailed structural neuroimaging methods have facilitated important steps in elucidating the cortical basis of clinical heterogeneity in HD (Montoya, et al., 2006). For example, several authors have demonstrated evidence for regional and progressive thinning of the cortical grey matter in both symptomatic and premanifest HD patients which correlates with the clinical expression of the disease (Jernigan et al., 1991; Rosas, et al., 2002; Kassubek, et al., 2004; Rosas et al., 2005; Douaoud et al., 2006; Nopoulos et al., 2007; Rosas, et al., 2008; Paulsen, 2009; Tabrizi et al., 2009; Nopoulos, et al., 2010). Importantly, Rosas and colleagues (2002) showed widespread cortical thinning of 11 HD patients with varying clinical severity. The thinning of the cortex appeared to be progressive, and followed a posterior to anterior regional pattern of cortical degeneration. Cortical thinning also occurred early in the disease and showed specific regional thinning in different cases. The greatest amount of thinning was observed in the sensorimotor cortex in patients at all stages of the disease. In the following study, MRI investigation of 33 HD patients showed varying degree of thinning in different cortical areas (Rosas, et al., 2008). The primary motor (BA 4), sensory (superior portions of BA 3, 2, 1) and visual cortical
regions were the most affected, and the thinning was extended to other regions that include posterior superior frontal, posterior middle frontal, superior parietal, and the parahippocampal gyrus, with an apparent thickening of the anterior cingulate cortex. Furthermore, the thinning in the different cortical areas correlated with the varying cognitive deficits and motor disorder of the different individuals with HD.

However, while it is agreed that cortical degeneration plays a major role in HD, it is uncertain whether there is a differential or selective vulnerability of the neuronal subtypes involved in the pathogenesis of HD in the cerebral cortex, and the pattern of these neuronal changes in the different regions. Studies on the areal and laminar specific neuronal degeneration in the HD cortex have indicated that there was a significant loss of pyramidal projection neurons in layers III and V in the superior frontal cortex (Cudkowicz & Kowall, 1990a; Hedreen, et al., 1991) and dorsolateral prefrontal cortex of HD patients with shrunken dendritic trees and sparse spines in advanced stages (Sotrel, et al., 1991; Sotrel, et al., 1993; Selemon, et al., 2004). Another study by Rajkowska and colleagues (1998) has shown neuronal degeneration in BA 9 and 17 with decreased neuronal size. These studies have focused mainly on the prefrontal cortex, as its role in behaviour suggests that changes in the prefrontal cortex may contribute to the behavioural aspects of HD (Watkins et al., 2000). A more detailed quantitative study using stereological cell counting has been addressed by Heinsen and colleagues (1994), and the authors found a pronounced pyramidal cell loss in the supragranular layers in the primary sensory areas including primary somatosensory cortex (BA 3, 1, 2), primary visual cortex (BA 17), primary auditory cortex (BA 41), and association areas of the frontal, parietal, and temporal lobes. Similarly, Macdonald and colleagues (1997) reported a significant reduction of pyramidal cells across layers III and V, and also found atrophy of cell bodies of the remaining cells in the angular gyrus of the parietal lobe. In the following studies, Macdonald and Halliday (2002) investigated cellular changes in the motor cortical regions, i.e., primary motor cortex (BA 4), supplementary and premotor region (BA 6), and cingulate motor cortex (posterior part of BA 24), and observed a significant reduction of total neuronal number in the primary motor cortex (58% loss) and the premotor region (51% loss) in HD. No significant change was observed in the cingulate motor region. In addition, there was a significant loss of pyramidal cells (59% loss) in the primary motor cortex compared to controls with relative sparing of interneurons.
In a recent pathologic study, the variable neuropathology in the cerebral cortex has been correlated with specific symptoms and progression of HD (Thu, 2006; Thu, et al., 2010). Detailed stereological cell counts in our laboratory of the total neuronal number (using NeuN marker) in 8 cortical regions of 14 HD cases have shown a variation in the total number of neurons (NeuN) in the primary motor cortex (24% loss), primary somatosensory cortex (27% loss), superior frontal cortex (42% loss), superior parietal cortex (36% loss), middle temporal cortex (27% loss), primary (27% loss) and secondary (27% loss) visual cortices, and anterior cingulate cortex (36% loss). In addition, the number of SMI32+ pyramidal neurons was also affected in these regions which generally followed the pattern of total neuronal loss, with 27-41% reduction in the pyramidal cell number in all cortical regions, except for the primary visual cortex which showed no overall significant pyramidal cell loss (Thu, 2006; Nana, 2009; Thu, et al., 2010). Interestingly, the loss of all neuronal types (NeuN) and SMI32+ pyramidal neurons varied between HD cases which expressed different clinical symptoms (Thu, et al., 2010). In particular, a significant cell loss in the primary motor cortex (28% loss in total neuronal population (NeuN); 45% loss of SMI32+ pyramidal neurons) was associated with HD cases with predominant motor abnormalities but no significant cell loss was observed in HD cases with major mood symptoms. In contrast, the significant cell loss in the anterior cingulate cortex (54% loss in total neuronal population (NeuN); 40% loss of SMI32+ pyramidal neurons) was associated with HD cases with major mood dysfunction but no significant loss was observed in HD cases with a predominant motor symptom profile. These studies clearly indicate the basis of cortical degeneration in which neuronal loss in the specific functional brain region correlates with the variation in the symptom profiles exhibited by different HD cases.

2.6. Involvement of Cortical Interneurons in HD

The degeneration of cortical pyramidal neurons in layers III, V, and VI have been well documented (Cudkowicz & Kowall, 1990a; Hedreen, et al., 1991; Sotrel, et al., 1991; Sotrel, et al., 1993; Heinsen, et al., 1994; Jackson, et al., 1995; Macdonald, et al., 1997; Rajkowska, et al., 1998; Gutekunst, et al., 1999; Sapp, et al., 1999; Sieradzan & Mann, 2001; Macdonald & Halliday, 2002; Selemon, et al., 2004; Thu, 2006; Nana, 2009; Thu, et al., 2010), although relatively few interneuron studies have been conducted in the HD cortex. Pathological studies on the cortical interneurons have shown that there were relative sparing
of parvalbumin (PV) and neuropeptide Y (NPY) expressing interneurons in the superior frontal cortex of HD patients (Cudkowicz & Kowall, 1990a, 1990b). In addition, Macdonald and Halliday (2002) showed no significant change in the interneuron populations defined by calbindin (CB), calretinin (CR), and parvalbumin (PV) in the motor cortical regions of 5 HD cases examined in their study. In contrast, Ferrer and colleagues (1994) observed a significant decrease in PV expressing interneurons in the frontal cortex, but significant difference was not observed in the occipital and temporal lobes. These results show that there is a heterogeneous topographical pattern of interneuron loss in the different functional regions of the cortex in HD.

Although the majority of studies on the interneuronal involvement in the cortex of the human brain show relative sparing, dysfunction in these cells are indicated by several animal studies in HD. More recently, Cre/LoxP conditional HD mice expressing mutant huntingtin with 103 glutamine repeats, either in all neurons of the brain or restricted to the vulnerable cortical pyramidal neurons, have been generated (Gu et al., 2005). Interestingly, the motor deficits and cortical neuropathology were observed only when the mutant huntingtin was expressed in multiple neuronal types, including cortical interneurons, but not when it was restricted to the cortical pyramidal neurons alone. This suggests that the pathological interactions between interneurons and pyramidal neurons may contribute to the cortical manifestation of HD. In addition, the authors have shown an early deficit in cortical inhibition, e.g., electrophysiological studies showed a reduction of GABAergic inhibitory input onto the cortical pyramidal neurons which occurred early in disease progression. This is significant because this study suggests that the cortical hyperexcitability as a result of deficits in GABAergic interneuronal inhibition could be an early event in HD. Another study by Spampanato and colleagues (2008) showed specific synaptic pathology of parvalbumin (PV) interneurons and pyramidal neurons in the upper layers II/III in a BAC transgenic mouse model of HD. In addition, a study in the somatosensory cortex of three HD mouse models (R6/2, YAC128, CAG140 knock-in) demonstrated alterations in the IPSP patterns and frequency of the cortical GABAergic interneurons (Cummings et al., 2009). The progressive synaptic dysfunction resulting in altered cortical excitation and the loss of inhibition onto pyramidal neurons further strengthens the hypothesis that the GABAergic interneurons are important in the pathogenesis of HD.
2.7. Aims of the Study

Huntington’s disease (HD) is characterised by major degeneration in the basal ganglia and the cerebral cortex. Recent studies indicate that the variable clinical HD symptomatology is related to the pathology in specific regions of the cerebral cortex. In particular, Thu (2006) and Thu and colleagues (2010) have provided evidence that the pattern of pyramidal cell loss in the cerebral cortex correlates with the symptom profile in HD. However, to date, there have been no studies to determine whether the GABAergic interneurons are also affected in the cerebral cortex and their relation to symptom heterogeneity in HD. Indeed, animal models of HD have suggested a possible role of cortical GABAergic interneurons in the pathogenesis of HD. Hence, it was hypothesised that dysfunction of distinct GABAergic interneuronal populations in different functional regions of the cerebral cortex might contribute differentially to the variable symptomatology in HD. Therefore, clarification of this question is of major importance for gaining a better understanding of the pathogenesis in the cerebral cortex in HD and its relation to the variable symptom profiles in HD.

This study is directed towards determining whether the GABAergic interneurons in the cerebral cortex are involved in the pathogenesis in HD. The specific aims of this study are to:

- Investigate the immunohistochemical staining patterns of the three major interneuronal populations defined by calcium-binding proteins, calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) in two functionally diverse cortical regions (primary motor cortex and anterior cingulate cortex) of the human brain in Huntington’s disease and neurologically normal cases.

- Quantify the total number of interneurons (CB, CR, PV interneurons) using unbiased stereological cell counting techniques in the two cortical regions in Huntington’s disease and normal control cases.

- Investigate the pattern of interneuron loss and correlate this pattern with the clinical data of each HD case such as the dominant HD symptom profile, HD striatal neuropathological grade, CAG repeat length, post-mortem interval, and age in the two cortical regions in HD.
CHAPTER 3. MATERIALS AND METHODS

3.1. General Introduction
In this study, post-mortem human brain tissue was used to investigate the GABAergic interneurons in two functionally diverse regions of the cerebral cortex - primary motor cortex (Brodmann area 4) and anterior cingulate cortex (Brodmann area 24) - in both Huntington’s disease (HD) and neurologically normal control brains. First, immunohistochemistry was performed using antibodies raised against the three calcium-binding proteins (calbindin-D28k, calretinin, parvalbumin) to identify the different interneuronal subpopulations in the two cortical regions, and secondly, light microscopy was used to view and compare the immunostaining patterns of the interneurons. Finally, the unbiased stereological cell counting techniques were used to compare the total number of interneurons in a subvolume of the entire Brodmann areas (4 and 24) in the HD and normal control cases. A subvolume of the entire tissue block was sampled instead of the entire region due to the constraints and availability of the human tissue. In addition, western blot analysis was performed to validate the specificity of the antibodies, and confocal laser scanning microscopy to quantify double-labelled neurons for the selected control cases.

3.2. Human Brain Tissue
The human brain tissue was obtained from the New Zealand Neurological Foundation Human Brain Bank in the Centre for Brain Research, The University of Auckland. The study commenced with the full consent of all families and the approval of research protocols from the University of Auckland Human Participants Ethics Committee. A total of 28 brains were used in this study with 13 cases of Huntington’s disease (HD) and 15 cases of neurologically normal control brains matched for age, gender and post-mortem interval. The cases included 17 males and 11 females, aged 35-83 years of age (mean age ± SD = 62.3 ± 11.1 years), with post-mortem interval (PMI) prior to perfusion between 3 to 24 hours (mean PMI = 12.8 ± 5.6 hours).
Note: The cases used in the present study were the same cases as the one used in previous cortical pathological studies in HD (Thu, 2006; Thu, et al., 2010).

3.2.1. Normal Control Brain Tissue

The normal control brains were obtained from cases with no history of neurological or psychiatric disorder and showed no indication of neuropathology on microscopic examination by a neuropathologist. A total of 15 control cases were used in this study, which were closely matched for age, gender and post-mortem interval to the selected HD cases (see next Section 3.2.2). The cases included 10 males and 5 females, aged 46-83 years of age (mean age = 64.5 ± 10.6 years), and post-mortem interval (PMI) between 5 and 21 hours (mean PMI = 11.7 ± 4.0 hours). The description of the normal control cases is given below (Table 3.1).

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Gender</th>
<th>PMI (hours)</th>
<th>CAG Repeat Length</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>H108</td>
<td>58</td>
<td>M</td>
<td>16</td>
<td>15/17</td>
<td>Coronary atherosclerosis</td>
</tr>
<tr>
<td>H110</td>
<td>83</td>
<td>F</td>
<td>14</td>
<td>17/17</td>
<td>Ruptured aortic aneurysm</td>
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<td>M</td>
<td>10</td>
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<tr>
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<td>79</td>
<td>M</td>
<td>8</td>
<td>14/15</td>
<td>Bleeding stomach ulcer</td>
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<tr>
<td>H115</td>
<td>61</td>
<td>M</td>
<td>12</td>
<td>17/19</td>
<td>Hypertensive heart disease</td>
</tr>
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<td>H118</td>
<td>57</td>
<td>M</td>
<td>10</td>
<td>15/16</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>H120</td>
<td>62</td>
<td>M</td>
<td>11</td>
<td>18/22</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>H121</td>
<td>64</td>
<td>F</td>
<td>5</td>
<td>18/23</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>H127</td>
<td>59</td>
<td>F</td>
<td>21</td>
<td>15/17</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>H129</td>
<td>48</td>
<td>M</td>
<td>12</td>
<td>20/21</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>H131</td>
<td>73</td>
<td>F</td>
<td>13</td>
<td>17/17</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>H132</td>
<td>63</td>
<td>F</td>
<td>12</td>
<td>15/19</td>
<td>Ruptured aortic aneurysm</td>
</tr>
<tr>
<td>H136</td>
<td>75</td>
<td>M</td>
<td>13</td>
<td>N/A</td>
<td>Ruptured aortic aneurysm</td>
</tr>
<tr>
<td>H139</td>
<td>73</td>
<td>M</td>
<td>5.5</td>
<td>N/A</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>H330</td>
<td>66</td>
<td>M</td>
<td>13</td>
<td>15/15</td>
<td>Cor pulmonale</td>
</tr>
</tbody>
</table>
3.2.2. HD Brain Tissue

The Huntington’s disease (HD) brains were obtained through a donor program where fully informed consent has been granted by the patient and/or the family in advance of the person’s death. The HD cases showed clinical symptoms characteristic of HD and had a known family history of HD. The brain tissue for HD cases were disease confirmed through gene testing (number of CAG repeats in both alleles of the *HD* gene), and examined by a neuropathologist and graded according to the standard Vonsattel striatal grading criteria (Vonsattel, et al., 1985; Vonsattel, et al., 2008). The HD cases used in this study were also categorised into three symptom groups according to their main clinical symptom profile (Tippett, et al., 2007; Thu, et al., 2010). A total of 13 HD cases were used in this study which included 7 males and 6 females, aged 35-75 years of age (mean age = 59.7 ± 11.5 years), and post-mortem interval (PMI) between 3 and 24 hours (mean PMI = 14.0 ± 7.0 hours). The description of the HD cases is given below (Table 3.2).

Table 3.2 List of Huntington’s disease cases used in this study

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Gender</th>
<th>PMI (hours)</th>
<th>CAG Repeat Length</th>
<th>Striatal Grade</th>
<th>Symptom Category</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC60</td>
<td>64</td>
<td>M</td>
<td>23</td>
<td>18/43</td>
<td>3</td>
<td>Mixed</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>HC68</td>
<td>65</td>
<td>F</td>
<td>11</td>
<td>17/42</td>
<td>1</td>
<td>Motor</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>HC72</td>
<td>63</td>
<td>F</td>
<td>24</td>
<td>17/42</td>
<td>2</td>
<td>Motor</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>HC73</td>
<td>47</td>
<td>M</td>
<td>4</td>
<td>19/49</td>
<td>2</td>
<td>Motor</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>HC79</td>
<td>56</td>
<td>F</td>
<td>4</td>
<td>17/42</td>
<td>1</td>
<td>Mixed</td>
<td>Cardio-resp failure</td>
</tr>
<tr>
<td>HC82</td>
<td>74</td>
<td>M</td>
<td>16</td>
<td>15/42</td>
<td>2</td>
<td>Mood</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>HC85</td>
<td>61</td>
<td>F</td>
<td>19</td>
<td>24/44</td>
<td>3</td>
<td>Mood</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HC86</td>
<td>46</td>
<td>M</td>
<td>18</td>
<td>17/37</td>
<td>0-1</td>
<td>Mixed</td>
<td>Head injury</td>
</tr>
<tr>
<td>HC93</td>
<td>56</td>
<td>F</td>
<td>17</td>
<td>20/43</td>
<td>3</td>
<td>Mixed</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>HC95</td>
<td>66</td>
<td>F</td>
<td>12</td>
<td>20/39</td>
<td>2</td>
<td>Mood</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HC99</td>
<td>68</td>
<td>M</td>
<td>13</td>
<td>21/41</td>
<td>2</td>
<td>Motor</td>
<td>Broncho-pneumonia</td>
</tr>
<tr>
<td>HC101</td>
<td>35</td>
<td>M</td>
<td>18</td>
<td>17/44</td>
<td>1</td>
<td>Mood</td>
<td>Asphyxia</td>
</tr>
<tr>
<td>HC107</td>
<td>75</td>
<td>M</td>
<td>3</td>
<td>19/43</td>
<td>3</td>
<td>Mixed</td>
<td>Broncho-pneumonia</td>
</tr>
</tbody>
</table>
3.2.2.1. Assessment of CAG Repeat Length

For each HD case, the number of CAG repeats in both alleles of the \textit{HD} gene was determined by polymerase chain reaction (PCR) amplification of DNA as previously described (Whitefield et al., 1996). The DNA for amplification was isolated either from cerebellar brain tissue or blood samples from the same HD cases.

3.2.2.2. Assessment of Striatal HD Grade

The neuropathologic “striatal” grading of the HD cases were carried out according to the standard Vonsattel grading criteria (Grades 0-4) (Vonsattel, et al., 1985; Vonsattel, et al., 2008) by a neuropathologist Dr. B. Synek with extensive experience in HD neuropathology. The striatal pathological grades of HD cases used in this study ranged from Grade 0-3 (Table 3.2).

3.2.2.3. Assessment of Clinical Symptomatology

Clinical data to assess symptom profiles for each case was collected retrospectively from the family members of the 13 individuals who had died with HD and whose families had kindly requested donation of the brain tissue to the Neurological Foundation of New Zealand Human Brain Bank. The total clinical data was collected as part of a larger study using a \textit{Semi-structured interview} followed by the \textit{Clinical HD questionnaire}. For detailed description, see Tippett and colleagues (2007), and Thu and colleagues (2010).

The \textit{Semi-structured interview} was designed to collect accurate information about the age of clinical onset and the patterns of symptom change related to the disease for each HD case. The interview was designed to contain open and broad questions initially, followed by more specific questions. This enabled the interview to be fluid and progress naturally to allow interviewees to describe their version of events before being exposed to the prompts of the specific questions.
The **Clinical HD questionnaire** was developed specifically for the HD research in our laboratory to provide a comprehensive account of all reported changes observed in HD and to provide an empirical method to evaluate the clinical severity of “motor” and “mood” impairment, both at clinical onset and at near end-stage. The assessment of “cognitive” domain was not feasible due to the retrospective aspect of data collection. The content of the questionnaire was based on the information derived from comprehensive review of the HD literature and consultation with a neurologist (Dr. J. Simcock) and a clinical geneticist (Dr. I. Winship) experienced with the HD population. The questionnaire consisted of 49 items, using language readily understandable to the lay-person and was conducted with the assistance of the researcher who clarified the content of individual items. For each item, the interviewees indicated whether or not “motor” or “mood” symptoms were seen in their family member with HD at two stages of the disease (at clinical onset, and in the period near death) and scored on a five-point severity scale. For specific details, see Tippett and colleagues (2007), and Thu and colleagues (2010).

Total clinical data for each case, i.e., responses from the *Semi-structured interview* and the *Clinical HD questionnaire* were then viewed independently by two psychologists, Dr. L. Tippett and V. Hogg in the Department of Psychology, Centre for Brain Research, The University of Auckland. For any inconsistencies, the case materials were reviewed by both psychologists until a formal consensus was reached. Subsequently, carefully assessed HD cases were categorised into three main symptom groups according to the dominant symptom profile. Definitions used for these classifications are outlined as follows:

- **HD Motor**: Individuals that clearly displayed movement disorder with no significant presence of mood symptoms during the disease course.

- **HD Mood**: Individuals that demonstrated predominant mood disturbance during the course of the disease, however, some degree of motor symptoms was present which were either very mild or only emerged in the very late stages of the disease.

- **HD Mixed (motor and mood)**: Individuals that clearly exhibited significant levels of both motor and mood symptom domains during a large part of the disease.
3.3. Brain Tissue Processing

The human brains were prepared by two methods. First, the brains were fixed by perfusion through the internal carotid and basilar arteries with phosphate-buffered saline (PBS) containing 1% sodium nitrite (NaNO₂) to dilate and clear blood out from the blood vessels for 30 min, followed by 15% formalin fixative in 0.1 M phosphate buffer at pH 7.4 for 60 mins. Following perfusion, the brain was carefully dissected into smaller tissue blocks containing specific functional regions located on particular gyri of the brain (see next Section 3.3.1). The tissue blocks were post-fixed by immersion in fresh fixative at room temperature for 24 h. The blocks were then stored in a cryo-protective solution, consisting of 20% sucrose in 0.1 M phosphate buffer with 0.1% sodium azide at pH 7.4 for 2-3 days, and then equilibrated in 30% sucrose in 0.1 M phosphate buffer with 0.1% sodium azide at pH 7.4 at 4 °C for a further 7 days. For long-term storage, the tissue blocks were then rapidly frozen using powdered dry ice, double wrapped in aluminium foil, and stored in a -80 °C freezer until required for immunohistochemical processing (see Section 3.3.2). Unless otherwise stated, the fixed tissue blocks used were obtained from the right hemisphere of the brain. Second, the brains were frozen as fresh unfixed tissue blocks for western blot analysis (see Section 3.3.4). The fresh unfixed brain was also carefully dissected into the same functional regions as the fixed hemisphere and stored in a -80 °C freezer. Unless otherwise stated, the fresh tissue blocks were obtained from the left hemisphere of the brain. For more details, see Waldvogel and colleagues (2006, 2008).

3.3.1. Cortical Regions of Interest

The two regions of the cerebral cortex investigated in this study include: the primary motor cortex and the anterior cingulate cortex. Due to the constraint on the availability of human tissue, only a particular block representing the cortical region was studied instead of the whole gyrus. In addition, a cortical block of each gyrus may contain more than one cytoarchitectural areas of Brodmann. In order to ensure the validity and rigour of this study, it was decided at the outset of the study to investigate only the specific Brodmann areas associated with each of the functional cortical regions, e.g., Brodmann area 4 of the primary motor cortex and Brodmann area 24 of the anterior cingulate cortex.
3.3.1.1. Primary Motor Cortex (Brodmann Area 4)

The primary motor cortex is located on the precentral gyrus, immediately anterior to the central sulcus. The entire pre- and postcentral gyrus was carefully dissected out during the tissue blocking step and was cut equally into 4 blocks in the dorso-ventral axis of the gyrus, and labelled 0, 1, 2 and 3 from the dorsal end. The tissue blocks were recognised by cutting a small wedge in the white matter within each block (no wedge for block 0, one wedge for block 1, two wedges for block 2, and three wedges for block 3). The variation in the size of the tissue blocks ranged from 0.75 to 1.5 cm depending on the size of the whole brain. In this study, primary motor cortex block 1 (SM1) was used which corresponds to the region just above the mid dorso-ventral portion of the precentral gyrus, i.e., the area corresponding topographically to the cortical areas of the upper limb (Figure 3.1).

Figure 3.1 Location of the primary motor cortex block used in this study
Diagram showing (A) the location of SM 1 block of the primary motor cortex, and the (B) cross-section of the SM 1 block with specific Brodmann area 4 cytoarchitectural region.
Cytoarchitectonically, **Brodmann area 4** (BA 4) is an agranular cortex that is characterised by a prominent layer V (Va and Vb) containing giant motor neurons known as Betz cells (Brodmann, 1909; Marín-Padilla, 1970; Sherwood, et al., 2003). Brodmann (1903, 1909) defined the caudal border of area 4 by lack of an inner granular layer from the adjoining area 3a in the fundus of the central sulcus. The rostral border of area 4 is more difficult to delineate. Other characteristics of area 4 include large cortical thickness, inconspicuous lamination pattern (indistinctive boundary between external pyramidal layer III and internal pyramidal layer V, lack of internal granular layer IV, a gradual transition from the multiform layer VI to the subjacent white matter), sparsely distributed cells and decreasing size of the Betz cells along the medio-latero-basal extent (Matelli, et al., 2004; Zilles, 2004; Brodmann, 2006). In addition, area 4 in humans is further subdivided into the anterior (4a) and the posterior (4p) regions (Geyer, et al., 1996).

3.3.1.2. Anterior Cingulate Cortex (Brodmann Area 24)

The cingulate gyrus is located immediately dorsal to the corpus callosum and extends along its entire length in the rostro-caudal axis. The human cingulate gyrus can be divided into: the *anterior cingulate cortex* (ACC) which can be further separated into the perigenual anterior cingulate cortex (pACC) and the midcingulate cortex (MCC); the *posterior cingulate cortex* (PCC); and the *retrosplenial cortex* (RSC). The ACC comprises Brodmann areas 25, 33, 24 and 32 at the rostral end. The MCC is located in the middle one-third of the cingulate gyrus which consists of Brodmann areas 33’, 24’, and 32’. The PCC is composed of Brodmann areas 23 and 31 whereas the RSC comprises areas 29 and 30 which extend around the splenium of the corpus callosum, and the gyrus curves inferiorly into a narrow isthmus and becomes continuous with the parahippocampal gyrus of the temporal lobe (Vogt, et al., 1995; Paus, 2001; Vogt, et al., 2004; Vogt et al., 2005). The entire cingulate gyrus was carefully dissected out during the tissue blocking step and were cut equally into 5 blocks in the rostro-caudal axis of the gyrus, and labelled 0, 1, 2, 3 and 4 from the rostral end. The variation in the size of the tissue blocks ranged from 0.7 to 1.3 cm depending on the size of the whole brain. In this study, **cingulate gyrus block 1** (CG1) was consistently used that correspond to the region of the anterior cingulate cortex immediately dorsal to the genu of the corpus callosum (Figure 3.2).
Cytoarchitectonically, Brodmann (1909) described the anterior to middle cingulate gyrus consisting of mainly area 24, but also includes cingulofrontal transition area 32 superiorly, and area 33 near the junction with the corpus callosum in the depths of the rostral callosal sulcus (Vogt, et al., 1995; Paus, 2001; Vogt, et al., 2004; Zilles, 2004). **Brodmann area 24** within the anterior cingulate cortex (ACC) is an agranular cortex characterised by cell-dense layer Va, and a neuron-sparse layer Vb with frequent clumps of medium to large neurons. The ACC in this respect has similar laminar specialisations to the adjacent motor areas of the neocortex (i.e., primary motor cortex). Area 24 occupies most of the ACC and can be further divided into 24a, 24b and 24c. Area 24a is located adjacent to area 33 and is partially in the depths of the callosal sulcus. A feature of area 24b is the magnocellular anterogennual field of Braak (1976) in layer Va that contains many small and large neurons. These cells have shown to form aggregates (Vogt, et al., 2004; Vogt, et al., 2005). In addition, there are spindle neurons of Nimchinsky that are unique to humans and nonhuman primates (Nimchinsky et al., 1995; Nimchinsky et al., 1999). Area 24c lies in the rostral depths of the cingulate sulcus lateral to area 24b and has thin layers II-III and thinner layer V. Due to the difficulty in distinguishing one area from another, all three sub-regions of area 24 were included in the study.

**Figure 3.2 Location of the anterior cingulate cortex block used in this study**

Diagram showing (A) the location of CG 1 block of the anterior cingulate cortex, and the (B) cross-section of the CG 1 block with specific Brodmann area 24 cytoarchitectural region.
3.3.2. Immunohistochemistry

3.3.2.1. Sectioning of Tissue Blocks and Sampling Method

For each brain, the specific fixed tissue blocks from the two cortical regions selected for detailed immunohistochemical and stereological cell counting analyses were removed from the -80 °C storage freezer and were serially sectioned on a Zeiss freezing microtome - at a thickness of 50 μm in the coronal plane perpendicular to the longitudinal axis of the gyrus. The sections were collected in a strict serial order into the 6-well tissue culture plates containing PBS with 0.1% sodium-azide (PBS-azide) and stored in the 4 °C coldroom until used for further immunohistochemical processing. For stereological purposes, immunohistochemistry was performed on a series of 10 sections from the first 100 sections selected by a systematic random sampling (SRS) protocol, i.e., every 10th section with a randomly determined starting point (1-10) was collected from the first 100 sections of each case which results in a total of ten sections equally spaced apart (for more details, see Section 3.4.3). Before the stereological analyses all cases were coded and blinded to the investigator.

3.3.2.2. Single Immunoperoxidase Labelling

The immunohistochemical procedures for single biotin-peroxidase labelling were processed free-floating in tissue culture plates (Waldvogel et al., 2006; Waldvogel et al., 2008). The sections stored at 4 °C in PBS-azide were transferred into a clean set of 6-well plates filled with PBS containing 0.2% Triton-X100 (PBS-T) and incubated overnight at 4 °C (to wash off PBS-azide used for storage). Standard washing of the sections involved three 10 min rinses in PBS-T (3 x 10 min PBS-T) unless otherwise specified. The sections were incubated in a solution containing 50% methanol with 1% H₂O₂ for 20 min to block endogenous peroxidase activity in the tissue and also to prevent non-specific background staining. The sections were then washed (3 x 10 min PBS-T) before primary antibody incubation. All primary antibodies were diluted in immunobuffer consisting of 1% normal goat serum (NGS) in PBS with 0.2% Triton-X and 0.04% Merthiolate (Sigma) to prevent any non-specific binding of the antibodies. The free-floating sections were then incubated with 1.5 ml of diluted primary antibody in each tissue culture well on a shaker at 4 °C for 72 h. A dilution series was performed for each antibody in order to determine their optimal concentration for staining (Table 3.3).
Table 3.3 List of primary antibodies used for single immunoperoxidase labelling

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin-D28k</td>
<td>Rabbit Polyclonal</td>
<td>1:2000</td>
<td>Piers Emson (Babraham Institute)</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Rabbit Polyclonal</td>
<td>1:5000</td>
<td>Swant</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Mouse Monoclonal</td>
<td>1:10000</td>
<td>Piers Emson (Babraham Institute)</td>
</tr>
<tr>
<td>Neuronal N</td>
<td>Mouse Monoclonal</td>
<td>1:1000</td>
<td>Chemicon (Millipore)</td>
</tr>
</tbody>
</table>

The primary antibodies were washed off (3 x 10 min PBS-T) the following day and the sections were incubated with biotinylated secondary antibodies specific to the host species of the corresponding primary antibodies diluted in 1% NGS on a shaker at room temperature overnight (Table 3.4). The secondary antibodies were then washed off (3 x 10 min PBS-T) and tertiary antibody was applied and incubated on a shaker at room temperature for 4 h. For sections labelled with primary antibodies generated in the mouse and rabbit, ExtrAvidin peroxidase conjugate and Streptavidin-HRP (horse radish peroxidise) conjugate was applied, respectively (Table 3.4). The tertiary antibodies were washed off (3 x 10 min PBS-T) and subsequently, the sections were developed with 0.05 M 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 0.01% H₂O₂ in 0.1 M phosphate buffer, pH 7.4, for 10-15 min to produce a brown reaction product. The sections were again thoroughly washed (3 x 10 min PBS-T) and gelatine-mounted onto chrome alum-coated glass slides and left to air-dry. The mounted sections were rinsed in distilled water and dehydrated through a graded alcohol series to xylene. It consisted of 5 min sequential washes in ascending concentrations of 75%, 85% and 95% alcohol baths; followed by two 10 min washes in 100% alcohol baths and three steps of xylene washes each 20 min long. Each slide was removed from the xylene and coverslipped with DPX mounting medium (BDH Laboratory Supplies, Poole, England). Some sections were also processed as negative controls to determine non-specific staining by following the same procedures detailed above except omission of either the primary or secondary antibodies at appropriate stages during the protocol.
Table 3.4 List of secondary and tertiary antibodies for single immunoperoxidase labelling

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Mouse</td>
<td>Goat</td>
<td>1:500</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-Rabbit</td>
<td>Sheep</td>
<td>1:500</td>
<td>Chemicon (Millipore)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tertiary Antibody</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ExtrAvidin-peroxidase conjugate</td>
<td>-</td>
<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Strepavidin-HRP conjugate</td>
<td>-</td>
<td>1:1000</td>
<td>Chemicon (Millipore)</td>
</tr>
</tbody>
</table>

3.3.2.3. Double Immunofluorescent Labelling

To study colocalisation of the three calcium-binding proteins (calbindin-D28k, calretinin and parvalbumin) in the human primary motor and anterior cingulate cortices (see Chapters 4 and 6), the three markers in pairs of combinations were examined using double immunofluorescence labelling. The procedure for double immunofluorescent labelling was carried out as described for single immunoperoxidase labelling, except that two primary antibodies raised in different species were applied collectively. All primary antibodies were diluted in immunobuffer consisting of 1% NGS in PBS with 0.2% Triton-X and 0.04% Merthiolate (Sigma) and incubated on a shaker at 4 °C for 72 h. The primary antibodies were washed off (3 x 10 min PBS-T) and fluorescently labelled secondary antibodies diluted in 1% NGS specific to the host species of the corresponding primary antibodies were added and left to incubate overnight at room temperature (Table 3.5). The secondary antibodies were selected that emit fluorescence in the different spectra. All mouse monoclonal antibodies were tagged with green fluorescent anti-mouse Alexa Fluor 488 conjugate while the rabbit polyclonal antibodies were tagged with red fluorescent anti-rabbit Alexa Fluor 594 conjugate. Once the secondary antibodies had been applied, sections were kept in the dark for the duration of all subsequent processing. The sections were then mounted in 0.1 M phosphate buffer, coverslipped using Prolong Gold antifade (Invitrogen), and nail polish was put around the coverslip to prevent drying of the tissue. The sections were then stored in the dark at 4 °C overnight before being visualised and imaged under a confocal laser scanning microscope (see Section 3.4.2). Negative control sections were processed identically to each of the experimental protocols described above, except either one or both primary antibodies were omitted from the incubating solution in the presence of both secondary antibodies.
Table 3.5 List of antibodies used for double immunofluorescent labelling

<table>
<thead>
<tr>
<th>Mixture of primary antibodies</th>
<th>Dilution</th>
<th>Supplier / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse CB / Rabbit CB</td>
<td>1:500 / 1:1000</td>
<td>Swant / Piers Emson (Babraham Institute)</td>
</tr>
<tr>
<td>Mouse CB / Rabbit CR</td>
<td>1:500 / 1:1000</td>
<td>Swant, for both antibodies</td>
</tr>
<tr>
<td>Rabbit CB / Mouse PV</td>
<td>1:1000 / 1:5000</td>
<td>Piers Emson (Babraham Institute) / Swant</td>
</tr>
<tr>
<td>Rabbit CR / Mouse PV</td>
<td>1:1000 / 1:5000</td>
<td>Swant, for both antibodies</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mixture of secondary antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat Anti-Mouse Alexa 488 / Goat Anti-Rabbit Alexa 594</td>
<td>1:500</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

3.3.3. Histochemistry (Nissl staining)

In this study, every 10th section of each case (justification below, Section 3.4.3.3) was histochemically stained using cresyl violet for the entire block of interest to aid visualisation of laminar and areal boundaries based on the cytoarchitecture. Cresyl violet staining reveals the Nissl substance of the endoplasmic reticulum in the cytoplasm of neurons which stains a pale pink-purple colour, and the nucleus and nucleolus a blue-purple colour. The 50 μm sections were first gelatine-mounted on chrome-alum glass slides, air dried, and dehydrated in an ascending series of graded alcohol (75%, 85% and 95% alcohol for 5 min each; twice in 100% baths for 10 min each), and were cleared in xylene for 10 min. The sections were then rehydrated by descending through the same series of alcohol to distilled water. Subsequently, the sections were placed in a 0.1% cresyl violet staining solution for 20-30 min. The mounted sections were then rinsed in distilled water and dehydrated in a graded alcohol series as described above. The sections were differentiated at the 70% alcohol stage and checked microscopically for optimal staining. Finally, the sections were washed in xylene (3 x 20 min each) and coverslipped with DPX mountant.
3.3.4. Western Blotting

3.3.4.1. Preparation of Brain Tissue Homogenates

Fresh frozen brain tissue from the primary motor cortex (BA 4) and the anterior cingulate cortex (BA 24) were obtained from the New Zealand Neurological Foundation Human Brain Bank. The regions of the BA 4 and BA 24 were carefully dissected out and the tissue samples (approximately 0.2-0.5 g) were collected into centrifuge vials with 0.5-1 ml of ice cold homogenisation buffer (150 mM sucrose, 60 mM KCl, 15 mM HEPES pH 7.9, 5 mM EDTA pH 8, 1 mM EGTA pH 8). Each sample was homogenised using a tissue homogeniser until the tissue was finely ground and produced homogenates of even consistency. This was followed by addition of Triton X-100 to a final concentration of 1% and incubated on ice for 1 h. Each sample was then centrifuged at 14,000 rpm, 4 °C for 10 min, and the supernatant was aliquoted into microcentrifuge tubes for storage at -80 °C. Protein concentrations of each brain sample were determined from a standard curve generated by a BSA (bovine serum albumin) dilution series using the Bio-Rad DC protein assay as per instructions (Bio-Rad Laboratories).

3.3.4.2. Gel Electrophoresis

Protein samples were separated using the SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) method. Unless otherwise stated 20 µg of total soluble protein homogenate was loaded per well. Homogenates were denatured in 2x Laemmli loading buffer (Sigma) at 95 °C for 5 min on a heating block. Precast NuPAGE Novex 4-12% Bis-Tris 10-well gels (Invitrogen) were used to separate the protein samples using the Invitrogen XCell SureLock™ Mini-Cell module (Invitrogen). Samples were separated at a constant voltage of 200 V for 30 min (or until desired separation) in gel specific 1x MOPS SDS running buffer (Invitrogen) with 0.5 ml NuPAGE antioxidant in the inner central core chamber. MagicMark™ XP protein standard ladder (Invitrogen) was run alongside the samples on each gel. Following electrophoresis, gels were transferred onto a polyvinylidene fluoride (PVDF) transfer membrane (Amersham) pre-activated by sequential immersion in methanol (30 s), water (2 min), and equilibrated in 1x NuPAGE transfer buffer (Invitrogen). Protein transfer was performed as per manufacturer’s instructions in a semi-dry blot module at 30 V for 90 min.
3.3.4.3. Immunodetection

The PVDF transfer membranes were blocked in Tris-buffered saline with 0.5% Tween (TBS-T) containing 5% w/v non-fat milk powder on an orbital shaker at room temperature for 1 h. The membranes were then washed three times with TBS-T (3 x 10 min TBS-T) followed by overnight incubation at 4 °C with primary antibodies diluted in 1% w/v non-fat milk powder. After primary antibody incubation, the membranes were washed (3 x 10 min TBS-T) and incubated at room temperature for 2 h with species specific horseradish peroxidise (HRP)-conjugated secondary antibody diluted in TBS-T containing 1% w/v non-fat milk powder. The list of primary and secondary antibodies used is listed in Table 3.6. Following a further wash (3 x 10 min TBS-T), the membranes were incubated with ECL Plus (Amersham) at room temperature for 3 min. The excess detection agent was drained off and the membranes were placed between clean plastic sheets and the chemiluminescence of antibody labelling was detected using the Fujifilm Global LAS-3000 developer at 1 min exposure (precision high) unless otherwise specified. A positive loading control of monoclonal mouse β-actin [AC-15] (abcam) at 1:10,000 dilution was used to indicate the amount of protein in each sample in the well and processed in the same way as described above. Where more than one protein was required to be investigated on the same blot, membranes were stripped of their antibodies and re-probed. Membranes were incubated in the stripping solution (2% SDS, 62.5 mM Tris pH 6.8, 0.7% β-mercaptoethanol) in a 60 °C water bath for 30 min. Blocking, probing and visualisation of the membrane were then performed as previously described.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin-D28k</td>
<td>Rabbit Polyclonal</td>
<td>1:2000</td>
<td>Piers Emson (Babraham Institute)</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Rabbit Polyclonal</td>
<td>1:1000</td>
<td>Swant</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Mouse Monoclonal</td>
<td>1:500</td>
<td>Swant</td>
</tr>
</tbody>
</table>

**Secondary Antibody**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Mouse HRP conjugate</td>
<td>Sheep</td>
<td>1:5000</td>
<td>Chemicon (Millipore)</td>
</tr>
<tr>
<td>Anti-Rabbit HRP conjugate</td>
<td>Sheep</td>
<td>1:5000</td>
<td>Chemicon (Millipore)</td>
</tr>
</tbody>
</table>
3.4. Data Analysis

3.4.1. Bright Field Light Microscopy

Single immunoperoxidase stained sections were viewed with a conventional Nikon Eclipse E800 bright field light microscope coupled to a high-resolution digital camera (DXM1200F, Nikon). The microscope is also equipped with a precision automated motorised stage which allowed measurement of movement in the x, y directions and a microcator on the focus control to enable movement in the z-axis for stereological cell counting analysis (Ludl Electronic Products Ltd). Low power images were acquired using 4×/0.13 NA objective for laminar and areal distribution of the immunostained sections, and high power images were acquired using 40×/0.75 NA and 100×/1.4 NA oil immersion objectives for detailed morphological examination of the immunoreactive cells.

3.4.2. Confocal Laser Scanning Microscopy and Colocalisation Cellular Quantification

Double immunofluorescence stained sections were viewed and imaged using a Zeiss LSM 710 inverted confocal laser scanning microscope with ZEN 2008 software (Carl Zeiss). The images were acquired using a 20×/0.8 NA dry objective (Plan-Apochromat) in sequential scanning mode to eliminate detection of bleed through and other artificial fluorescent signals (Bolte & Cordelieres, 2006). Also, the images were acquired in slices of 1-2 μm apart through the z-direction of the specimen to reconstruct a three dimensional maximal intensity projection of the z-series to confirm double-labelling. In addition, the number of immunoreactive cells and the percent of double-labelled cells were quantified in a 425 μm width x 3400 μm length cortical strip (area of each montage = 1445000 μm²) that were captured by a tiling procedure to montage together adjacent images that encompasses all cortical layers from the cortical white matter to the pia in areas 4 and 24 for the selected normal control cases (see Chapters 4 and 6). Two montaged strips perpendicular to the pial surface were obtained from the gyral crest and midpoint between crest and sulcus per section (Figure 3.3). The quantitative analysis was manually carried out using the particle analysis cell counter plugin on Image J software. Only cells with clear nuclear, cytoplasmic, and dendritic profiles that could be discerned were included in the analysis. In addition, high
power images were taken using 63×/1.4 NA oil immersion objective (Plan-Apochromat) for detailed morphological examination of immunostained sections.

Figure 3.3 Cortical strips collected for double-labelling cellular quantification

3.4.3. Design-Based Stereological Cell Counting (Nv:Vref Method)

Design-based stereology is a quantification method for a precise and unbiased estimate of the total cell number in a well-defined region of the brain. There are two comparable ways of stereological counting methods, i.e., the Nv:Vref and the optical fractionator counting methods. In this study, the total absolute number (N) of GABAergic interneurons immunolabelled with calbindin, calretinin and parvalbumin in a well-defined block of the primary motor (BA 4) and anterior cingulate cortices (BA 24) of HD and normal control cases were determined using the Nv:Vref principles as previously described (Thu, 2006; Thu, et al., 2010). There are three factors that have advanced to allow for an unbiased estimate of the total cell number in a region of interest. The first is the application of a three-dimensional probe called the optical disector (Sterio, 1984; West et al., 1991), which can be used to obtain unbiased estimates of numerical density (Nv) that is free of assumptions about size, shape, orientation and distribution of the entities being counted. The second of these is a highly efficient probe that is used to estimate the reference volume (Vref) called the Cavalieri estimator from areas of the sectional profiles of the layers and the known distance between the sections. The third is the application of systematic random sampling (SRS) to account for the inhomogeneous nature of biological tissue (Gundersen et al., 1999). The total number of
interneurons \((N)\) is derived by the product of the numerical density \((N_v)\) by the total reference volume \((V_{ref})\) of each region, hence the **N_v:V_{ref} method** (Equation 1). In addition, the **optical fractionator** method was used in part to validate the accuracy of the **N_v:V_{ref}** counting method. The methodological overview is presented in Figure 3.5.

\[
N = N_v \cdot V_{ref}
\]

*Where, \(N = \text{the total number of neurons}\)*

*\(N_v = \text{numerical density}\)*

*\(V_{ref} = \text{total reference volume}\)*

Stereological cell counting (\(N_v\) and \(V_{ref}\)) and systematic random sampling were performed using Stereoinvestigator software version 7.0 (MBF Bioscience) to obtain an unbiased estimate of the total number of interneurons in the SM1 block of the primary motor cortex and CG1 block of the anterior cingulate cortex. The boundaries of the cortical region of interest must be precisely defined to ensure accurate calculation of the total number of neurons in the defined region (Schleicher et al., 2000). The border of BA 4 and BA 24 and delineations of the neuron-containing layers were identified using serially adjacent sections labelled with neuronal N (NeuN; Chemicon) antibody and cresyl violet (Nissl) staining. NeuN is a 46-48 kDa soluble neuron-specific nuclear protein that distinctly labels neuronal cells (both pyramidal and nonpyramidal neurons) in the human brain and provide excellent delineation of cytoarchitectural borders in addition to cresyl violet staining (Mullen et al., 1992). These sections were labelled immunohistochemically and histochemically as described above (Sections 3.3.2 and 3.3.3). Slides were marked on the underside using a fine point waterproof marker pen to indicate the limit of BA 4 and BA 24 based on cytoarchitecture.

3.4.3.1. Numerical Density (\(N_v\)) using the Optical Disector

All sampled sections were viewed with 100×/1.4 NA oil immersion objective on the Nikon bright field light microscope equipped with an automated mechanical stage (\(x, y, z\)). For each sampled area, the numerical density (\(N_v\)) within each cortical region was estimated by
using the optical disector probe. The optical disector is a design-based stereological probe that samples isolated objects in a three-dimensional space with an equal probability, regardless of its size, shape, orientation or distribution in the tissue. This involves placing an unbiased three-dimensional virtual counting frame within the section, defining the disector volume in which cells are counted. The counting frame consists of a rectangle with two types of boundaries: inclusion (green) and exclusion lines (red). At each sampling site, any cells which fell within the counting space or touched the inclusion lines were counted, provided they did not touch the exclusion lines. Any cells which touched the exclusion lines or fell outside the counting space were not counted (Figure 3.4).

![Figure 3.4 Optical disector probe](image)

Three-dimensional virtual counting frame (optical disector probe) within the section with inclusion (green) and exclusion (red) lines.

The number of cells counted ($\sum Q$) per total sampling sites ($\sum F$) was substituted into Equation 2. These rules were extended in three dimensional thickness of the section, i.e., the distance between the upper and lower surface of the section measured by focusing up and down through the section. Neurons were counted as they came fully into focus within the disector height (T), with reference to the counting frame (West, 2002; Schmitz & Hof, 2005). The disector height was defined as the central 8 $\mu$m of each section. This value was smaller than the thickness of each section, allowing for 1-2 $\mu$m guard zones at the top and bottom to ensure that the cut edges of the sections which may be physically distorted or contain lost caps, warping and tearing, did not fall within the counting space (Glaser et al., 2007). The site of counting was selected using a counting frame that was overlaid on the image at each grid point, delineating the x, y boundaries of the unbiased virtual counting space. Therefore, the $V_{\text{dis}}$ can be determined by multiplying the area of the sampling frame,
a(frame), which is corrected for magnification by the disector height (T). Then, the total value of neuronal density (Nv) in the region of interest for each case was determined from the formula, i.e., the number of neurons (\( \sum Q' \)) in a disector volume (\( V_{\text{dis}} \)) in the section obtained as outlined below (Equation 2).

**Equation 2**

\[
Nv = \frac{\sum Q'}{\sum V_{\text{dis}}}
\]

\[
\sum Q' = \sum_{F} Q
\]

\[
\sum V_{\text{dis}} = T \cdot a(\text{frame})
\]

*Where, \( \sum Q' \) = total number of neurons in all disector planes
*\( \sum V_{\text{dis}} \) = sum of the disector volumes
*\( \sum Q \) = total number of neurons per sampling site
*\( \sum F \) = total sampling sites
*\( T \) = height of the disector
*a(frame) = area of the counting frame*

The counting frame dimensions, disector height and grid size were optimised in a pilot study to ensure that the coefficient of error (CE) was within acceptable limits, i.e., CE \( \leq 0.10 \). This was kept constant for all cases. The area of the unbiased sampling frame, disector height and the sampling interval used throughout each case for each marker (CB, CR and PV) in the two cortical regions are listed in Appendix I.

### 3.4.3.2. Reference Volume (Vref) using the Cavalieri Estimator

The total reference volume (Vref) of each cortical region of interest was determined using Cavalieri’s direct volume estimate (Gundersen & Jensen, 1987). The *Cavalieri estimator* is a design-based stereological probe for determining the total reference volume (Vref) using point counting method. The same set of sections that were used for Nv were used to estimate the Vref within each region. A regularly spaced grid was superimposed at 2×/0.06
NA objective lens on each slide at random grid rotation, and the points falling within the boundary of BA 4 of the motor and BA 24 of the cingulate cortices were counted separately for each case. The area of each section was determined from the number of points and the area represented by each point, a(p). The distance between sampled sections (d) was 10, i.e., every 10th section. The mean section thickness (t) was measured using a 100×/1.4 NA oil immersion objective by focusing through the section and recording the distance moved by the stage with a z-axis encoder during the Nv procedure. These values were substituted into Equation 3, together with the number of points falling on each sampled section (∑P) to calculate the total reference volume (Vref), according to the Cavalieri’s principle.

\[
V_{\text{ref}} = \sum P \cdot a(p) \cdot t \cdot d
\]

Where, \( \sum P \) = sum of points falling on the region of interest for each section
\( a(p) \) = the real area represented by each point
\( t \) = average thickness of the sections
\( d \) = distance between the sampled sections

A pilot study was performed to optimise the grid size for the Cavalieri estimator. This was kept constant for all cases. For each region, 100-200 points (∑P) per case were required to produce a coefficient of error (CE) within acceptable limits (West, 1999; Slomianka & West, 2005). The actual parameters used for calculating Vref are listed in Appendix I.

3.4.3.3. Systematic Random Sampling

To investigate the total number of cells in a well-defined block in the two cortices using stereological counting techniques, the entire tissue block of interest was sectioned at a thickness of 50 μm and systematic random sampling (SRS) protocol was carried out to pick a serial section, i.e., with a fixed and known periodicity from a random starting point. Every nth section series, that is, a series of sections at equal intervals sampled in a systematic random fashion would be collected and stained with a marker of interest. The nth section series, is determined upon the size of the block. For statistic viability, 10-15 sections per
block from each case are sufficient for a stereological counting study (Gundersen, et al., 1999; West, 1999; Slomianka & West, 2005). For example, if there are 300 sections per block on an average case, every 30th section (i.e., 1/30 series) is sampled systematically, collected and stained with a specific cell marker.

Due to the lack of tissue availability and age of stored tissue, immunohistochemical studies encompassing the entire cortical block of the primary motor and anterior cingulate cortices could not be undertaken. Hence, the first 100 sections (subvolume) of each case were immunostained and sampled for the average neuronal density (Nv) estimation of the two cortices (the total number of sections per block ranged from 150-300 sections for SM1 block of the primary motor cortex and 120-260 sections for CG1 block of the anterior cingulate cortex). Accordingly, every 10th section of the first 100 sections of each block was collected and stained with the specific GABAergic interneuronal markers (calbindin-D28k, calretinin, and parvalbumin). To calculate the total reference volume (Vref) of the entire cortical block (that extended beyond the first 100 sections, i.e., the whole block), a separate series of every 10th section from the entire tissue block was selected (at a random starting point from numbers, 1 to 10) and stained with cresyl violet.

To calculate the total number of interneurons in the entire block where the first 100 sections were immunolabelled, the following procedure was employed (Equation 4). The Vref of the first 100 sections of the two cortical regions immunolabelled with GABAergic interneuronal markers (CB, CR, PV) for each case was found using the Cavalieri estimator (Vref_{marker first100}) and compared with the Vref calculated for the first 100 Nissl sections of each case (Vref_{nissl first100}). A ratio of Marker:Nissl was obtained. The Cavalieri estimator was then used to find the Vref of the remaining Nissl sections (Vref_{nissl residual}). Using this volume, and the previously calculated Marker:Nissl ratio, it was possible to predict the reference volume that would have been obtained in the remaining unlabelled sections (Vref_{marker residual}). The total reference volume (Vref_{total}) for each marker was thus determined from the sum of the Vref for the first 100 sections and the predicted Vref for the remainder of the series, i.e., Vref_{total} = Vref_{marker first100} + Vref_{marker residual}. Once the volume of the entire tissue block (Vref_{total}) was determined, the total number of interneurons (N) in the entire tissue block can be calculated by multiplying the average neuronal density (Nv) obtained using the optical
**3.4.3.4. Coefficient of Error (CE)**

The precision or reproducibility in stereology is termed the **coefficient of error (CE)** of the estimate (Slomianka & West, 2005). It is desirable to have a low CE value as possible to ensure that any variation seen between and within cases is due to the real biological differences rather than contributed by experimental procedures. The precision and accuracy of the values obtained by both the *optical disector* and the *Cavalieri estimator* can be derived using a revised Matheron’s quadratic approximation formula (Gundersen & Jensen, 1987; Gundersen, et al., 1999). For the quantification of neurons in the cortex, the CE should be less than half the total observed variation (coefficient of variation, CV), or $CE^2 / CV^2 < 0.5$. The CV is calculated as the standard deviation ($SD_{n-1}$) divided by the mean number of cells. Previous stereological counting studies have shown that the CV of the human cerebral cortex was ~0.2, therefore a CE of $\leq 0.1$ was considered to be acceptable (Gundersen & Jensen, 1987). The average CE for each volume, and for the total number estimate, for Huntington’s disease and normal control groups were each calculated using the formula $CE = (1/n \cdot \sum_i CE_i^2)^{1/2}$, where n is the number of cases (Gundersen, et al., 1999). The CE is automatically calculated by StereoInvestigator for both the *optical disector* and the *Cavalieri estimator* separately or manually calculated using Equations 5 and 6. No valid mathematical formula for calculating the combined CE of total cell number ($N$) exists for the $Nv$:Vref method (Schmitz & Hof, 2005). Calculation on CE in both optical disector and Cavalieri estimator are outlined below:
**CE in optical disector**

The precision, CE, of the estimate of $N_v$ is expressed as a ratio of the standard error of the mean (SEM) to the mean of the repeated estimates, i.e., $CE = SEM / \text{mean}$. The CE of the estimate in the optical disector probe is influenced by two independent factors of variance. One is the variability of the estimates made within each of the individual sections, i.e., intra-section contribution to the error ($\text{VAR}_{\text{section noise}}$). The second is the variability between sections attributable to the systematic random sampling scheme, i.e., inter-section contribution to the error ($\text{VAR}_{\text{SRS}}$).

### Equation 5

$$
\begin{align*}
\text{VAR}_{\text{section noise}} \text{ of } (\sum Q^-) &= \sum_{i=1}^{n} Q^- = S^2 \\
\text{VAR}_{\text{SRS}} \text{ of } (\sum Q^-) &= \frac{3(A - \text{VAR}_{\text{section noise}}) - 4B + C}{240}, m = 1 \\
\Rightarrow \text{Total VAR} &= \text{VAR}_{\text{section noise}} + \text{VAR}_{\text{SRS}} \\
\therefore CE_{\text{total}} &= \sqrt{\frac{\text{Total VAR}}{\text{VAR}_{\text{section noise}}}} = \sqrt{\frac{3(A - S^2) - 4B + C}{240 + S^2}}
\end{align*}
$$

Where “A” is the sum of the squares of the number of points counted on each section ($Q_i \cdot Q_i$), “B” is the sum of the product of the number of points counted on each section and the number of points counted on the next section in the series ($Q_i \cdot Q_{i+1}$), and “C” is the sum of the products of the number of points on each section and the number counted on the second next section in the series ($Q_i \cdot Q_{i+2}$).

### Note:

The counts from individual optical dissectors are assumed to originate from a Poisson distribution. The variance of Poisson distribution is equal to the mean; and sum of two Poisson distributions is itself a Poisson distribution. Therefore, the sum of all dissectors is equal to the mean of the Poisson distribution thus equal to the variance of the Poisson of distribution.
The precision, CE, of the estimate of Vref is expressed as a ratio of the standard error of the mean (SEM) to the mean of the repeated estimates, i.e., $CE = \frac{SEM}{mean}$. The CE of the estimate in the Cavalieri estimator probe is influenced by two independent factors of variance. One is the variability in the number of points that counted when the point counting grid is placed repeatedly over the same section in a random manner, i.e., intra-section contribution to the error ($VAR_{section\ noise}$). The second is the variability between sections attributable to the systematic random sampling scheme, i.e., inter-section contribution to the error ($VAR_{SRS}$).

**Equation 6**

\[
VAR_{section\ noise} \text{ of } (\sum P) = 0.0724 \cdot \frac{b}{\sqrt{a}} \cdot \sqrt{n} \cdot \sum_{i=1}^{n} P^i = S^2
\]

\[
VAR_{SRS} \text{ of } (\sum Q^i) = \frac{3(A - VAR_{section\ noise}) - 4B + C}{240}, m = 1
\]

\[
\Rightarrow \text{Total } VAR = VAR_{section\ noise} + VAR_{SRS}
\]

\[
\therefore CE_{total} = \frac{\sqrt{\text{Total } VAR}}{VAR_{section\ noise}} = \sqrt{\left[\frac{3(A - S^2) - 4B + C}{240} + S^2\right] \sum_{i=1}^{n} Q^i}
\]

Where “A” is the sum of the squares of the number of points counted on each section ($P_i \cdot P_i$), “B” is the sum of the product of the number of points counted on each section and the number of points counted on the next section in the series ($P_i \cdot P_{i+1}$), and “C” is the sum of the products of the number of points on each section and the number counted on the second next section in the series ($P_i \cdot P_{i+2}$).

3.4.3.5. Optical Fractionator Method to Estimate the Total Number

The total number of interneurons ($N$) can be also estimated using the **optical fractionator** method using the StereoInvestigator version 7.0 software (MBF Bioscience). This method was used additionally to further assess the validity of the counting results provided by the
**Nv:Vref** method in the first 100 sections within the same cortical block. The total number estimation in the first 100 sections was compared between the two methods as the entire block of cortical tissue was unavailable for immunohistochemical staining (the percentage difference is outlined in Appendix IV). The optical fractionator involves counting neurons in a known fraction of the section thickness, under a known fraction of the areas of the section, in a known fraction of the sections that pass through the region of interest, i.e., one directly counts the number of neurons in a known fraction of the volume of interest. Sampling with the optical fractionator method was performed on live images of the section on a Nikon E800 microscope equipped with a digital camera (Optronics) and automated mechanical stage (Ludl Electronic Products Ltd). To achieve unbiased sampling, each cortical region was sampled in a systematically random manner. The area of interest on each section was first traced under low magnification (2×/0.06 NA objective) and a grid of known dimensions was then randomly put onto each tracing. Pilot studies were carried out to select an optimal grid size for each marker and each cortical region. Each section was then sampled at the top left hand corner of each square in the grid, using a 100×/1.4 NA oil immersion objective for cell counting. The thickness of the section was found by focusing through the tissue; it was taken as the distance, in micrometers, between the first point of the tissue to come into focus and the last point to come into focus as the stage was moved in the z-direction. On each sampling site, a three dimensional optical disector counting frame was superimposed on the image and neurons were counted using the same principle - inclusion and exclusion lines as described above for the Nv:Vref method. Pilot studies were conducted to determine the counting frame dimensions and the disector height required to produce the desired number of observations for each neuronal marker and cortical regions. These measurements were kept constant for all cases within each cortical region. A total of ~200 cells were counted for a set of ten sections. Data collected from each case for the number of neurons counted in a disector frame (ΣQ), average measured section thickness (t), disector height (h), area sampling fraction (asf), and section sampling fraction (ssf) were entered into Equation 7 by the StereoInvestigator to obtain an unbiased estimate of the total neuron number (N).

\[
N = \sum Q \cdot \frac{1}{h} \cdot \frac{1}{\text{asf}} \cdot \frac{1}{\text{ssf}}
\]

**Equation 7**
3.4.4. Statistical Analyses

*Mean difference using ANOVA and Bonferroni’s post hoc test*

The mean, standard deviation (SD) and standard error of the mean (SEM) of the total interneuron cell numbers from the stereological counting analysis was calculated for all HD and normal control cases, as well as HD cases subgrouped into the dominant symptom and striatal grade groups. A statistical package for social scientists, SPSS 14.0 was used to perform a one-way analysis of variance (ANOVA) to compare all HD cases and the normal control group, and the HD symptom and HD grade subgroups. The value of the error mean squares (EMS) from the within-groups comparison was obtained from SPSS output and analysed using the Bonferroni’s post-hoc test (Equation 8). The degrees of freedom were obtained from SPSS output and used, together with the Bonferroni value, to find the p-value from a 2-tailed Student’s t-distribution table. A p-value of <0.05 was considered to be significant (statistical significance is expressed as *p<0.05, **p<0.01, and ***p<0.001).

**Equation 8**

\[
\text{Bonferroni post hoc test} = \frac{(\mu_1 - \mu_2)}{\sqrt{\text{EMS}[(1/n_1) + (1/n_2)]}}
\]

Where, \(\mu_1\) is the larger and \(\mu_2\) is the smaller of the group means, which correspond to the sample size \(n_1\) and \(n_2\), respectively. The error mean square (EMS) was derived by: \(\text{EMS} = \text{error of the sum of squares/degrees of freedom for this error}\). This value of error mean square was obtained from the SPSS 14.0 computer output.

*Correlation of interneuron cell number and CAG repeat length, PMI, and age*

The correlation between the mean total interneuron cell number and the CAG repeat length, post-mortem interval (PMI), and age of HD cases were analysed using the Pearson regression test (Chapters 5 and 7).
Figure 3.5 Overview of the methodology

**Human brain tissue**
- Normal control cases
- Huntington's disease cases
  - CAG repeat length assessment
  - Striatal pathological grading
  - Clinical symptomatology (Motor, Mood, Mixed)

**Human brain tissue processing**
- Two cortical regions
  - Perfusion-fixed / Fresh
  - Blocking
  - Sectioning at 50 µm

**Immunohistochemistry**
- Single immunoperoxidase labelling
- Double immunofluorescent labelling

**Western blotting**
- Antibody specificity

**Microscopy**
- Bright field light microscopy
- Confocal laser scanning microscopy

**Stereological cell counting** ($N = N_v \times V_{ref}$)
- Systematic random sampling
- Numerical density ($N_v$) - Optical dissector
- Reference volume ($V_{ref}$) - Cavalieri estimator

**Statistical analyses**
- CE estimators (for $N_v$, $V_{ref}$)
- ANOVA and Bonferroni’s post-hoc test
- Pearson correlation
CHAPTER 4. RESULTS

DISTRIBUTION OF INTERNEURONS
IN THE PRIMARY MOTOR CORTEX
OF NORMAL HUMAN BRAIN

4.1. Introduction

The human agranular frontal cortex can be subdivided into two broad regions based on the
cytoarchitectural areas 4 and 6 of Brodmann (1909, 2006). Functionally, area 4 corresponds
to the primary motor cortex which lies immediately anterior to the central sulcus. It is
caracterised by the lack of granular layer IV and the presence of giant pyramidal or Betz
cells in layer V that are mainly responsible for the control of kinematic and dynamic
properties of voluntary movements (Naidich et al., 2001; Binkofski, et al., 2002; Sherwood,
et al., 2003; Matelli, et al., 2004; Zilles, 2004; Chouinard & Paus, 2006). The diverse
cytoarchitecture of the human motor cortex has been assessed with Golgi- and Nissl-stained
preparations from the early works of Brodmann (1909) and von Economo and Koskinas
(1925). Although these preparations continue to be useful for the parcellation and
identification of different neuronal types in the cortex, detailed anatomical analyses to
delineate different neuronal populations, especially the local-circuit \textbf{GABAergic
interneurons}, have not been studied in detail in the primary motor cortex of the human
brain.

The cortical intrinsic inhibitory network comprises distinct subpopulations of local-circuit \(\gamma\)-aminobutyric acidergic (GABAergic) interneurons. The complexity of cortical networks is
due to the vast diversity of these interneurons that include more than 10 morphological
classes, and express various types of molecular and biochemical markers (DeFelipe, 2002;
Markram, et al., 2004). Thus, the identification of subclasses according to their respective
distribution of neurochemicals has been a vital method for differentiating distinct
subpopulations of GABAergic interneurons (Kubota, et al., 1994; McBain & Fisahn, 2001;
Markram, et al., 2004; Petilla Interneuron Nomenclature Group, 2008; Kubota, et al., 2011). Of these, the three calcium-binding proteins, namely calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) that belong to the EF-hand domain family have been useful neurochemical markers for differentiating subpopulations of GABAergic interneurons in the neocortex (Baimbridge, et al., 1992; Andressen, et al., 1993; Gifford, et al., 2007; Schwaller, 2009). Besides classifying interneurons on the basis of morphological and biochemical features, different subpopulations also reveal discrete patterns of synaptic connectivity with the pyramidal neurons. For example, CB and CR are mainly expressed by interneurons that form inhibitory axo-dendritic contacts with its neighbouring pyramidal neurons, whereas PV is expressed by interneurons that make axo-somatic and axo-axonic synapses with the pyramidal neurons. Accordingly, the inhibitory interneurons are critical determinants of neural activity in the neocortex.

In the present chapter, the distribution pattern of the GABAergic interneurons defined by the three calcium-binding proteins are investigated in Brodmann area 4 (BA 4 or area 4) of the primary motor cortex in neurologically normal human brains. We tested the hypothesis that the differential expression of these three interneuronal markers may define distinct, non-overlapping interneuronal populations. Firstly, western blot analysis was performed to validate the antibody specificity (Section 4.2) and secondly, immunohistochemical analyses using single immunoperoxidase and double immunofluorescent labelling were carried out to investigate the laminar distribution, cellular morphological characteristics and the extent of cellular colocalisation of the three interneuronal markers (Sections 4.3 and 4.4).
4.2. Western Blot Analysis

The western blot analysis on fresh human tissue homogenates prepared from the primary motor cortex of 7 normal cases (H108, H111, H112, H115, H118, H121, H127; case details are listed on Table 3.1; see Chapter 3) revealed clear and specific protein bands of antibodies raised against the three calcium-binding proteins. Rabbit polyclonal CB, CR, and mouse monoclonal PV antibodies were used for the analysis (details are listed in Table 3.6). The blots were detected using the avidin-biotin immunoperoxidase method and visualised with enhanced chemiluminescence signal. The CB, CR, and PV antibodies recognised a prominent band at approximately 28, 29, and 12 kDa, respectively (Figure 4.1A, B, C), which were the expected bands on the molecular weight of these proteins (Schwaller, 2009). All blots were probed with β-actin antibody for positive loading control (~42 kDa) to ensure consistency of protein loading for each sample (Figure 4.1D).

![Figure 4.1 Western blots showing the expression of calcium-binding proteins in the primary motor cortex of the normal human brain](image)

The blots show specific bands at approximately (A) 28 kDa (CB), (B) 29 kDa (CR), (C) 12 kDa (PV), and (D) 42 kDa (β-actin) in the normal human brain of the primary motor cortex. Apparent molecular weights of protein bands are expressed in kilodaltons (kDa).
4.3. Description of Single Immunohistochemical Staining Patterns in the Normal Human Primary Motor Cortex

In order to study the distribution pattern and distinct morphology of the GABAergic interneurons in BA 4 of the primary motor cortex, different interneuronal markers were used to label specific cell populations (Figure 4.2). The three major types of GABAergic interneurons were immunohistochemically labelled using antibodies raised against the three calcium-binding proteins: calbindin-D28k (CB); calretinin (CR); and parvalbumin (PV). In addition, an adjacent series of sections were stained with cresyl violet (Nissl) histological staining and neuronal N (NeuN) immunohistochemical staining to determine, qualitatively, the specific laminar distribution of each marker and to also delineate exact boundaries of the BA 4 region of the primary motor cortex. The detailed description of the methods is described in Chapter 3. The normal human brain cases used for this study are listed in Table 3.1.

4.3.1. Distribution Pattern of Interneurons (CB, CR, PV)

*Calbindin-D28k*

Calbindin-D28k (CB) immunoreactivity in the primary motor cortex showed a distinctive layer specific staining pattern (Figure 4.2C). The most prominent CB immunopositive (CB+) neurons were intensely labelled small nonpyramidal cells that were confined to two horizontal bands - in the upper layers II/III and in deeper layers V/VI. The CB+ neurons in the upper layers II/III comprised the largest number of intensely labelled cells, with fewer more lightly labelled nonpyramidal neurons that were present in the deeper layers. The limits of the layer II/III border and the layer VI and subjacent white matter were difficult to delineate with the CB antibody. Therefore, the precise limits of the horizontal layers were estimated from the cortical depth in comparison with other, adjacent Nissl-stained or NeuN-immunostained sections. The neuropil staining was heaviest in layers I to III. There was punctuate CB+ immunoreactivity with a plexus of labelled fibres in layer I, although no cell body labelling was observed. A few, thin, axon-like processes were found in layer VI and the subjacent white matter with small, round cells.
When viewed at high magnification (Figure 4.2c), both the nucleus and the cytoplasm of the cell somata were darkly stained and cell processes in the proximal region were clearly visible. Some cells were characterised by staining of long and vertically oriented cellular processes. CB staining labelled a heterogeneous group of small to medium sized cells (7-25 μm in cell body diameter) comprising mainly of two groups - nonpyramidal neurons showing heavily labelled round or fusiform cell soma with multipolar or bipolar dendritic trees, and a smaller group made up of lightly labelled layer III and V pyramidal neurons. These small to medium sized pyramidal neurons were exclusively excluded from the stereological cell counting analysis in the primary motor cortex of Huntington’s disease (see next Chapter 5).

Calretinin

Calretinin (CR) immunoreactivity in area 4 also showed a layer specific pattern of staining (Figure 4.2D). Calretinin stained (CR⁺) neurons were found throughout all layers of the cortex from layer I through to VI. These CR⁺ neurons were mostly concentrated in the upper cortical layers II and III, and a smaller population were found in the deeper layers, followed by fewest cells that were sparsely spread in the molecular layer I. CR staining of the neuropil was densest in the upper portion of layer II and intermediate levels of neuropil staining were found in lower part of layer III and deep layers V and VI.

At higher magnification (Figure 4.2d), CR immunoreactivity was present in neuronal cell bodies, proximal processes (axons and dendrites), and revealed detailed morphology of the individual neurons that were nonpyramidal in shape. The majority were small to medium sized (6-15 μm in cell body diameter). The most common morphology was spindle shaped soma that gave off vertically oriented bipolar branches from the upper and lower poles. A small number of CR⁺ neurons also appeared to have multipolar or bitufted morphologies. Layer I contained a few CR⁺ neurons with medium to long horizontal dendrites that extended for ~1 mm within layer I. Another type included a round cell body that gave rise to short dendrites with multipolar morphology.
**Parvalbumin**

Parvalbumin immunoreactive (PV+) neurons were found sparsely distributed throughout all layers of the cortex with the exception of layer I (Figure 4.2E). The majority of PV+ neurons were concentrated in a band extending through lower layer III to upper layer V, and a few were present in layers II and VI. Neuropil labelling was most intense in the middle cortical layers while the staining was virtually absent in layer I-II.

PV antibody resulted in highly selective staining of the neuronal cell bodies and proximal dendrites in area 4 of the motor cortex (Figure 4.2e). In addition, a light to medium nuclear labelling was also observed. PV+ cells were exclusively nonpyramidal in shape and formed a morphologically heterogeneous group with somal sizes ranging from less than 10 μm to greater than 25 μm. The PV+ neurons can be broadly classified into three subclasses, based primarily on the size of cell soma and dendritic configuration. First, small sized cells with ovoid or round soma that lacked sufficient dendritic staining to further classify them morphologically were spread throughout layers II-VI. A second group was comprised of a small percentage of cells that were small to medium in size (approximately 10-15 μm) that possessed vertically oriented dendritic branches. A third group of PV+ cells were made up of medium to large cell bodies and were distributed in layers II/III to VI. These cells were more frequent in layers III and V. The PV+ cell bodies were mostly multipolar neurons with 3 to 5 primary dendrites radiating out from the cell body in all directions. In some instances, dense PV+ perisomatic puncta surrounding large, unstained pyramidal cells in layer Vb of the motor cortex were observed.

**4.3.2. Distribution of Neurons identified by Nissl and NeuN**

*Cresyl Violet*

Cresyl violet (Nissl) staining labelled the cell bodies of all neuronal types (pyramidal and nonpyramidal neurons) and glial components (microglia, oligodendrocytes, astrocytes). Nissl-stained sections differentiated area 4 of the motor cortex by a prominent layer V, which contains many large, darkly stained pyramidal cells and a lack of layer IV that usually contains many densely packed granular cells found in other cortical regions. The
border between layers II and III was more difficult to distinguish and these upper layers consisted of many small to medium sized cells. Layer VI was composed of many, densely packed, small to medium sized pyramidal and granular cells (Figure 4.2A, a).

**Neuronal N**

Neuronal N (NeuN) is a 46-48 kDa soluble neuron-specific nuclear protein that distinctly labels the cell nuclei, cytoplasm, and some proximal processes of neuronal cells in the human brain (Mullen, et al., 1992). NeuN antibody immunolabelled all neuronal populations in the primary motor cortex (Figure 4.2B). The NeuN staining was most dense in the nuclei of neurons while the cytoplasm and processes of neurons showed lighter, granular staining. Based on their morphology, size and distribution patterns, these cells can be divided into two major neuronal types - pyramidal and nonpyramidal neurons. Both types were heterogeneously distributed throughout the six cortical layers. The pyramidal neurons were mainly observed in layers III, V, and VI (Figure 4.2b) and were characterised by their conical shape that gave rise to both apical and proximal basal dendrites. The apical dendrites radiated out from the apex of the cell body whereas the basal dendrites emanated laterally from the base of the body. In addition, thin to medium sized axons and axon initial segment were observed at the base of the cell body. Layer III consisted of mainly small to medium sized (12-25 μm in cell body diameter) pyramidal neurons, whereas large sized pyramidal neurons were concentrated in layer V (30-50 μm). There were also lighter stained very large cells (>50 μm) that were previously described as Betz cells (Marin-Padilla, 1970; Sherwood, et al., 2003). Layer VI consisted of smaller neurons (9-15 μm) that merged with the underlying white matter. The nonpyramidal neurons were identified by a dark band in layers II/III which was distinguished from layer I. The primary motor cortex lacked layer IV but layer V was much enlarged and subdivided into Va and Vb. The deepest layer VI contained relatively few spindle shaped interneurons.
Figure 4.2 Representative photomicrographs showing the distribution pattern of neurons in the primary motor cortex (Brodmann area 4) of the normal human brain

This figure shows the distribution pattern and morphology of different cell types in the primary motor cortex (area 4) of neurologically normal brains in 50 μm coronal sections. The overall distribution of cells are shown by sections stained for (A, a) cresyl violet (Nissl) and (B, b) neuronal N (NeuN). The distribution patterns of the different types of GABAergic interneurons are shown on adjacent series of sections immunostained for (C, c) calbindin-D28k (CB), (D, d) calretinin (CR), and (E, e) parvalbumin (PV).

**A-E:** Low magnification photomicrographs of the distribution pattern of neurons in the normal human primary motor cortex.

**A:** Area 4 histochemically stained for cresyl violet (Nissl).

**B:** Area 4 immunohistochemically stained for neuronal N (NeuN).

**C:** Area 4 immunohistochemically stained for calbindin-D28k (CB). CB⁺ neurons are found prominently in the upper layers II-III.

**D:** Area 4 immunohistochemically stained for calretinin (CR). CR⁺ neurons are found in layers I-VI. CR⁺ neurons are more abundant in the supragranular layers than the infragranular layers.

**E:** Area 4 immunohistochemically stained for parvalbumin (PV). PV⁺ neurons are absent from molecular layer I. PV⁺ neurons are more numerous in the deeper layers than in superficial layers. Fibre staining is most intense in the middle layers of the cortical depth.

**a-e:** High magnification photomicrographs showing the morphology of neurons in the human primary motor cortex (area 4) stained for: (a) cresyl violet (Nissl) cells in layer III; (b) neuronal N (NeuN) cell in layer III; (c) calbindin-D28k (CB) cells in layers II-III; (d) calretinin (CR) cells in layers II-III; and (e) parvalbumin (PV) cells in layer V.

Scale bars: **A-E = 500 μm; a-e = 50 μm**
Figure 4.2

A. Nissl staining
B. NeuN staining
C. CB staining
D. CR staining
E. PV staining

Layers:
- I
- II
- III
- Va
- Vb
- VI
4.4. Double Immunofluorescent Labelling

To assess the degree of overlap between the different subpopulations of GABAergic interneurons that express different calcium-binding proteins, the colocalisation of calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) in all possible pairs of combinations were examined using double immunofluorescence labelling in 4 normal cases (H111, H115, H120, H330; case details are listed in Table 3.1, see Chapter 3). All mouse monoclonal antibodies were tagged with green fluorescent Alexa Fluor 488 label while the rabbit polyclonal antibodies were tagged with red fluorescent Alexa Fluor 594 label. Any indication of colocalisation in the merged images appeared yellow in colour. The qualitative analysis on the laminar distribution and cellular staining was observed using the Zeiss LSM 710 inverted confocal laser scanning microscope. In addition, quantitative analysis was performed on images acquired using a tiling method to obtain a cortical strip montage to work out the numerical density and the relative proportions of the interneurons in the primary motor cortex (for detailed description of methodology, see Chapter 3, Sections 3.3.2.3 and 3.4.2). Each montaged cortical strip encompasses all six horizontal cortical layers with dimensions of 425 µm width x 3400 µm length (area of each montage = 1445000 µm²). Only cells with clearly visible nucleus, cytoplasm, and proximal dendrites within the entire cortical strip montage were marked and counted in the collected z-stack.

4.4.1. Calbindin (CB) mouse monoclonal / Calbindin (CB) rabbit polyclonal

To confirm the reliability and consistency of CB antibodies raised in two different species, the mouse monoclonal CB (mCB) and rabbit polyclonal CB (rCB) antibodies were used to test whether these markers were colocalised to the same type of interneurons (Figure 4.3A, a). In general, the rabbit polyclonal CB antibody labelled cells with more clear and distinct neuronal processes that assisted in the identification of the different morphological classes of cortical interneurons.

When the high resolution images of mCB and rCB immunolabelling were merged, there were 115 clearly identified mCB/rCB double-labelled cells from two cortical strip montages of 1 normal motor cortex case (H115) (Figure 4.3A, a), which indicates colocalisation of
these two markers in the same clearly labelled cells. The weakly stained CB⁺ pyramidal
neurons were excluded from the counting analysis. This suggests that mCB and rCB are
expressed by the same type of GABAergic interneurons in the BA 4 region of the primary
motor cortex.

4.4.2. Calbindin-D28k (CB) / Calretinin (CR)

The pattern of laminar distribution and numerical density of the calbindin (CB) expressing
cells were comparable to the calretinin (CR) expressing cells. Intensely labelled mouse
monoclonal CB immunoreactive (CB⁺) neurons were mainly found in high numbers in the
upper layers II/III, with fewer cells in the deeper layers V and VI (Figure 4.3B). These cells
were small to medium in size (8-18 μm cell body diameter) and typically had multipolar and
bitufted morphologies. CB⁺ cells were absent from layer I. In contrast, CR
immunoreactivity showed neuronal staining in all cortical layers from I to VI (Figure 4.3B).
CR immunopositive (CR⁺) cells comprised exclusively nonpyramidal neurons with bipolar
and bitufted dendritic morphologies that were generally smaller than CB⁺ cells. The density
of CR⁺ cells was lower in layers II/III than that for CB, but higher number of cells were
observed in the middle and deeper layers. The layer I CR⁺ cells had axons and dendrites that
spread horizontally.

In a total of 469 cells counted from the six cortical strip montages of 3 normal human motor
cortex cases (H111, H120, H330), there were clearly identified single-labelled 68 CB⁺ cells
(14.5%), 401 CR⁺ cells (85.5%), and no double-labelled cells (0%). This suggests that CB
and CR are found in distinct, non-overlapping populations of GABAergic interneurons in
the BA 4 region of the primary motor cortex (Figure 4.3b).

4.4.3. Calbindin-D28k (CB) / Parvalbumin (PV)

The rabbit polyclonal CB antibody showed similar staining patterns with the mouse
monoclonal CB antibody described earlier (see above; also Figures 4.3A and B). The mouse
monoclonal parvalbumin (PV) antibody showed intense labelling of nonpyramidal cell
bodies in layers II to VI. PV immunoreactive (PV+) cells were absent from layer I (Figure 4.3C). The PV neuropil staining was highest in the middle layers. PV+ cells showed cell bodies of varying size (small, medium and large; 12-35 μm in cell body diameter) and intensely labelled dendrites and axons that showed bipolar and multipolar morphologies.

In a total of 653 cells counted from the six cortical strip montages of 3 normal human motor cortex cases (H111, H120, H330), there were clearly identified single-labelled 166 CB+ cells (25.4%), 486 PV+ cells (74.4%), and 1 double-labelled cell (0.2%). This suggests that CB and PV are generally found in distinct, non-overlapping populations of GABAergic interneurons in the BA 4 region of the primary motor cortex (Figure 4.3c).

4.4.4. Calretinin (CR) / Parvalbumin (PV)

The laminar distribution of PV+ cell staining showed a higher number in the middle and deeper layers than CR+ cells (Figure 4.3D). Both antibodies exclusively stained nonpyramidal neurons. However, the cell bodies stained for CR were generally smaller in size. In addition, CR+ cells showed a similar mixture of neurons with bipolar and multipolar dendritic morphologies, while multipolar shaped cells were more abundant in cells that express PV.

In a total of 896 cells counted from the six cortical strip montages of 3 normal human motor cortex cases (H111, H120, H330), there were clearly identified single-labelled 439 CR+ cells (49%), 457 PV+ cells (51%), and no double-labelled cells (0%). This suggests that CR and PV are expressed by separate groups of GABAergic interneurons in the BA 4 region of the primary motor cortex (Figure 4.3d).
This figure shows the distribution pattern and morphology of the different types of interneurons that differentially express calcium-binding proteins, calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) in the primary motor cortex of neurologically normal human brains. The pairs of double-labelled immunofluorescent photomicrographs of the different types of interneurons is shown using sections immunostained for (A, a) mCB / rCB, (B, b) mCB / rCR, (C, c) rCB / mPV, and (D, d) rCR / mPV.

**A-D:** Low magnification photomicrographs of the interneurons in different pairs of combinations in the human primary motor cortex.

**a-d:** High magnification photomicrographs of the representative interneurons in different pairs of combinations in the human primary motor cortex.

**A, a:** Area 4 immunohistochemically stained with mCB / rCB arrangement.

**B, b:** Area 4 immunohistochemically stained with mCB / rCR arrangement.

**C, c:** Area 4 immunohistochemically stained with rCB / mPV arrangement.

**D, d:** Area 4 immunohistochemically stained with rCR / mPV arrangement.

Scale bars: **A-D** = 100 μm; **a-d** = 20 μm
Figure 4.3
4.4.5. Proportions of the Three Interneuronal Subpopulations in the Primary Motor Cortex of the Human Brain

To assess the relative proportions of the interneuronal subpopulations identified by the three calcium-binding proteins, the cell counts obtained from the double immunofluorescence study above which showed no significant overlap were collected and the ratio between the three non-overlapping subpopulations of interneurons was determined.

Among 2019 cells counted from the 3 normal human brains (H111, H120, H330), there were clearly identified single-labelled 235 CB⁺, 840 CR⁺, and 944 PV⁺ cells. The relative proportions of these cells were 11.6%, 41.6%, and 46.8%, respectively (Figure 4.4).
4.5. Discussion

The calcium-binding proteins, calbindin D-28k (CB), calretinin (CR), and parvalbumin (PV) are reliable markers that permit the discrimination of the different interneuronal subtypes in the cortex (McBain & Fisahn, 2001; DeFelipe, 2002; Petilla Interneuron Nomenclature Group, 2008; Kubota, et al., 2011). Functionally, the calcium-binding proteins serve to regulate intracellular calcium (Ca\textsuperscript{2+}) levels and buffer Ca\textsuperscript{2+} transients resulting from a multitude of cellular processes (Schwaller, et al., 2002; Schwaller, 2009). A number of studies describe these proteins in the cortex of various species but there is little information on their distribution patterns in the human primary motor cortex. Using these markers, interneurons in the BA 4 region of the normal human primary motor cortex have been characterised using immunohistochemical methods and found that these calcium-binding proteins are expressed in morphologically distinct and non-overlapping classes of GABAergic interneurons. Thus, what follows is a summarised description of the staining patterns of the three calcium-binding proteins and the possible functional significance of these interneurons in the cortical neuronal circuitry.

4.5.1. Cortical GABAergic Interneurons identified by Calcium-Binding Proteins

*Calbindin-D28k*

Calbindin-D28k (CB) is a calcium-binding protein that belongs to the EF-hand family and has been known to modulate intracellular calcium (Kojetin, et al., 2006; Schwaller, 2009). In the brain, CB is colocalised with the L-type calcium channels (Celio, 1990) and recent studies have shown that CB is involved in reducing calcium influx through the voltage dependent L-type calcium channels as well as enhancing the sensitivity of these channels to evoked calcium transients (Lee, et al., 2006; Christakos, et al., 2007). CB was identified as a protein of 28 kDa in the rabbit cerebellum and kidney (Celio et al., 1990). The western blot analysis using homogenates from the normal human primary motor cortex showed a clear, single band at approximately 28 kDa (immediately below the 30 kDa ladder band) which indicates the size and specificity of the antibody (Figure 4.1A).
The immunohistochemical results showed that CB staining was most prominent in the upper layers II/III with intensely labelled small nonpyramidal cells that have the bipolar and multipolar morphological characteristics, determined by the shape of axonal and dendritic branching. The bipolar shaped interneurons are likely to represent a class of nonpyramidal neurons termed *double bouquet* cells as indicated in previous Golgi studies (Somogyi & Cowey, 1981, 1984). These cells were first named by Cajal in 1899 as *células bipenachadas* (or *cellules à double bouquet dendritique/protoplasmique* in French; *bitufted cells* in English) and are characterised by smooth dendrites with prominent long, vertically oriented axon bundles (Yanez et al., 2005). CB has been previously reported to be expressed by *double bouquet* cells in the monkey and human cortex (Kubota, et al., 1994; Gabbott & Bacon, 1996). Although the typical *double bouquet* cells are observed in monkeys and humans, these cells are less numerous in cats (Demeulemeester et al., 1989; Demeulemeester et al., 1991) and rarely identified in the rodent cortex (van Brederode et al., 1991; Kubota, et al., 1994). This suggests that the distribution of these cells may represent fundamental species differences in the neuronal organisation of the cortex. These cells are distributed at regular intervals (~30 μm), and therefore are also suggested to be involved in the inhibitory action of minicolumnar organisation of the cortex (DeFelipe et al., 1990; del Rio & DeFelipe, 1997b; Mountcastle, 1997; Buxhoeveden & Casanova, 2002; Mountcastle, 2003; Horton & Adams, 2005; Rockland, 2010).

The small, round multipolar shaped neurons in layers III-VI are likely to represent *neurogliaform* and *Martinotti* morphological cell types (Fairén, et al., 1984; Jones, 1984b). The *neurogliaform* cells (NGCs) have been found to express calbindin (CB), choline acetyl transferase (ChAT), and GABA in area 4 of the human motor cortex which were mainly localised in layers II and VI (Kalinichenko et al., 2006). The NGCs are small, round nonpyramidal neurons that resemble glial cells. The NGCs are thought to be an important neuromodulatory component via acetylcholine activity due to their expression of ChAT. Cholinergic afferents originating from the basal forebrain nuclei are found in all layers of the neocortex, where they modulate the activity of pyramidal and nonpyramidal cells (Lewis, 1991; Mrzljak et al., 1995; Xiang et al., 1998). Cholinergic depolarisation is known to mainly involve CB⁺ interneurons, while PV⁺ interneurons remain unresponsive to acetylcholine activity (Kawaguchi, 1997). In some GABAergic synapses, acetylcholine acts as a neuromodulator by binding to pre- and extrasynaptic receptors to regulate GABA
release (Zhang et al., 2002). Some CB$^+$ cells are also expressed by Martinotti cells that coexpress neuropeptides such as somatostatin (SMT), nitric oxide synthase (NOS) and neuropeptide Y (NPY) (Kawaguchi & Kubota, 1996; Smiley et al., 2000). These cells are found in layers II-VI and send their axons to layer I and synapse onto dendritic tufts of pyramidal neurons in layer I (Kawaguchi, 1997), as well as contacting neighbouring pyramidal neurons (Wang et al., 2004; Silberberg & Markram, 2007). In addition, a small population of pyramidal neurons have been shown to contain CB. These pyramidal neurons were mainly found in layer III as lightly stained neurons. This observation has also been described in a previous report in area 4 of the human cortex (Hayes & Lewis, 1992). Interestingly, a colocalisation study between SMI32$^+$ pyramidal neurons and CB$^+$ pyramidal neurons in layer III in areas 18, 20, 41, and 42 of the human brain showed varying degrees, which indicates a regional difference of the overlap between SMI32$^+$ pyramidal and CB$^+$ interneurons. For example, the colocalisation was greatest in area 20 (30% of SMI32$^+$ neurons and 42% of CB$^+$ neurons were double-labelled), and least in area 18 (only 1% of SMI32$^+$ neurons and 1% of CB$^+$ neurons were double-labelled) (Hayes & Lewis, 1992).

**Calretinin**

Calretinin (CR) is a calcium-binding protein that shares close homology to calbindin-D28k (Rogers, 1987). CR has been shown to protect against calcium-induced excitotoxicity (Lukas & Jones, 1994; D'Orlando, et al., 2002) and is also implicated as a modulator for neuronal excitability (Gall, et al., 2003). CR was identified as a protein of 29 kDa in the rat brain (Pochet et al., 1985). The results showed a similar prominent band at approximately 29 kDa from the western blot analysis using homogenates from the normal human primary motor cortex (Figure 4.1B).

The immunohistochemical results showed that calretinin (CR) labelled cells which were nonpyramidal in shape. The CR$^-$ cells were found throughout all layers of the cortex from layers I-VI but were more abundant in the supragranular layers than in the infragranular layers. The horizontal cells observed in the molecular layer I are likely to be Cajal-Retzius cells (Marin-Padilla, 1984, 1998). The Cajal-Retzius cells are small bipolar or multipolar neurons that are found in layer I and are thought to make contacts with the terminal tufts of
pyramidal neurons (Hestrin & Armstrong, 1996). There were also copious amounts of bipolar shaped cells and small multipolar shaped neurons. These cells are likely to correspond to bipolar and double bouquet morphological cell classes that form axo-dendritic contacts with its neighbouring pyramidal neurons (Peters, 1984a; Somogyi & Cowey, 1984). The bipolar cells occur in layers II-VI and form axonal trajectories that extend vertically up and down towards layer I and VI. These cells form inhibitory synapses but may also exert excitatory action by releasing neuropeptides such as vasoactive intestinal polypeptide (VIP) onto both spiny and smooth neuronal types (Peters & Harriman, 1988; Peters, 1990; Markram, et al., 2004).

**Parvalbumin**

Parvalbumin (PV) is a small protein (9-12 kDa) that is known to modulate intracellular calcium transients in neurons (Augustine et al., 2003; Schwaller, 2009). The western blot analysis shows that there is a single band below the 20 kDa ladder at approximately 10-12 kDa using protein homogenates from the normal human primary motor cortex (Figure 4.1C).

In the motor cortex of normal cases stained for parvalbumin (PV), neurons were distributed throughout all cortical layers, except for molecular layer I. PV showed a complementary staining with CB and CR as PV+ cells were most abundant in the middle and deeper cortical layers. The majority of PV+ cells were multipolar shaped with a smaller population of cells that were bipolar shaped. These neurons are likely to be basket and chandelier cells as previously identified in Golgi-preparations (Jones & Hendry, 1984; Peters, 1984b). The basket cells in the cortex are medium to large sized neurons found in layers II-VI that are involved in intracolumnar, lateral, and callosal inhibition (Kisvarday et al., 1993; Kisvarday et al., 1994; Okhotin & Kalinichenko, 2002). The basket cells are known to form basket-like axonal arborisations that surround the pyramidal cell soma (Jones & Hendry, 1984). Therefore, these cells are suggested to form specialised inhibitory axo-somatic contacts to control spike behaviour of pyramidal neurons by forming rhythmically active and interconnected interneuronal network (Buzsaki & Chrobak, 1995; Wang et al., 2002; Buzsaki & Draguhn, 2004; Markram, et al., 2004). Among cortical GABAergic
interneurons, the *chandelier* cells exhibit unique synaptic specificity which enables them to exert a strong inhibitory influence on pyramidal cells. These specialised nonspiny stellate nonpyramidal neurons with smooth dendrites and short axons were first identified by Szentágothai and Arbib (1974). The *chandelier* cells are named due to the axons that form short vertical rows of boutons resembling “candles” (Fairén & Valverde, 1980) and target the axon initial segment of pyramidal neurons (Szentagóthai & Arbib, 1974; Fairén & Valverde, 1980; Peters, 1984b; DeFelipe, 1997; Somogyi, et al., 1998; Zhu et al., 2004). Previous studies in the primate motor and somatosensory cortices have indicated that PV is also expressed in layer V large pyramidal neurons (Preuss & Kaas, 1996). However, the PV⁺ pyramidal cells were not observed in the staining patterns in the results section above (Figure 4.2E, e). This observation was also noted in another study in the human motor cortex where the authors also did not observe PV⁺ pyramidal neurons in both layers III and V (Ince et al., 1993).

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**Figure 4.5** Schematic diagram to show target specificity of cortical GABAergic interneurons and pyramidal neurons (Pyr) in the primary motor cortex
4.5.2. Colocalisation of the Three Types of Calcium-Binding Proteins

The immunofluorescent double labelling results showed very little or no overlap between the cell types identified with the three interneuronal markers, CB, CR, and PV (Section 4.4; Figure 4.3). The clear parcellation of CB, CR, and PV immunoreactivity in the human primary motor cortex suggests that each of these calcium-binding proteins is preferentially associated with a subset of GABAergic interneurons. The relative proportions of CB, CR, and PV were 11.6%, 41.6%, and 46.8%, respectively (Section 4.4.5; Figure 4.4). CB and CR are structurally closely related (Rogers, 1987); however, they are expressed in separate neuronal populations. In addition, although CB and CR share close homology, these proteins have been shown to contain different structural domain organisations (Palczewska, et al., 2003). Previous colocalisation studies for CB and CR revealed little or no overlap in the rat cortex (Rogers, 1992; Rogers & Resibois, 1992; Kubota, et al., 1994) and in the human medial prefrontal and temporal cortices (Ferrer et al., 1992; del Rio & DeFelipe, 1995; Fonseca & Soriano, 1995; del Rio & DeFelipe, 1996; Gabbott et al., 1997). However, few studies have indicated that the axons of the double bouquet cells that express CB also contained CR in the human temporal cortex, although only a small percentage of colocalisation of cell soma was observed (~4-11%) (del Rio & DeFelipe, 1996, 1997b). Thus, double bouquet cells exhibit a chemically heterogeneous expression of CB and CR in the fibre bundles within the same class of interneurons. As for the expression between CB and PV, several studies have reported a small overlap between the two markers. For example, a study in the rat frontal cortex showed a small percentage of colocalisation between CB and PV in layers V and VI (6-7%), although a relatively high percentage of CB+ cells in layers II/III were immunoreactive for PV (91%) (Kubota, et al., 1994). In addition, previous colocalisation studies of CB and PV have shown little or no overlap in the cat visual cortex (Demeulemeester, et al., 1989; Demeulemeester, et al., 1991) and in the monkey visual and prefrontal cortices (Conde, et al., 1994; Carder et al., 1996). The colocalisation of CB and PV in the present study showed a small 0.2% overlap in the human primary motor cortex. This suggests that there is a diverse neurochemical expression of the calcium-binding proteins which display regional and species differences, and that these various types of calcium-binding protein expressing interneurons represent an important feature of cortical organisation.
4.6. Conclusion

The local-circuit GABAergic interneurons play a critical role in the modulation of cortical output. These cells express a unifying set of morphological, neurochemical and physiological features that support the proposal to subdivide GABAergic cells into at least three non-overlapping groups of inhibitory neurons. The clear parcellation of CB, CR, and PV immunoreactivity in the human primary motor cortex suggests that each of these calcium-binding proteins is preferentially associated with specific interneuronal subpopulations. The primary motor cortex was selected for this study because of the wealth of information on cortical connections in many different species, and because of this area’s clinical relevance in Huntington’s disease with respect to its role in voluntary movements which is described in the next chapter (Chapter 5).
CHAPTER 5. RESULTS
INTERNEURONAL CHANGES IN THE
PRIMARY MOTOR CORTEX
IN HUNTINGTON'S DISEASE

5.1. Introduction

Huntington’s disease (HD) is characterised by major degeneration in the basal ganglia and the cerebral cortex (Vonsattel & DiFiglia, 1998; Vonsattel, et al., 2008). Several lines of evidence implicate the cerebral cortex in the pathophysiology of HD (Cudkowicz & Kowall, 1990a; Hedreen, et al., 1991; Sotrel, et al., 1993; Heinsen, et al., 1994; Jackson, et al., 1995; Macdonald, et al., 1997; Rajkowska, et al., 1998; Macdonald & Halliday, 2002; Selemon, et al., 2004; Thu, 2006; Nana, 2009; Thu, et al., 2010). Also, recent advances in neuroimaging techniques have demonstrated evidence for regional and progressive thinning of the cortex in premanifest and symptomatic HD patients, and more importantly, these studies have shown a correlation between heterogeneous pattern of cortical thinning and dysfunction with different clinical expression of the disease (Jernigan, et al., 1991; Sax, et al., 1996; Rosas, et al., 2002; Kassubek, et al., 2004; Rosas, et al., 2005; Douaud, et al., 2006; Nopoulos, et al., 2007; Rosas, et al., 2008; Tabrizi, et al., 2009; Nopoulos, et al., 2010).

The motor deficits are a classical hallmark of the disease (Huntington, 1872; Berardelli, et al., 1999; Huntington, 2003; Walker, 2007). Patients with HD suffer from a wide range of movement disorder which includes involuntary, hyperkinetic choreiform movements that are progressively replaced by a more hypokinetic syndrome. The somatotopically organised primary motor cortex plays a major role in the control and execution of movement (He, et al., 1993; Matelli, et al., 2004; Geyer & Zilles, 2005; Chouinard & Paus, 2006). Hence, it was hypothesised that cell loss in this region would clearly disrupt motor function, and may also relate to the motor symptoms of HD. Previous in vivo imaging studies have observed...
alterations in the motor cortex of HD patients (Nardone et al., 2007; Rosas, et al., 2008). However, it is not determined at the cellular level, whether there is a differential vulnerability of specific cortical interneurons - specifically the GABAergic interneurons - that are affected in the human primary motor cortex in HD and their relation to motor symptom dysfunction.

The neuronal population in the cerebral cortex is principally comprised of two main functional classes: the pyramidal projection neurons; and the locally-projecting GABAergic interneurons. Previous studies have clearly shown loss of pyramidal neurons in the HD cortex (Cudkowicz & Kowall, 1990a; Hedreen, et al., 1991; Sotrel, et al., 1991; Sotrel, et al., 1993; Heinsen, et al., 1994; Macdonald, et al., 1997; Rajkowska, et al., 1998; Gutekunst, et al., 1999; Sapp, et al., 1999; Sieradzan & Mann, 2001; Macdonald & Halliday, 2002; Selemon, et al., 2004; Thu, 2006; Nana, 2009; Thu, et al., 2010). More interestingly, detailed stereological analyses in our laboratory have shown a significant reduction of SMI32+ pyramidal neurons in the primary motor cortex in HD cases that were predominantly affected by a movement disorder, while the HD cases with major mood disorder were relatively unaffected in the motor cortex (Thu, 2006; Thu, et al., 2010). This is the first study in the primary motor cortex in HD to show that the loss of pyramidal neurons in HD is associated with the motor symptom profile in HD. However, although there was one previous study (Macdonald & Halliday, 2002) on the GABAergic interneurons in the motor cortex of post-mortem HD brain, there has been no study that relates the cellular and morphological changes of the GABAergic interneurons to symptom subtypes in the human primary motor cortex in HD. The cellular abnormalities of the key modulators of cortical output - the GABAergic interneurons - must obviously influence GABAergic neurotransmission, and hence cortical function. Therefore, this research aims to investigate: (1) the detailed cellular changes of the inhibitory GABAergic interneurons in the human primary motor cortex in HD; and (2) whether there is any relation between GABAergic interneuron loss and symptom profile in HD. The research performed in this study is novel, as few laboratories have access to HD tissue extensively characterised according to symptom profiles.
In order to study the pattern of interneuron cell loss in Brodmann area 4 (BA 4) of the primary motor cortex, immunohistochemistry was first used to label specific populations of interneuron cell types identified with the three calcium-binding proteins - calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) interneurons - in 13 HD (HC60, HC68, HC72, HC73, HC79, HC82, HC85, HC86, HC93, HC95, HC99, HC101, HC107) and 14 neurologically normal control cases (H108, H110, H111, H115, H118, H120, H121, H127, H129, H131, H132, H136, H139, H330) (for case details, see Chapter 3, Table 3.1 and 3.2). Secondly, unbiased stereological cell counting methods were used to obtain the total number of the three different GABAergic interneurons (CB, CR, PV) in a subregion (SM1 block) of the primary motor cortex (see Chapter 3 for detailed methodology). The stereological counting results in this chapter are presented as a percentage of the mean number of cells of normal cases with standard error indicated as (%mean ± SEM), and the statistical significance are expressed as *p<0.05, **p<0.01, and ***p<0.001. In addition, morphological changes in these cells were analysed using bright field light microscopy. The results are compared and correlated to the dominant HD symptomatology, striatal neuropathological grade, CAG repeat lengths, post-mortem interval (PMI) and age of each individual HD cases.

5.2. The Pattern of Interneuron Loss in All HD and Normal Control Cases in the Primary Motor Cortex

The stereological counting results showed that in the primary motor cortex (BA 4), there was no overall significant loss in all three types of interneurons when all 13 HD cases were grouped together and compared to the 14 normal controls (Figure 5.1). The results showed that there was an average loss of 9% of CB+, 19% of CR+, and 4% of PV+ interneurons; however, these were not statistically significant (p>0.05 for all markers; single-factor ANOVA test).

Most importantly, it was interesting to note that there was a considerable variation in the number of interneurons lost between the 13 individual HD cases. As shown in Figure 5.2, the total loss of CB+ interneurons varied from 55-59% reduction in four cases (HC68, HC72, HC73, HC99) to 2-37% loss in four cases (HC60, HC79, HC85, HC95), with five
cases (HC82, HC86, HC93, HC101, HC107) showing no loss. The total loss of CR⁺ interneurons varied from 41-61% loss in four cases (HC60, HC73, HC82, HC85) to 2-33% loss in six cases (HC68, HC72, HC86, HC93, HC101, HC107), with three cases (HC79, HC95, HC99) showing no loss. The total loss of PV⁺ interneurons varied from 64% loss in one case (HC73) to 3-29% loss in six cases (HC60, HC68, HC72, HC85, HC93, HC107), and other six cases (HC79, HC82, HC86, HC95, HC99, HC101) showing no loss.

For all stereological analyses of the HD and control cases, the average coefficient of error (CE) for the total number of interneurons (N) for each marker in the Nv and Vref methods [i.e., CE(Nv) and CE(Vref)] was always less than 0.10 (see Appendix II). This indicates that the estimates of Nv, Vref and the total number N, were generally reliable (i.e., CE ≤0.10). In addition, for all analyses, the observed mean variance of the individual total number estimates (i.e., CE²) was less than half of the observed mean variance of the group (coefficient of variation, CV²; CV=SDn-1 / mean), or CE²/CV² <0.5. This indicates that the variability is due to a true difference between cases in the total cell number rather than a lack of precision in the stereological counting methods employed (Gundersen, et al., 1999; West, 2002; Glaser, et al., 2007).
Figure 5.1 Stereological counting of the total number of interneurons in the normal primary motor cortex (BA 4) compared to all HD cases in the human brain

The graph shows the total number of interneurons defined by the three calcium-binding proteins (CB⁺, CR⁺, and PV⁺ interneurons) in the primary motor cortex of normal and Huntington’s disease brains, as determined using stereological cell counting methods and expressed as a percentage of normal controls. There was no significant change in the mean total number of interneurons in all 13 HD cases compared to 14 normal controls in the primary motor cortex.

Figure 5.2 Variation of the total number of interneurons in the primary motor cortex (BA 4) of individual HD cases

The graph shows the variation of the total number of three interneuronal populations between HD cases in the human primary motor cortex. The variation was most pronounced in the CB⁺ interneurons. The four HD cases (HC68, HC72, HC73, HC99) showed 55-59% CB⁺ cell loss, whereas the other nine HD cases (HC60, HC79, HC82, HC85, HC86, HC93, HC95, HC101, HC107) showed a varying loss of CB⁺ interneurons that ranged from no loss to 37% loss.
5.3. The Pattern of Interneuron Loss Correlated with the Dominant HD Symptom Profile in the Primary Motor Cortex

We next investigated whether the variation in the pattern of interneuron cell loss in the primary motor cortex was related to the variation in the dominant symptom profile of HD. The symptom profiles of all HD cases used in the present study were carefully examined by two neuropsychologists (Dr. L. Tippett and V. Hogg) and were categorised into three main symptom groups: four cases were classified as mainly “motor” (HC68, HC72, HC73, HC99); four were classified as mainly “mood” (HC82, HC85, HC95, HC101); and five were classified as “mixed” (motor/mood) symptoms (HC60, HC79, HC86, HC93, HC107). These 13 HD cases for which we had detailed symptomatology records are listed in Table 3.2, Chapter 3. For more details on the symptom classification methods, see Chapter 3, and also Tippett and colleagues (2007) and Thu and colleagues (2010).

5.3.1. Stereological Analyses of the Total Number of Interneurons

The total interneuron loss between different symptom subgroups showed a significant 57% loss of CB⁺ interneurons (**p=0.0037) in the HD motor symptom subgroup, but no significant loss in the other two interneuron populations (average loss of 14% CR⁺, and 12% PV⁺ interneurons; p>0.05) (see Figure 5.3 and Table 5.1). In addition, the counting results showed no significant change in the total interneuron numbers in the HD mixed and HD mood symptom subgroups for all three interneuron populations compared to normal controls (p>0.05 for all markers for both symptom subgroups). In the HD mixed cases, there was no significant change of CB⁺ interneurons, a non-significant 16% loss of CR⁺, and a non-significant 7% loss of PV⁺ interneurons. In the HD mood cases, there was no significant change of CB⁺ interneurons, a non-significant 26% loss of CR⁺, and no significant change of PV⁺ interneurons (Figure 5.3). These results therefore indicate that a significant loss of interneurons was only observed in CB⁺ interneuron subtype in the HD motor cases in the primary motor cortex.

Most importantly, testing for significance of calbindin-D28k (CB) interneuron loss between symptom subgroups showed that there was a significant difference of the numbers of CB⁻ interneurons between the HD motor and HD mixed cases (**p=0.0025), and, between the
**HD motor** and **HD mood** cases (**p=0.0048**) (Figure 5.4). This significance between symptom subgroups clearly establishes that the validity of the major loss of CB+ interneurons in the motor symptom subgroup.

The average coefficient of error (CE) for the total number of interneurons [i.e., CE(Nv) and CE(Vref)] was less than 0.10 for all the normal controls, and for all HD cases of the three symptom subgroups in all three interneuronal populations. These data indicate that the estimate of the total interneuron number was a reliable estimate (CE ≤0.10) (Appendix III). In addition, for the normal controls and the three HD symptom subgroups, the observed mean variance of the individual total interneuron number estimates (i.e., CE2) was less than half the observed mean relative variance of the group (i.e., CV2), or CE2/CV2 <0.5. This indicates that the variability is due to a true difference between cases in total interneuron number, and not the precision of the estimates made with the stereological counting techniques employed (Gundersen, et al., 1999; West, 2002; Glaser, et al., 2007).

### 5.3.2. Morphological Changes of Interneurons in the HD Primary Motor Cortex

In terms of cellular morphology, the calbindin-D28k immunoreactive (CB+) GABAergic interneurons in lamina II/III and V of the primary motor cortex in HD showed major differences between the cases showing mainly “motor” symptomatology, compared with normal control cases (Figure 5.5). In these cases, light microscopy observations showed that the CB+ interneurons had abnormal morphology and exhibited shrinkage of the cell bodies and extensive loss of dendrites. In contrast, the other two interneuron populations (CR and PV) showed minor shrinkage of cell bodies and loss of dendritic processes in the remaining cells in the cortex. The CR+ neurons were affected in the HD motor cases but to a lesser extent than the CB+ interneurons, and PV+ interneurons were the least affected in HD motor cases in the primary motor cortex. In the HD mixed and HD mood cases, all three interneuronal populations showed little variation in cellular morphology. The density and cell morphology in these two symptom subgroups closely resembled the normal control cases, with only minor apparent reduction in their somal size and some loss of dendritic staining. However, the cases with higher striatal neuropathological grades showed some shrinkage in cell soma and moderate to severe dendritic changes (Figure 5.5). These
changes are suggestive of an ongoing progressive neuronal dysfunction in the remaining motor cortical interneurons.

In summary, there was a selective reduction in the number of CB⁺ interneurons (57% loss) in the cases with dominant HD motor symptomatology in the primary motor cortex compared to the cortex of normal control brains. Furthermore, a considerable proportion of the surviving CB⁺ neurons in the primary motor cortex in HD cases with mainly “motor” symptoms showed marked dystrophic changes. There was a small reduction in the other two interneuron populations (CR, 14% loss; and PV, 12% loss) in the same HD motor cases, but no significant difference was observed compared to the normal control cases. In addition, the HD mixed and HD mood cases showed no significant reduction in all three interneuronal populations. There was a slight increase in the CB⁺ interneurons in the HD mixed and HD mood cases, but no statistical difference was observed compared to the normal control cases.
Figure 5.3 Total number of interneurons in the primary motor cortex (BA 4) according to the dominant HD symptom profile

The graph shows the total number of interneurons defined by the three calcium-binding proteins (CB⁺, CR⁺, and PV⁺ interneurons) in the primary motor cortex of normal and Huntington's disease brains as determined using stereological cell counting methods and grouped according to the dominant symptom profile. The HD cases with mainly “motor” symptoms showed a significant loss of CB⁺ interneurons (57% loss), a non-significant loss in the other two interneuronal populations (14% loss of CR⁺ and 12% loss of PV⁺ interneurons). The HD cases with “mixed” and “mood” cases showed no significant loss in all three populations of interneurons.

Table 5.1 Variation in the mean total interneuron number between normal controls and dominant HD symptom subgroups in the primary motor cortex

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HD Motor</th>
<th>HD Mixed</th>
<th>HD Mood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of CB⁺ interneurons (± SEM)</td>
<td>746532 ± 69281</td>
<td>322304 ± 7172</td>
<td>849246 ± 145411</td>
<td>834423 ± 99012</td>
</tr>
<tr>
<td>Total mean number of CR⁺ interneurons (± SEM)</td>
<td>2094553 ± 244203</td>
<td>1798807 ± 51094</td>
<td>1758596 ± 311625</td>
<td>1545539 ± 270297</td>
</tr>
<tr>
<td>Total mean number of PV⁺ interneurons (± SEM)</td>
<td>1556931 ± 128005</td>
<td>1364435 ± 393304</td>
<td>1445313 ± 132216</td>
<td>1694662 ± 254519</td>
</tr>
<tr>
<td>Number of cases (n)</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Percentage loss (%)</td>
<td>-</td>
<td>CB: 57% loss</td>
<td>CR: 14% loss</td>
<td>PV: 12% loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CB: ↑14%</td>
<td>CR: 16% loss</td>
<td>PV: 7% loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CB: ↑12%</td>
<td>CR: 26% loss</td>
<td>PV: ↑9%</td>
</tr>
</tbody>
</table>

Note: Significant cell loss is indicated in red text.
Figure 5.4 Total number of interneurons in the primary motor cortex (BA 4) according to the dominant HD symptom profile showing statistical difference between HD subgroups

The graph shows the statistical difference between three HD symptom subgroups and normal controls. The results show a significant loss of CB⁺ interneurons between: the normal and HD motor group (57% loss, **p=0.0038); the HD motor and HD mixed groups (**p=0.0026); and the HD motor and HD mood groups (**p=0.0048). No significant difference was found in the total number of CR⁺ and PV⁺ interneurons between the different HD symptom subgroups and to the normal controls.
Figure 5.5 Representative photomicrographs showing the morphology of GABAergic interneurons in the primary motor cortex (BA 4) of normal and HD cases of varying symptom profile

The figure shows the morphological changes of the three interneuronal populations (CB, CR, PV) in the primary motor cortex of representative normal and HD cases with different symptom profiles ("motor", "mixed", and "mood" symptoms). Note the severe degeneration of CB⁺ cells in the HD motor case.

A-D: Area 4 immunohistochemically stained for calbindin-D28k (CB) cells in layers II/III in the (A) normal and HD cases of dominant symptom profiles: (B) HD Motor, (C) HD Mixed, and (D) HD Mood cases. (B) Shows a clear degeneration of CB⁺ cells in the HD motor cases.

E-H: Area 4 immunohistochemically stained for calretinin (CR) cells in layers II/III in the (E) normal and HD cases of dominant symptom profiles: (F) HD Motor, (G) HD Mixed, and (H) HD Mood cases. There is no apparent loss of CR⁺ cells in the HD symptom subgroups.

I-L: Area 4 immunohistochemically stained for parvalbumin (PV) cells in layer V in the (I) normal and HD cases of dominant symptom profiles: (J) HD Motor, (K) HD Mixed, and (L) HD Mood cases. There is no apparent loss of PV⁺ cells in the HD symptom subgroups.

Scale bar: A-L = 50 μm
Figure 5.5

Normal | HD Motor | HD Mixed | HD Mood

Calbindin-D28k

A | B | C | D

H115 CAG 17/19 | HC68 CAG 17/42 | HC85 CAG 17/42 | HC85 CAG 24/44

Calretinin

E | F | G | H

H115 CAG 17/19 | HC68 CAG 17/42 | HC79 CAG 17/42 | HC95 CAG 20/39

Parvalbumin

I | J | K | L

H115 CAG 17/19 | HC99 CAG 21/41 | HC93 CAG 20/43 | HC82 CAG 15/42
5.4. The Pattern of Interneuron Loss Correlated with HD Striatal Neuropathological Grade in the Primary Motor Cortex

In order to investigate the relationship between the pathology in the striatum and the primary motor cortex, the 13 HD cases were also independently examined by a neuropathologist (Dr. B. Synek) and each case was graded according to the striatal neuropathology using the Vonsattel grading criteria (Vonsattel, et al., 1985; Vonsattel & DiFiglia, 1998). The grades of the 13 HD cases ranged from 0 to 3. There were four HD grade 0-1 cases (HC68, HC79, HC86, HC101), five HD grade 2 cases (HC72, HC73, HC82, HC95, HC99), and four HD grade 3 cases (HC60, HC85, HC93, HC107) (case details are listed on Table 3.2, Chapter 3).

The total number of interneurons determined by stereological counting analysis in the primary motor cortex showed no significant overall reduction in all three HD striatal grade subgroups compared to the normal controls (p>0.05 for all three interneurons for all grade subgroups), and no significant difference was found between grade subgroups. However, there was a general trend towards more interneuron loss with increasing striatal grade for CR⁻ and PV⁺ interneurons. On average, there was 5% loss of CB⁺, 4% loss of CR⁺, and no significant change of PV⁺ GABAergic interneurons in the HD grade 0-1 cases compared to normal controls. In HD grade 2 cases, there was an average 30% loss of CB⁺, 17% loss of CR⁻, and 3% loss of PV⁺ interneurons compared to normal controls. The total number of interneurons in HD grade 3 cases showed, on average, no significant change (↑15%) in CB⁺ interneurons, however, 36% loss of CR⁺ and 21% loss of PV⁺ interneurons were observed although these were not statistically significant (Figure 5.6).

In summary, analyses of the total loss of GABAergic interneurons in the primary motor cortex revealed a variable non-significant interneuron loss in the different striatal neuropathological grades of HD cases. In general terms, the trend in the pattern of average interneuron loss in two populations (CR and PV) appeared to parallel the overall increasing striatal neuropathological grades, although statistical significance was not observed.
The average coefficient of error (CE) for the total number of neurons [i.e., CE(N_v) and CE(\text{Vref})] was less than 0.10 for all normal controls, and for all HD grade subgroups (grade 0-1, grade 2 and grade 3) in all three populations of interneurons (see Appendix III). These data indicate that the estimate of the total interneuronal number was reliable (CE ≤0.10). In addition, for the normal and the three HD grade groups, the observed mean variance of the individual total interneuronal number estimates (i.e., CE^2) was less than half the observed mean relative variance of the group (i.e., CV^2), or CE^2/CV^2 <0.5. This indicates that the variability is due to a true difference between cases in total interneuronal number, rather than a lack of precision in the stereological counting methodology employed (Gundersen, et al., 1999; West, 2002; Glaser, et al., 2007).
Figure 5.6 Total number of interneurons in the primary motor cortex (BA 4) according to the HD striatal neuropathological grade

The graph shows the total number of interneurons defined by the three calcium-binding proteins (CB⁺, CR⁺, and PV⁺ interneurons) in the primary motor cortex of normal and Huntington’s disease brains as determined using stereological counting methods and grouped according to the different striatal neuropathological grade. There was no significant loss of interneurons for all grade subgroups. However, in general terms, the average interneuron number showed a decreased trend with higher striatal pathological grades, except for CB⁺ interneurons which showed a small increase (↑15%) in the HD grade 3 group, although this was not significant.

Table 5.2 Variation in the mean total interneuron number between different striatal pathological grade groups in the primary motor cortex in Huntington’s disease

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HD Grade 0-1</th>
<th>HD Grade 2</th>
<th>HD Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of CB⁺ interneurons (± SEM)</td>
<td>746532 ± 69281</td>
<td>708763 ± 210260</td>
<td>523023 ± 153682</td>
<td>855743 ± 129770</td>
</tr>
<tr>
<td>Total mean number of CR⁺ interneurons (± SEM)</td>
<td>2094553 ± 244203</td>
<td>2019472 ± 185328</td>
<td>1747333 ± 412395</td>
<td>1338954 ± 286396</td>
</tr>
<tr>
<td>Total mean number of PV⁺ interneurons (± SEM)</td>
<td>1556931 ± 128005</td>
<td>1744332 ± 131719</td>
<td>1517160 ± 329708</td>
<td>1224956 ± 79318</td>
</tr>
<tr>
<td>Number of cases (n)</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Percentage loss (%)</td>
<td>-</td>
<td>CB: 5% loss</td>
<td>CB: 30% loss</td>
<td>CB: ↑15%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CR: 4% loss</td>
<td>CR: 17% loss</td>
<td>CR: 36% loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PV: ↑12%</td>
<td>PV: 3% loss</td>
<td>PV: 21% loss</td>
</tr>
</tbody>
</table>
5.5. The Pattern of Interneuron Loss Correlated with CAG Repeat Length, PMI, and Age in the HD Primary Motor Cortex

We next investigated whether the total number of interneurons in the three interneuronal populations (CB, CR, PV) was correlated with the CAG repeat lengths in the HD gene, post-mortem interval (PMI), and age of the 13 HD cases in the primary motor cortex (Figure 5.7).

**CAG repeat length**

As shown in Figure 5.7 (panel A, D, G), there were no significant correlations between the total number of interneurons and the CAG repeat number in HD cases, although there appeared to be a general trend towards a decrease in interneuron number with increasing CAG repeat length. A Pearson regression statistical analysis demonstrated that there was no significant deviation in the CB + (r²=0.0554, p=0.4387), and CR + interneurons (r²=0.3038, p=0.0509). There was however, a significant correlation of the loss of PV + interneurons with the number of CAG repeats (r²=0.3412, *p=0.0361).

**Post-mortem interval**

No significant correlation was found between the total interneuron number and PMI of the CB + (r²=0.0508, p=0.4591), CR + (r²=0.135, p=0.2168), and PV + (r²=0.0216, p=0.6319) interneurons in the HD primary motor cortex (Figure 5.7, panel B, E, H).

**Age**

There was no significant correlation between the total interneuron number and age at death of the CB + (r²=0.046, p=0.4817), CR + (r²=0.0076, p=0.7764), and PV + interneurons (r²=0.0006, p=0.9352) in the HD primary motor cortex (Figure 5.7, panel C, F, I).
The figure shows the correlation of the total interneuron number with CAG repeat length, post-mortem interval, and age of the 13 HD cases in the primary motor cortex. In general terms, the average interneuron cell number decreased with increasing CAG repeat length. However, no significant correlation was observed between the total interneuron number and CAG repeat length, except for PV⁺ cells (*p=0.0361). No significant correlation was observed between the total interneuron number with post-mortem interval and age of HD cases used in this study.
5.6. Discussion

The present chapter provides a detailed account of the degeneration of the GABAergic interneurons in BA 4 of the primary motor cortex in HD using immunohistochemical and unbiased stereological counting techniques. The results show that there was no significant loss in all three interneuronal populations (average loss of 9% CB⁺, 19% CR⁺, and 4% PV⁺ interneurons) when all HD cases were grouped together (n=13) and compared to the neurologically normal controls (n=14) (Figure 5.1). These results were similar to a previously reported study in the motor cortex (Macdonald & Halliday, 2002) which showed no significant cell loss in all three calcium-binding protein expressing interneurons (CB, CR, PV) in the motor cortex of 5 HD cases.

However, most importantly, the present study has clearly demonstrated a considerable variation in the extent of interneuron cell death between HD cases in the primary motor cortex, and most notably in the calbindin-D28k (CB) interneurons in the HD motor symptom cases (Figure 5.2). This finding has prompted the hypothesis that the variable pattern of interneuron loss in the primary motor cortex may be related to the motor symptomatology in HD. Indeed, a previous study on the primary motor cortex in HD in our laboratory has shown that there was a major cell loss of all neuronal types (NeuN⁺ cells) and a subpopulation of pyramidal neurons (SMI32⁺ pyramidal neurons) in the motor cortex in HD cases that showed major “motor” symptoms, while cases characterised by “mood” symptoms showed no significant cell loss in this region, even between cases of the same neuropathological grade (Thu, 2006; Thu, et al., 2010). This was the first type of study to demonstrate that phenotypic variability in HD is related to the variable neuronal degeneration in the cortex. This suggests that a heterogeneous pattern of cortical cell loss is a key feature of the neuropathology of HD and that the variable neuronal loss in the motor cortex is associated with the variable motor symptoms in HD. Indeed, the results presented in this chapter also showed a differential pattern of GABAergic interneuron loss which correlates with the symptom profile of HD. In particular, there was a major selective loss of CB⁺ interneurons in the HD cases with major motor phenotype in the primary motor cortex.
In addition, an association between the pattern of interneuron loss with striatal neuropathological grades, CAG repeat length, post-mortem interval, and age was also investigated for each HD case. These novel findings are of major importance in gaining a better understanding of the pathophysiological basis of the variable symptomatology which characterises HD, and may provide a better appreciation of the structural-functional relationships in the human forebrain.

5.6.1. The Relation between the Extent of Interneuron Loss and the Dominant Symptom Profile of HD in the Primary Motor Cortex

Motor dysfunction is a characteristic symptom observed in HD patients. Since the primary motor cortex plays a major role in the control of movement, it was hypothesised that the greatest cell loss in the primary motor cortex in HD may occur in cases with predominantly motor symptomatology. To test whether the pattern of interneuron loss in the primary motor cortex was related to the different symptom subtypes of HD, the blinding of the clinical and anatomical assessments was removed, and the average total interneuron loss of the cases in each of the three HD symptom groups (motor, mood and mixed) was compared. These 13 HD cases were grouped according to their symptoms regardless of their “striatal” neuropathological grade and were compared to the normal controls (a statistical inference of symptom vs. grade is outlined under methodological considerations in Section 8.2 in the General Discussion, Chapter 8).

The results of the present study demonstrated an overall small but non-significant reductions of GABAergic interneurons in the primary motor cortex in the HD brain (Figure 5.1). However, comparison of the total interneuron loss in the subgroups of the three symptom profiles in the primary motor cortex showed a significant association between cell loss and symptom phenotype of interneurons labelled with calbindin-D28k (CB). In the group of HD cases who experienced mainly “motor” symptoms, there was a major selective loss of CB⁺ interneurons in the motor cortex, with an average total 57% reduction (**p=0.0037; Bonferroni post hoc test). There was a small but non-significant reduction in the other two populations (14% loss of CR⁺ and 12% loss of PV⁺ interneurons) in the same motor symptom cases. There was no significant loss of all three interneurons (CB, CR, PV) in the
**HD mood** or **HD mixed** cases which showed relative sparing of the GABAergic interneurons in these two symptom subgroups (Figure 5.3). Therefore, overall, these results represent a preferential loss of a subset of CB⁺ interneurons in the HD motor cases in the primary motor cortex. In addition, there was a significant difference between the numbers of CB⁻ interneurons between different HD symptom subgroups, i.e., between HD motor and HD mood cases (**p=0.0048), and between HD motor and HD mixed cases (**p=0.0025) (Figure 5.4), which clearly establishes that the loss of CB⁻ interneurons in HD motor cases contrasts significantly with non-significant reductions of CB⁺ interneurons in the HD mood and HD mixed cases.

Previously reported pathological studies on the GABAergic interneurons show in general a relative sparing of these cells in the HD cortex. For example, Macdonald and Halliday (2002) showed no significant cellular changes in the number, distribution and morphology of calcium-binding protein (CB, CR, PV) interneurons in the motor cortices of 5 HD cases examined in their study. In addition, there was relative sparing of PV⁺ interneurons in the superior frontal cortex of 11 HD cases (Cudkowicz & Kowall, 1990b). The changes in the present study showed overall, similar sparing of all three types of interneurons in all 13 HD cases in the primary motor cortex (Figure 5.1). However, a study by Ferrer and colleagues (1994) observed a significant decrease in PV⁺ interneurons in the frontal cortex, but no significant difference was observed in the occipital and temporal lobes. These results show that there is a heterogeneous pattern of interneuron loss in the different functional regions of the cortex in HD. In the present study, there was a selective loss of CB⁺ interneurons which was specifically correlated with severe motor symptomatology in the primary motor cortex. Other authors did not observe the loss of CB⁺ interneurons and this can be clearly explained by having HD cases not grouped according to the main clinical symptomatology. Detailed stereological cellular analyses in our laboratory (Thu, 2006; Thu, et al., 2010) on the same HD cases to the present study have shown that there was a clear association between the loss of SMI32⁺ pyramidal neurons (45% loss) with the identical HD motor cases analysed in the primary motor cortex, but no cell loss was observed in the HD mood cases (Figure 5.8). This suggests that motor symptomatology in HD is associated with a loss of pyramidal neurons and CB⁺ interneurons in the primary motor cortex.
Figure 5.8 Schematic diagram showing the association between the patterns of pyramidal and interneuron cell loss in the HD primary motor cortex according to the dominant symptom profiles

The heterogeneity in clinical symptomatology which characterises HD is associated with the pattern of SMI32⁺ pyramidal cell loss (45% loss) (Thu et al., 2010) and CB⁺ interneuron loss (57% loss) only in the HD motor cases, but not in the HD mood cases in the primary motor cortex.

As shown in Chapter 4, the calcium-binding protein calbindin-D28k (CB) labels local-circuit nonpyramidal neurons that form important axo-dendritic interactions with the pyramidal neurons which include double bouquet, Martinotti, and neurogliaform morphological cell types. Thus, loss of these different types of interneurons labelled with CB in the primary motor cortex in HD motor cases suggests that there is a lack of axo-dendritic inhibitory action onto the pyramidal neurons (Figure 5.8), and that the selective loss of CB interneurons in the motor cortex may underlie the cortical pathogenic basis for motor dysfunction observed in HD patients. The early loss of inhibition of the pyramidal neurons by the CB⁺ interneurons and the resultant hyperexcitability of the pyramidal neurons may be a contributing factor in motor cortical dysfunction in HD resulting in: (1) pyramidal cell degeneration; and (2) the “hyperkinetic” motor symptoms of HD. For example, it was most interesting to note that the pattern of interneuron cell loss paralleled pyramidal cell loss, i.e., CB⁺ interneuron loss was only observed in the HD motor cases in
the primary motor cortex where there was also major pyramidal cell loss. This suggests that the perturbation between the pyramidal-interneuron interactions is a critical factor in the pathogenesis in the cortex of HD. Indeed, a study in Cre/LoxP conditional HD mice showed that the interaction between interneurons and pyramidal neurons was necessary to produce motor deficits and cortical pathology (Gu, et al., 2005). In their study, restricting the expression of toxic mutant huntingtin fragment only to the pyramidal neurons was not sufficient to cause widespread cortical pathology or motor deficits; however, the expression of mutant huntingtin to all neuronal types (pyramidal and interneurons) elicited both progressive motor deficits and cortical pathology including neuronal degeneration and dysmorphic neurites. Therefore, this suggests that an early loss of interneurons together with pyramidal neuronal loss and their interactions in the motor cortex is a critical factor in the pathophysiology of HD and that this may also result in the early dysfunction of motor symptoms in HD. A study has hypothesised that an early loss of pyramidal neurons in the motor cortex which project to the putamen results in the production of early motor deficits such as clumsiness and falling, i.e., loss of projections to the putamen and subsequent loss of input to the GPe, causes elevated excitation to the cortex via the thalamus resulting in hyperkinetic choreiform movements (Koroshetz et al., 1992). Also, the continued dysfunction and the progressive loss of cortical neurons and their projections to the putamen and the subsequent projections to the GPi has been suggested to cause rigidity in later stages of the disease (Storey & Beal, 1993). Furthermore, a study using transcranial magnetic stimulation to assess cortico-cortical interactions in the motor cortex of HD have shown alterations in the intracortical inhibition and facilitation patterns which were postulated to result from impaired GABAergic interneurons (Abbruzzese et al., 1997). Interestingly, these changes were related to clinical rating of choreic dyskinesias. These studies indicate that an early loss of CB⁺ interneurons and ongoing degeneration of these neurons are likely to be involved in both hyperkinetic and dyskinetic movement disorder in HD. Indeed, coexistence of chorea and bradykinesia has been previously documented in HD (Thompson et al., 1988).

Also, constitutive CB⁻² null mutant mice demonstrated impairment in motor coordination (Airaksinen et al., 1997), although these changes are likely to act via altered transmission of cerebellar Purkinje cell signalling. CB is highly expressed in Purkinje cells and selective genetic deletion of CB from Purkinje cells resulted in distinct deficits in motor and sensory processing (Barski et al., 2002; Barski et al., 2003). It is also interesting to note that in
studies on the striatum, Tippett and colleagues (2007) showed a loss of CB immunoreactivity in the matrix compartment (which is linked to sensorimotor and related associative areas) in HD cases with severe motor impairment. These combined results show that the calcium-binding protein expressing cells, especially CB are differentially affected in both the cortex and the striatum, which is suggestive of possible similar mechanisms of cell death in both cortical and striatal brain regions in HD.

In conclusion, this is the first study to demonstrate that in the primary motor cortex, there is a major selective loss of CB+ interneurons in the HD cases with predominant “motor” symptoms, but not in cases with “mood” or “mixed” symptoms. In other words, the pattern of selective CB+ interneuron loss in the primary motor cortex is associated to the motor deficits in HD, suggesting that interneuron loss and degeneration in the primary motor cortex may play a major role in producing motor abnormalities in HD.

5.6.2. The Relation between the Extent of Interneuron Loss and the Striatal Neuropathological Grades of HD in the Primary Motor Cortex

In this study, the 13 cases of HD were also categorised into three groups based on the Vonsattel neuropathological grades - grade 0-1 (four cases), grade 2 (five cases) and grade 3 (four cases) for the data analysis. The detailed stereological counting studies have demonstrated that in general, a greater loss of interneurons in HD cases was observed with higher neuropathological grades, although a clear statistical association was not evident (Figure 5.6). A study by Macdonald and Halliday (2002) found no significant change in the morphology, number or distribution of calcium-binding protein immunoreactive neurons in the primary motor cortex in their study of five HD grade 2-3 cases. The results of our study showed on average, 5% loss of CB+, 4% loss of CR+, and no loss of PV+ interneurons in HD grade 0-1 cases. In HD grade 2 cases, there was reduction of 30% CB+, 17% CR+, and 3% PV+ interneurons, and in HD grade 3 cases, there was no loss of CB+, 36% CR+, and 21% PV+ interneurons; that is, the neuronal degeneration in the primary motor cortex generally parallels the Vonsattel grade of neuropathology which is based on the extent of striatal atrophy. This “trend” suggests that the interneuron degeneration in the primary motor cortex may be linked to the degeneration in the striatum in HD (see later under in Chapter 8
General Discussion). These findings are generally consistent with previous studies on the human primary motor cortex in our laboratory where neuronal loss in this region was associated with increasing striatal neuropathological grades (Thu, 2006; Thu, et al., 2010). In particular, the number of SMI32$^+$ pyramidal neurons decreased with higher striatal HD grades which may contribute to dysfunction in other cortical and subcortical brain regions. However, a small increase of CB$^+$ interneurons was observed in HD grade 3 case, although this was not statistically significant. The HD grade 3 cases in the present study were also in the category of mixed and mood symptom groups where no cell loss was observed, and this suggests that symptomatology of HD may also influence the degeneration of interneurons in the motor cortex in HD. In conclusion, the findings in our study shows a trend towards a greater loss of GABAergic interneurons with higher HD striatal grades which may suggest a link between cortical and striatal degeneration.

5.6.3. Correlation between Interneuron Loss and the CAG Repeat Length, Post-Mortem Interval, and Age in the HD Primary Motor Cortex

This study also investigated the differences in the CAG repeat length, post-mortem interval, and age of HD cases which may account for the variable differences observed in the human brain. Striatal pathology has been shown to be closely associated with the number of CAG repeats in HD (Penney, et al., 1997; Vonsattel, et al., 2008); however, it is not yet clear if there is a link between CAG repeat length and cortical pathology, especially with interneuron loss. The correlation between the length of CAG repeat and the total number of interneurons in HD showed no significant correlation using a Pearson regression analysis at the 5% level, although a general trend was observed with greater interneuron loss with increasing CAG repeat length. One exception included a significant correlation between the CAG repeat length and the number of PV$^+$ interneurons ($r^2=0.3412, *p=0.0361$) (Figure 5.7). This suggests that the number of PV$^+$ interneurons decrease with increasing CAG repeat length. The analysis of post-mortem interval (PMI) showed no overall correlation between the total interneuron number and PMI in the primary motor cortex of HD cases. This suggests that the differences in time interval between death and tissue processing, and the possible resultant modifications in neuronal immunoreactivity was not accountable in this study. Pakkenburg and Gundersen (1997) have found an association between the decreasing numbers of cortical neurons with increasing age. The analysis of age at death in
this study showed no significant overall correlation between the total interneuron number and age in the primary motor cortex in HD cases. In line with the present study, a previous study on the age-related changes of the three calcium-binding proteins (CB, CR, PV) in the human primary motor cortex also showed no significant reductions in the number of interneurons immunoreactive for CB, CR, and PV compared to normal controls, although a trend towards a decrease in the number of these neurons was observed (Bu et al., 2003). These outcomes indicate that in general, the results between the total interneuron number with the symptom profile and striatal disease grade described above can be regarded as separate from the influence of CAG repeat length, post-mortem interval, and age of the HD cases used in this study.

5.7. Conclusion

The major finding in the present study is the significant reduction of calbindin-D28k (CB) expressing interneurons in the human primary motor cortex in HD cases with major motor disorder. The local-circuit GABAergic interneurons form critical elements in the cortical organisation due to their presumed inhibitory functions. Therefore, the loss of CB\textsuperscript{+} interneurons that make axo-dendritic contacts with the distal dendrites of pyramidal neurons in HD motor cases may indicate a dysfunction in the intrinsic cortical circuitry. This observation suggests that there may be a selective loss of GABAergic modulation that contributes to the functional abnormalities of the motor cortex in HD. Also, the lack of any significant loss of CB\textsuperscript{+} interneurons in HD cases with major mood disorder in the motor cortex, which may well correlate to lack of any dominant motor abnormalities, suggests that the CB\textsuperscript{+} interneurons in the motor cortex play a prominent role in contributing to the generation of a motor phenotype in HD.
CHAPTER 6. RESULTS

DISTRIBUTION OF INTERNEURONS
IN THE ANTERIOR CINGULATE CORTEX
OF NORMAL HUMAN BRAIN

6.1. Introduction

The cingulate gyrus is located on the medial surface of the brain that comprises anatomically and functionally distinct areas. It forms a major component of the limbic system and has been shown to subserve a wide range of emotion, autonomic, cognitive and somatic motor functions (MacLean, 1993; Devinsky, et al., 1995; Mega et al., 1997; Morecraft & Van Hoesen, 1998; Bush, et al., 2000; Paus, 2001; Vogt, et al., 2004; Vogt, et al., 2005). In the human, the cingulate cortex has been shown to comprise an intricate organisation based on detailed cytoarchitectural, electrical stimulation, and imaging studies (Brodmann, 1909; von Economo & Koskinas, 1925; Rose, 1927; Vogt, et al., 1995; Whalen et al., 1998; Phan et al., 2002; Palomero-Gallagher, et al., 2008). The fundamental regions of the human cingulate cortex include: the anterior cingulate cortex (ACC) which is further subdivided into the perigenual anterior cingulate cortex (pACC) and midcingulate cortex (MCC); the posterior cingulate cortex (PCC); and the retrosplenial cortex (RSC). The region of the anterior cingulate cortex (ACC) used in this study comprises a heterogeneous region involved in emotion, fear, and autonomic control (Vogt, et al., 1995; Paus, 2001; Vogt, et al., 2004). These functions are maintained through reciprocal connections with the amygdala, ventral striatum, anterior and mediodorsal (MD) thalamic nuclei, and projections to the solitary tract and dorsal motor nucleus of the vagus (Cummings, 1993; Devinsky, et al., 1995; Vogt, et al., 2004).

The functional activity of each cortical area is governed by dynamic interface between the cortical afferents, intrinsic neuronal connectivity, and the efferent pathways. The structural
components involved in the local neuronal circuitry in the cortex are composed of interconnected populations of pyramidal and nonpyramidal neurons (Douglas & Martin, 2004). The latter provides the main source of inhibition and uses γ-aminobutyric acid (GABA) as the neurotransmitter. These local-circuit GABAergic interneurons account for 15-30% of all cortical neurons and are morphologically distinct from pyramidal neurons (Lorente de Nó, 1933; Peters & Jones, 1984; DeFelipe, 1997, 2002). In addition, the GABAergic interneurons may differ in their chemical phenotypes. In particular, the three calcium-binding proteins, calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) have been specific neurochemical markers for differentiating subpopulations of cortical GABAergic interneurons (Baimbridge, et al., 1992; Andressen, et al., 1993; Kubota, et al., 1994; Kawaguchi & Kubota, 1997; DeFelipe, 2002; Markram, et al., 2004; Gifford, et al., 2007; Schwaller, 2009). In addition, previous studies have shown that specific types of cortical inhibitory interneurons have defined laminar location and patterns of synaptic connectivity with particular regions of the pyramidal cells (e.g., axon initial segments, somata, and dendrites) as well as forming extensive connections between cortical interneurons. These studies show that cortical interneurons are morphologically, biochemically, and physiologically diverse and this suggests that different interneuronal subpopulations may play different functional roles in shaping cortical circuitry.

In view of the structural and functional heterogeneity of the GABAergic inhibitory interneurons, a detailed assessment of these neurons defined by the three calcium-binding proteins, CB, CR, and PV in the human anterior cingulate cortex are investigated and described in this chapter. Firstly, western blot analysis was performed to validate the specificity of the antibodies raised against the three calcium-binding proteins (Section 6.2). Secondly, morphological and distribution patterns of the three types of interneurons were investigated using single immunoperoxidase and double immunofluorescent labelling methods (Sections 6.3 and 6.4). Such information will provide a base upon which to analyse cellular changes in the anterior cingulate cortex of the human brain in disease states such as Huntington’s disease which is described in the next chapter (Chapter 7).
6.2. Western Blot Analysis

The western blot analysis on fresh human tissue homogenates prepared from the anterior cingulate cortex of 7 normal cases (H108, H111, H112, H115, H118, H121, H127; case details are listed on Table 3.1; see Chapter 3) revealed clear and specific protein bands by antibodies raised against the three calcium-binding proteins. Rabbit polyclonal CB, CR, and mouse monoclonal PV antibodies were used for the analysis (details are listed in Table 3.6). The blots were detected using avidin-biotin immunoperoxidase method and visualised with enhanced chemiluminescence signal. The CB, CR, and PV antibodies recognised a prominent band at approximately 28, 29, and 12 kDa, respectively (Figure 6.1A, B, C), which were the expected bands on the molecular weight of these proteins (Schwaller, 2009). All blots were probed with β-actin antibody for loading control (~42 kDa) to ensure consistency of protein loading for each sample (Figure 6.1D).

![Western Blot Analysis](image)

**Figure 6.1 Western blots showing the expression of calcium-binding proteins in the anterior cingulate cortex of the normal human brain**

The blots show specific bands at approximately (A) 28 kDa (CB), (B) 29 kDa (CR), (C) 12 kDa (PV), and (D) 42 kDa (β-actin) in the normal human brain of the anterior cingulate cortex. Apparent molecular weights of protein bands are expressed in kilodaltons (kDa).
6.3. Description of Single Immunohistochemical Staining Patterns in the Normal Human Anterior Cingulate Cortex

The single immunoperoxidase staining pattern of interneurons defined by the three calcium-binding proteins, CB, CR, and PV in area 24 of the anterior cingulate cortex was comparable to the staining patterns in area 4 of the primary motor cortex (see Chapter 4). Both cortices were characterised by an agranular cortex, with lack of granular layer IV and enlarged layer V, subdividing it into Va and Vb. At the cellular level, the immunoreactivity was similar in the distribution and morphological cell types assessed with CB, CR, and PV. An adjacent series of sections were stained with cresyl violet (Nissl) and neuronal N (NeuN) immunohistochemistry to determine specific laminar staining and to delineate exact boundaries of the BA 24 region of the anterior cingulate cortex. For more details on the methodology, see Chapter 3. The details of the normal human brain cases used for this part of the study are listed under Table 3.1.

6.3.1. Distribution Pattern of Interneurons (CB, CR, PV)

*Calbindin-D28k*

Calbindin-D28k immunoreactive (CB⁺) neurons in area 24 of the anterior cingulate cortex were found as an intense band of cells on layers II and III (Figure 6.2C). At higher magnification (Figure 6.2c), the CB antibody labelled neuronal somata and proximal processes with intense immunoreactivity. The intensity of the immunoreactive somata varied from a moderately brown to a dark brown colour. The vast majority of these neurons were mainly small to medium sized (6-28 μm cell body diameter) nonpyramidal cells with bipolar or multipolar oriented dendritic trees. The majority of the bipolar/fusiform cells were characterised by the vertical ascending and descending tufts of axons and dendrites which emerged from the apical and basal poles of the cell body, but some were horizontally oriented. A small population of these cells were also observed in the deeper infragranular layers. In addition, a lightly stained population of cells that showed features of small pyramidal cell morphology were found sparsely scattered throughout layers III and V. These pyramidal shaped cells were identified and exclusively excluded from stereological cell counting analysis in the anterior cingulate cortex of Huntington’s disease in Chapter 7.
**Calretinin**

Calretinin immunoreactive (CR⁺) neurons also showed a layer specific staining pattern (Figure 6.2D). The CR⁺ neurons were mainly concentrated in the supragranular layers II and III, with fewer cells localised in layer I. The cells in layer I often had cell bodies running parallel to the pial surface. More cells were found in layers II/III than in infragranular layers V and VI. The majority of these cells were small to medium sized (5-15 \( \mu m \) in cell body diameter) nonpyramidal neurons with spindle shaped cell soma that gave off vertically oriented bipolar branches from upper and lower poles (Figure 6.2d). Occasionally, bipolar shaped cells with long descending processes were observed reaching as far as the white matter. A smaller population of multipolar cells were also found. Many ascending dendritic branches reached layer I where they ran parallel to the pial surface.

**Parvalbumin**

Parvalbumin immunoreactive (PV⁺) neurons were distributed sparsely throughout layers II-VI (Figure 6.2E). The majority of PV⁺ neurons were located within layers III and V, and a few were present in layers II and VI. These cells were mainly multipolar neurons with multiple dendrites emanating from the cell soma (Figure 6.2e). In addition, there was a sparse distribution of cells that showed features of bipolar dendritic morphology. No cells were observed in layer I. The PV⁺ cells identified in the upper layers had medium sized cell somata (12-23 \( \mu m \) in cell body diameter). The deeper layers had somatic cell sizes that were significantly larger (>30 \( \mu m \)). The multipolar shaped cells had dendrites that radiated out of the cell body with no polarity. Most large multipolar neurons in layers III and V had, however, longer vertical than horizontal dendrites. One of the most notable features of PV immunoreactivity was the presence of a dense plexus of fibres which extended from the midportion of layer III as far as the limit with layer Vb. The plexus was made up of PV⁺ fibres, among which lay immunopositive neurons mentioned above.
6.3.2. Distribution of Neurons identified by Nissl and NeuN

Cresyl Violet

Cresyl violet (Nissl) histological staining labelled the cell bodies of all neuronal types (pyramidal and nonpyramidal neurons) as well as glial cells (microglia, oligodendrocytes and astrocytes). Nissl-stained sections differentiated area 24 of the anterior cingulate cortex by the lack of layer IV and a prominent layer V. The exact border between layers II and III, and layer VI and the subjacent white matter were more difficult to differentiate (Figure 6.2A, a).

Neuronal N

Neuronal N (NeuN) immunohistochemistry stained all cortical neuronal populations. The nuclei of neurons was most densely stained while the cytoplasm and processes of neurons showed lighter, granular staining (Figure 6.2b). The area 24 of the anterior cingulate cortex is an agranular cortex that consist of layers I, II, III, Va, Vb and VI (Figure 6.2B). Layer I contained very few neurons, while layers II and III consisted primarily of small pyramidal cells, with cell processes directed vertically towards the superficial layers as well as small nonpyramidal neurons. Layer V consisted of a small number of large pyramidal neurons with laterally extending basal dendrites that were visible. The deepest layer VI adjacent to the white matter contained relatively few cells. These were mostly small pyramidal cells and spindle shaped interneurons.
Figure 6.2 Representative photomicrographs showing the distribution pattern of neurons in the anterior cingulate cortex (Brodmann area 24) of the normal human brain

This figure shows the distribution pattern and morphology of different cell types in the anterior cingulate cortex (area 24) of neurologically normal brains in 50 μm coronal sections. The overall distribution of cells are shown by sections adjacently stained for (A, a) cresyl violet (Nissl) and immunostained for (B, b) neuronal N (NeuN). The pattern of the distribution of the different types of GABAergic interneurons is shown on an adjacent series of sections immunostained for (C, c) calbindin-D28k (CB), (D, d) calretinin (CR), and (E, e) parvalbumin (PV).

A-E: Low magnification photomicrographs of the distribution pattern of neurons in the normal human anterior cingulate cortex.

A: Area 24 histochemically stained for cresyl violet (Nissl).

B: Area 24 immunohistochemically stained for neuronal N (NeuN).

C: Area 24 immunohistochemically stained for calbindin-D28k (CB). The CB⁺ neurons predominate in the upper layers II-III that have bitufted and multipolar morphologies.

D: Area 24 immunohistochemically stained for calretinin (CR). The CR⁺ neurons are found in layers I-VI but are more numerous in the supragranular layers.

E: Area 24 immunohistochemically stained for parvalbumin (PV). The PV⁺ neurons are found in layers II-VI. In layer II, small bipolar and multipolar cells can be seen. In layer V, the predominant cell type is multipolar shaped cells with long, radiating dendrites.

a-e: High magnification photomicrographs showing the morphology of neurons in the human anterior cingulate cortex (area 24) stained for: (a) cresyl violet (Nissl) cells in layer III; (b) neuronal N (NeuN) cells in layer III; (c) calbindin-D28k (CB) cells in layers II-III; (d) calretinin (CR) cells in layers II-III; and (e) parvalbumin (PV) cells in layer V.

Scale bars: A-E = 500 μm; a-e = 50 μm
Figure 6.2

Nissl NeuN CB CR PV

A B C D E

I II/III Va Vb VI

a b c d e
6.4. Double Immunofluorescent Labelling

To assess the degree of overlap between the different subpopulations of GABAergic interneurons that express the three calcium-binding proteins, the colocalisation of CB, CR, and PV in all possible pairs of combinations were investigated using double immunofluorescent labelling method (Figure 6.3). The details on the 4 normal human brain cases used (H111, H115, H120, H330) are listed in Table 3.1, see Chapter 3. All mouse monoclonal antibodies were tagged with an Alexa Fluor 488 label that emits green fluorescence and rabbit polyclonal antibodies were tagged with an Alexa Fluor 594 label that emits red fluorescence. Any indication of colocalisation in the merged images appeared yellow in colour. The fluorescent images were acquired using the Zeiss LSM 710 inverted confocal laser scanning microscope. The immunofluorescent sections were qualitatively observed for distribution patterns and morphological features and quantitative analysis was also carried out to determine the numerical density and the relative proportions of the three markers in the anterior cingulate cortex. The cellular quantification was performed on images that were acquired using a tiling method to montage together a cortical strip encompassing all six horizontal layers with dimensions of 425 μm x 3400 μm (area of each montage = 1445000 μm²). The cells with clearly visible profiles of the nucleus, cytoplasm, and proximal dendrites that appeared in the collected z-stack within the entire cortical strip montage were included in the counting analysis (for detailed description of methodology, see Chapter 3, Sections 3.3.2.3 and 3.4.2).

6.4.1. Calbindin (CB) mouse monoclonal / Calbindin (CB) rabbit polyclonal

To confirm the reliability and consistency of CB antibodies raised in two different species, the mouse monoclonal CB (mCB) and rabbit polyclonal CB (rCB) antibodies were used to test whether the same type of interneurons were double-labelled (Figure 6.3A, a). A distinctive feature of both mCB and rCB antibodies was the presence of numerous neurons, particularly in the superficial layers. The laminar distribution of CB staining showed a higher number of cells in the upper layers II/III and fewer cells in the deeper layers V and VI. No neurons were present in layer I. In addition, a weakly labelled population of typical pyramidal cells were distributed throughout layer III. In general, the rabbit polyclonal CB
antibody labelled the cell body, axons and dendritic arborisations more distinctively than the mouse CB antibody.

When the high resolution images of mCB and rCB immunolabelling were merged, there were 49 clearly identified mCB/rCB double-labelled cells from two cortical strip montages of 1 normal case (H115), which indicates colocalisation of these two markers in the same clearly labelled cells. The weakly stained CB⁺ pyramidal neurons were excluded from the counting analysis. This suggests that mCB and rCB antibodies recognise the same type of GABAergic interneurons in the BA 24 region of the anterior cingulate cortex (Figure 6.3A, a).

6.4.2. Calbindin-D28k (CB) / Calretinin (CR)

The mouse monoclonal calbindin expressing (CB⁺) cells were confined mainly to intensely labelled cells that showed features of bipolar and multipolar morphologies. These cells were numerous in the superficial layers and formed a distinctive band made of cell clusters in layers II/III (Figure 6.3B, b). Also, weakly labelled neurons were typically found in layers II/III and V/VI, which comprised both pyramidal and nonpyramidal neurons. The rabbit polyclonal calretinin immunoreactive (CR⁺) neurons were found throughout all cortical layers, with the predominance of these neurons in the upper layers II and III (Figure 6.3B). Fewer nonpyramidal neurons were present in the deeper cortical layers. With higher magnification, CR immunoreactivity was homogeneously distributed throughout the cell cytoplasm and proximal dendritic processes of neurons (Figure 6.3b). Layer I contained a few spindle shaped neurons, with some of them having the major axis oriented parallel to the pial surface. In general, the CR⁺ cells were smaller than the CB⁺ cells in the population of nonpyramidal neurons.

In a total of 465 cells counted from the six cortical strip montages of 3 normal human cingulate cortex cases (H111, H115, H120), there were clearly identified single-labelled 112 CB⁺ cells (24.1%), 353 CR⁺ cells (75.9%), and no double-labelled cells (0%). This suggests
that CB and CR are found in distinct, non-overlapping populations of GABAergic interneurons in the BA 24 region of the anterior cingulate cortex (Figure 6.3B, b).

6.4.3. Calbindin-D28k (CB) / Parvalbumin (PV)

The staining patterns of rabbit polyclonal calbindin immunoreactive (CB+) neurons were the same as described above (Figure 6.3A and B). The mouse monoclonal parvalbumin (PV) immunoreactivity revealed a morphologically heterogeneous population of cells that were exclusively nonpyramidal in shape. PV+ cells were found in all layers of the cortex except layer I and reached their peak density in the middle layers (Figure 6.3C, c). One group of cells possessed an ovoid or round soma with robust dendritic arbours with vertical orientations. A second category of PV+ cells were larger in size and primarily located in layers III and V. These cells were multipolar neurons with three to five primary dendrites radiating out from the cell body in all directions.

In a total of 739 cells counted from the eight cortical strip montages of 4 normal human cingulate cortex cases (H111, H115, H120, H330), there were clearly identified single-labelled 204 CB+ cells (27.6%), 529 PV+ cells (71.6%), and 6 double-labelled cells (0.8%). The colocalisation study shows that CB and PV are generally expressed by separate populations of GABAergic neurons, however, a small number of CB+ neurons in layers II and III coexpressed PV in the BA 24 region of the anterior cingulate cortex (Figure 6.3C, c).

6.4.4. Calretinin (CR) / Parvalbumin (PV)

The immunohistochemical staining of CR and PV showed distinct, non-overlapping populations of GABAergic interneurons (Figure 6.3D, d). In the upper layers II and III, the number of CR+ neurons was considerably higher than PV. Conversely, the density of PV+ neurons was considerably higher in the deeper layers V and VI. Also, CR+ neurons were present in a small but reproducible number in layer I. Compared with PV+ neurons, cell bodies stained for CR were in general smaller in size. CR and PV expression patterns were
further differentiated in the neuropil; PV staining fibres were most intense in the middle layers while CR staining was associated with vertical descending fibres from layer II to VI.

In a total of 1122 cells counted from the eight cortical strip montages of 4 normal human cingulate cortex cases (H111, H115, H120, H330), there were clearly identified single-labelled 653 CR$^+$ cells (58.2%), 469 PV$^+$ cells (41.8%), and no double-labelled cells (0%). The diverse staining patterns and the numerical density suggests that the CR$^+$ neurons are distinct from PV$^+$ cells and that they constitute separate subpopulations of GABAergic interneurons in the BA 24 region of the anterior cingulate cortex (Figure 6.3D, d).
**Figure 6.3 Photomicrographs showing the distribution pattern of CB, CR, and PV interneurons in the normal human anterior cingulate cortex (Brodmann area 24)**

This figure shows the distribution pattern and morphology of the different types of interneurons that differentially express calcium-binding proteins, calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) in the anterior cingulate cortex of neurologically normal human brains. The pairs of double-labelled immunofluorescent photomicrographs of the different types of interneurons is shown using sections immunostained for (A, a) mCB / rCB, (B, b) mCB / rCR, (C, c) rCB / mPV, and (D, d) rCR / mPV.

**A-D:** Low magnification photomicrographs of the interneurons in different pairs of combinations in the human anterior cingulate cortex.

**a-d:** High magnification photomicrographs of the representative interneurons in different pairs of combinations in the human anterior cingulate cortex.

**A, a:** Area 24 immunohistochemically stained with mCB / rCB arrangement.

**B, b:** Area 24 immunohistochemically stained with mCB / rCR arrangement.

**C, c:** Area 24 immunohistochemically stained with rCB / mPV arrangement.

**D, d:** Area 24 immunohistochemically stained with rCR / mPV arrangement.

Scale bars: **A-D = 100 μm; a-d = 20 μm**
Figure 6.3
6.4.5. Proportions of the Three Interneuronal Subpopulations in the Anterior Cingulate Cortex of the Human Brain

To assess the relative proportions of the interneuronal subpopulations defined by the three calcium-binding proteins, the cell counts obtained from the double immunofluorescence study above which showed no significant overlap was used to determine the ratio between the three types of non-overlapping interneurons (normalised for the number of cases).

Among 2487 cells counted from 4 normal human brain cases (H111, H115, H120, H330), there were clearly identified single-labelled 359 CB⁺, 1124 CR⁺, and 1004 PV⁺ cells. The relative proportions of these cells (CB, CR, PV) were 14.4%, 45.2%, and 40.4%, respectively (Figure 6.4).
6.5. Discussion

The present chapter provides qualitative immunohistochemical description of the distribution pattern and morphology of the GABAergic interneurons in the anterior cingulate cortex (BA 24) of the human brain. In addition, colocalisation and the relative quantitative proportions of the interneurons defined by the three calcium-binding proteins, CB, CR, and PV was investigated using double immunofluorescent labelling and confocal laser scanning microscopy methods. In general, the results showed comparable staining patterns to several other investigations on the laminar distribution and morphology of interneurons in the cingulate and prefrontal cortices of the monkey and human (Hof & Nimchinsky, 1992; Conde, et al., 1994; Gabbott & Bacon, 1996; Nimchinsky et al., 1997). Thus, what follows is a brief summary of the laminar distributions and cellular morphologies of neurons that express CB, CR, and PV in the human anterior cingulate cortex and their possible functional roles in the cortical circuitry.

6.5.1. Cortical GABAergic Interneurons identified by Calcium-Binding Proteins

Calbindin-D28k

Calbindin-D28k (CB) is a 28 kDa protein that belongs to the EF-hand family and has been known to buffer intracellular calcium (Kojetin, et al., 2006; Schwaller, 2009). In the brain, CB has been shown to decrease calcium influx through L-type calcium channels and modulate intracellular calcium homeostasis (Lee, et al., 2006; Christakos, et al., 2007). CB was identified as a protein of 28 kDa in the rabbit cerebellum and kidney (Celio, et al., 1990). The western blot analysis using homogenates from the normal human anterior cingulate cortex showed a clear single band at approximately 28 kDa (immediately beneath the 30 kDa ladder band) which indicates the size and specificity of the antibody (Figure 6.1A).

The immunohistochemical staining results showed that CB labelled nonpyramidal neurons that were mainly confined to upper layers II and III. These neurons have bipolar or spindle shaped cell bodies with vertically oriented axonal and dendritic branches that traverse horizontally through the cortical layers. These neurons resembled double bouquet cells that
were described previously in Golgi studies (Somogyi & Cowey, 1981, 1984). The *double bouquet* cells are known to be distributed at regular intervals and produce long bundles of axons (mean spacing of \(~30 \mu m\)) and are therefore assumed to constitute a key inhibitory component of the minicolumnar organisation of the cortex (DeFelipe, et al., 1990; del Rio & DeFelipe, 1997a; Mountcastle, 1997; Buxhoeveden & Casanova, 2002; Mountcastle, 2003; Horton & Adams, 2005; Yanez, et al., 2005; Rockland, 2010). In addition, in the present study a small population of multipolar shaped neurons were found in layers II/III and layer V. These cells are likely to be *Martinotti* and *neurogliaform* cells identified in Golgi-prepared studies (Fairén, et al., 1984; Jones, 1984b). The *Martinotti* cells are found in layers II-VI and form axons that project to layer I, where they exert an inhibitory input on the dendritic tufts of pyramidal neurons (Kawaguchi & Kubota, 1997). These neurons also form inhibitory synapses on the dendrites of neighbouring pyramidal neurons (Wang, et al., 2004; Silberberg & Markram, 2007). The *neurogliaform* cells are small cells that form dense local axonal fields and radiate short, fine dendrites with minimal branching (Markram, et al., 2004). The nonpyramidal neurons labelled with CB described in the present study were also found in previous studies in the monkey and human cingulate cortex (Hof & Nimchinsky, 1992; Conde, et al., 1994; Gabbott & Bacon, 1996; Nimchinsky, et al., 1997). The mean somatic size of CB\(^+\) neurons in the monkey medial prefrontal cortex (areas 24, 25 and 32) was 11.9 \(\mu m\) (range: 5.2 to 25.3 \(\mu m\)) (Gabbott & Bacon, 1996). This was similar to the size (6-28 \(\mu m\)) observed in the present immunohistochemical studies (Section 6.3.1). Although CB antibody was expressed mainly by nonpyramidal neurons, a smaller population of pyramidal cells in layer III were also lightly immunoreactive for CB. This observation has also been reported previously in the monkey and human (Conde, et al., 1994; Gabbott & Bacon, 1996; Nimchinsky, et al., 1997). The functional significance is unclear, however, this may suggest that there is a chemically distinct population of pyramidal cells in layers III and V of the anterior cingulate cortex in the human brain.

**Calretinin**

Calretinin (CR) is a calcium buffering protein (Resibois & Rogers, 1992; Andressen, et al., 1993) that provides protection against calcium-induced excitotoxicity in the brain (Lukas & Jones, 1994; D'Orlando, et al., 2002), and is also implicated as a modulator for excitability in neurons (Gall, et al., 2003). CR was identified as a protein of 29 kDa in the rat brain.
(Pochet, et al., 1985). The results in the present human study showed a similar clear protein band at approximately 29 kDa from the western blot analysis using homogenates from the normal human anterior cingulate cortex (Figure 6.1B).

The immunohistochemical staining patterns showed that CR$^+$ cells were found throughout all cortical layers (I-VI) but were mainly confined to layers II and III. These cells were nonpyramidal neurons with bipolar or spindle shaped cell bodies that resembled bipolar or double bouquet cells (Peters, 1984a; Somogyi & Cowey, 1984). The bipolar cells occur in layers II-VI and send long axons which extend vertically up and down towards layer I and VI. These cells contain inhibitory GABA neurotransmitter but are also known to be excitatory by releasing vasoactive intestinal polypeptide (VIP) neuropeptide (Peters & Harriman, 1988; Peters, 1990; Markram, et al., 2004). In addition, the CR$^+$ neurons were found in layer I, and are presumed to represent Cajal-Retzius cells (Marín-Padilla, 1984, 1998). The Cajal-Retzius cells are small multipolar or bipolar inhibitory neurons in layer I and target the terminal tufts of pyramidal neurons (Hestrin & Armstrong, 1996). The cells labelled with CR described in the present study were also observed in the monkey and human cingulate cortices (Hof & Nimchinsky, 1992; Conde, et al., 1994; Gabbott & Bacon, 1996; Nimchinsky, et al., 1997). In the monkey medial prefrontal cortex (areas 24, 25 and 32), Gabbott and Bacon (1996) have found that the mean somatic size of CR$^+$ neurons was 9 μm (range: 5.1 to 13.4 μm). This was similar to the size (5-15 μm) observed in the immunohistochemical studies indicated above (Section 6.3.1). Also, studies in the anterior cingulate cortex of 13 primate species have shown that pyramidal neurons in the superficial part of layer V were immunoreactive for CR. These were found in numerous numbers in the hominids, especially in chimpanzees, gorillas, and high numbers in humans (Hof et al., 2001). These cells were not often observed in the posterior regions of the cingulate gyrus.

The authors concluded that CR$^+$ pyramidal neurons in the anterior region of the cingulate cortex may form a functionally discrete population to integrate complex emotional behaviours subserved by the anterior cingulate cortex which have developed and adapted in the great ape lineages, i.e., these cells represent pyramidal neurons in the cingulate motor areas (CMAs) that are inherent in the anterior cingulate cortex which regulate specific motor functions such as control of vocalisation, facial expression, or autonomic function. Another study also indicated the existence of lightly stained CR$^+$ pyramidal neurons in layer V and VI of the human cingulate cortex (areas 24 and 25) (Nimchinsky, et al., 1997). Both of these
studies noted a decrease in the density of the CR⁺ pyramidal neurons in the posterior regions of the cingulate cortex, indicating that these neurons are not directly involved in somatic motor function. The immunohistochemical results of the present study have not observed the presence of large pyramidal neurons, although a small population of lightly stained medium sized pyramidal neurons in layers III and V were detected in some cases (H120, H136, H139) in the anterior cingulate cortex of the human brain.

Parvalbumin

Parvalbumin (PV) is a small protein (9-12 kDa) that is known to modulate intracellular calcium levels in neurons (Augustine, et al., 2003; Schwaller, 2009). The western blot analysis showed a single band below the 20 kDa ladder at approximately 10-12 kDa using protein homogenates from the normal human anterior cingulate cortex (Figure 6.1C).

The immunohistochemical staining patterns showed that PV labelling was mainly found throughout layers II-VI, with the predominance of these cells in the lower part of layer III to the deeper layers V and VI. Intense neuropil staining was found in the middle layers III-V. The small bipolar and multipolar shaped cells with vertically oriented PV immunoreactive fibres may represent axonal cartridges or terminals of chandelier cells that target axon initial segments of layer III and V pyramidal neurons (Szentagothai & Arbib, 1974; Fairén & Valverde, 1980; Peters, 1984b; DeFelipe, 1997; Somogyi, et al., 1998; Zhu, et al., 2004). In addition to the chandelier cells, medium to large sized neurons were also found scattered throughout layers III and V and these cells are likely to represent basket cells that form axonal arborisations with basket-like appearance around pyramidal cell soma (Jones & Hendry, 1984). These cells are specialised in targeting the cell soma and proximal dendrites of the pyramidal neurons to exert distinctive inhibitory action (Kisvarday, et al., 1993; Kisvarday, et al., 1994; Okhotin & Kalinichenko, 2002; Wang, et al., 2002; Markram, et al., 2004). Both of these cell types were found in the monkey and human anterior cingulate cortex using PV antibody in previous reports (Hof & Nimchinsky, 1992; Gabbott & Bacon, 1996; Kalus & Senitz, 1996; Nimchinsky, et al., 1997). The mean somatic size of PV⁺ neurons in the monkey medial prefrontal cortex (areas 24, 25 and 32) was 12.9 μm (range: 6.1 to >30 μm) (Gabbott & Bacon, 1996). The immunohistochemical results in the present
study (Section 6.3.1) observed a slightly larger size of PV$^+$ neurons (12 to >30 μm) in the anterior cingulate cortex of the human brain.

Figure 6.5 Schematic diagram to show target specificity of cortical GABAergic interneurons and pyramidal neurons (Pyr) in the anterior cingulate cortex

6.5.2. Colocalisation of the Three Types of Calcium-Binding Proteins

The distribution pattern and morphological characteristics of GABAergic interneurons using double immunofluorescent labelling showed that CB, CR, and PV were expressed in well defined, non-overlapping subpopulations of interneurons in the human anterior cingulate
cortex (Figure 6.3). There were no colocalisation between the three markers, except for a small population of cells (0.8%) which showed coexpression of both CB and PV markers (Section 6.4.3). The relative proportions of CB, CR, and PV were 14.4%, 45.2%, and 40.4%, respectively (Section 6.4.5; Figure 6.4). Previous colocalisation studies of the GABAergic interneurons observed no indication of overlap between the three calcium-binding proteins in the monkey prefrontal cortex (Conde, et al., 1994; Zaitsev et al., 2005). The calcium-binding proteins, CB and CR are structurally closely related (Rogers, 1987), although CB and CR have been shown to possess different structural domains (Palczewska, et al., 2003). The colocalisation studies have shown a small percentage of overlap between CB and CR (~4-11%) in the cell somata in the human temporal cortex (del Rio & DeFelipe, 1996, 1997b), despite both antibodies showing similar labelling of vertically oriented axon bundles that are formed by neurons that resemble double bouquet cells. This indicates that the two calcium-binding proteins are differentially expressed only in the fibre bundles within the same class of interneurons, i.e., double bouquet cells. These observations were also observed in the rat cortex (Rogers, 1992; Rogers & Resibois, 1992) and in the human medial prefrontal and temporal cortices (Ferrer, et al., 1992; del Rio & DeFelipe, 1995; Fonseca & Soriano, 1995; del Rio & DeFelipe, 1996; Gabbott, et al., 1997).

Few studies have indicated a small overlap between the two markers, CB and PV, e.g., mRNA expression of CB and PV in the rat somatosensory cortex showed some overlapping profiles in basket cells (Wang, et al., 2002), and also PV cells were colocalised with CB in the upper layers II/III (11%) and in layers V/VI (6%) in the rat frontal cortex (Kubota, et al., 1994; Kawaguchi & Kubota, 1997). In addition, a certain population of chandelier cells in the human temporal cortex were colocalised with CB and PV antibodies (del Rio & DeFelipe, 1997a). The colocalisation results of the present study have shown a small overlap (0.8%) between the two markers, CB and PV. This suggests that there is a diverse neurochemical and morphological distinction of the different types of calcium-binding protein expressing interneurons in the mammalian neocortex and that these subsets of interneurons may play specific functional roles in the neocortical circuitry.
6.6. Conclusion

The anterior cingulate cortex in the human shows a diverse population of morphologically and biochemically heterogeneous group of local-circuit GABAergic interneurons. The distribution pattern and morphological characteristics of these interneurons were observed using immunohistochemical analyses with the three calcium-binding protein markers, CB, CR, and PV. These interneurons comprise distinct, non-overlapping populations and form crucial elements in the local cortical microcircuitry which furnishes strategic inhibitory action onto pyramidal neurons as well as shaping disinhibition of other interneurons. This proposed diversity may be relevant in the processing of information that the cingulate cortex conveys especially with the components of the limbic system. Therefore, the distinct subpopulations of CB, CR, and PV interneurons may serve as a means for further improving our knowledge of the detailed cortical circuitry in the healthy state as well as in neurodegenerative disorders involving the cingulate cortex such as Huntington’s disease, which is described in the next chapter (Chapter 7).
CHAPTER 7. RESULTS
INTERNEURONAL CHANGES IN THE
ANTERIOR CINGULATE CORTEX
IN HUNTINGTON'S DISEASE

7.1. Introduction

Huntington’s disease (HD) is a neurodegenerative disorder characterised by a triad of symptoms including motor, cognitive, behavioural abnormalities. The HD patients show widespread disturbances in mood and behaviour symptoms and these often occur in the early stages of HD (Rosenblatt & Leroi, 2000; Paulsen, et al., 2001a; Paulsen, et al., 2001b). Since the anterior cingulate cortex plays a major role in the regulation of limbic associated functions, e.g., emotion, attention, memory and learning processes (Posner, 1995; Vogt, et al., 1995; Mega, et al., 1997; Paus, 2001; Vogt, et al., 2004), it was hypothesised that major cell loss in the anterior cingulate cortex may contribute to mood and behavioural symptom expression in HD.

The pathological hallmark of HD is the major degeneration in the striatum of the basal ganglia and the cerebral cortex (Vonsattel, et al., 2008). The cortical degeneration in HD has been well documented in a number of previous reports (Cudkowicz & Kowall, 1990a; Hedreen, et al., 1991; Sotrel, et al., 1991; Sotrel, et al., 1993; Heinsen, et al., 1994; Jackson, et al., 1995; Macdonald, et al., 1997; Rajkowska, et al., 1998; Gutekunst, et al., 1999; Sapp, et al., 1999; Sieradzan & Mann, 2001; Macdonald & Halliday, 2002; Selemon, et al., 2004; Thu, 2006; Nana, 2009; Thu, et al., 2010). However, while it is agreed that cortical degeneration plays a major role in HD, it is unknown whether there is a differential vulnerability of specific neuronal subtypes, especially the GABAergic interneurons, which are involved in the pathophysiology of HD, and its relation to the variable symptoms.
The local neuronal circuitry is governed by a dynamic interaction between the cortical pyramidal neurons and the local-circuit interneurons. The inhibitory GABAergic interneurons play a key role in the regulation of cortical output and therefore are critical determinants of neural activity in the cortex. Few studies have examined specific cortical pyramidal cell loss in HD. In particular, detailed stereological analyses in our laboratory have shown a significant reduction in SMI32+ pyramidal neurons in HD cases in the human anterior cingulate cortex (Thu, et al., 2010). Interestingly, in this study, the pattern of pyramidal cell loss in the anterior cingulate cortex was highly associated with the symptom heterogeneity in HD, i.e., there was a major loss of pyramidal neurons in the anterior cingulate cortex in HD cases with major mood disorder, but not in HD cases with major motor dysfunction. This shows that the phenotypic variability is related to the variable pattern of pyramidal neuronal degeneration in the anterior cingulate cortex in HD. While specific pyramidal cell loss has been reported in previous studies, detailed studies on the cellular and morphological abnormalities of the inhibitory GABAergic interneurons in the HD anterior cingulate cortex have not been undertaken, and its relation to the variable symptom profile in HD.

Therefore, this chapter investigates the cellular changes of the GABAergic interneurons in Brodmann area 24 (BA 24) of the anterior cingulate cortex in 11 HD (HC60, HC68, HC73, HC79, HC82, HC85, HC93, HC95, HC99, HC101, HC107) and 14 neurologically normal control cases (H108, H110, H111, H112, H115, H118, H120, H121, H127, H131, H132, H136, H139, H330) (for case details, see Chapter 3). In order to study the pattern of interneuron cell loss in the anterior cingulate cortex in HD, the different classes of GABAergic interneurons were first immunohistochemically identified using three calcium-binding proteins, calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) (see previous Chapter 6 for immunostaining patterns in the normal human brain). The immunostained cells were then quantified using unbiased stereological counting methods. The counting results in this chapter are presented as a percentage of the mean number of cells of normal cases with standard error indicated as (%mean ± SEM), and the statistical significance is expressed as *p<0.05, **p<0.01, and ***p<0.001. In addition, morphological changes in these cells were analysed using bright field light microscopy. The pattern of interneuron loss was compared to the (1) pattern of the dominant HD symptom profile, (2) striatal
neuropathological grades and (3) the CAG repeat length in the *HD* gene, post-mortem interval, and age of the individual HD cases.

**7.2. The Pattern of Interneuron Loss in All HD and Normal Control Cases in the Anterior Cingulate Cortex**

The total interneuronal population in the anterior cingulate cortex (BA 24) of 11 HD and 14 matched control cases were determined using stereological counting techniques on sections immunostained for calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV). In the 11 cases of HD brains, a significant overall reduction in the average total number was observed in two interneuron populations (CR and PV) (Figure 7.1). The results showed that when all HD cases were grouped together and compared to normal controls, there was an average non-significant loss of 28% CB$^+$ interneurons (p>0.05; single-factor ANOVA test), however, a significant loss of 34% CR$^+$ interneurons (*p=0.011), and a significant loss of 45% PV$^+$ interneurons was observed (*p=0.019).

In addition to interneuron loss of the two populations (CR and PV) in the 11 HD cases, it was most interesting to note that there was a considerable variation of the total number of interneurons between 11 individual HD cases. As detailed in Figure 7.2, the total loss of CB$^+$ interneurons varied from 73-79% reduction in three cases (HC82, HC85, HC95) to 28-56% loss in four cases (HC79, HC93, HC101, HC107), with four cases (HC60, HC68, HC73, HC99) showing no loss. The total loss of CR$^+$ interneurons varied from 79% loss in one case (HC85) to 21-57% loss in eight cases (HC60, HC79, HC82, HC93, HC95, HC99, HC101, HC107), with two cases (HC68, HC73) showing no loss. The total loss of PV$^+$ interneurons varied from 73-86% loss in five cases (HC79, HC82, HC85, HC95, HC101) to 33-62% loss in four cases (HC60, HC93, HC99, HC107), with two cases (HC68, HC73) showing no loss.

For all stereological analyses of the HD and normal control cases, the average coefficient of error (CE) for the total number of interneurons (N) for each marker in the Nv and Vref methods [i.e., CE(Nv) and CE(Vref)] was always less than 0.10 (see Appendix II). In
individual cases the CE was mostly less than 0.10 and always less than 0.15. This indicates that the estimates of \( N_v \), \( V_{ref} \) and total number \( N \), were generally reliable (i.e., \( CE \leq 0.10 \)). In addition, for all analyses, the observed mean variance of the individual total number estimates (i.e., \( CE^2 \)) was less than half of the observed mean variance of the group (coefficient of variation, \( CV^2 \); \( CV = \frac{SD_{n-1}}{\text{mean}} \)), or \( CE^2/CV^2 < 0.5 \). This indicates that the variability is due to a true difference between cases in the total cell number rather than a lack of precision in the stereological counting methods employed (Gundersen, et al., 1999; West, 2002; Glaser, et al., 2007).
Figure 7.1 Stereological counting of the total number of interneurons in the normal anterior cingulate cortex (BA 24) compared to all HD cases in the human brain

The graph shows the total number of interneurons defined by the three calcium-binding proteins (CB⁺, CR⁺, and PV⁺ interneurons) in the anterior cingulate cortex of normal and Huntington’s disease brains, and expressed as a percentage of normal controls. There was no significant change in the CB⁺ interneurons, although a 28% reduction was observed. There were however significant changes in the mean total numbers of CR⁺ (34% loss, *p=0.011) and PV⁺ (45% loss, *p=0.019) interneurons in all 11 HD cases compared to 14 normal controls in the anterior cingulate cortex.

Figure 7.2 Variation of the total number of interneurons in the anterior cingulate cortex (BA 24) of individual HD cases

The graph shows the variation in the total number of three interneuronal populations between individual HD cases in the human anterior cingulate cortex. The variation between cases was prominent in all three interneurons. In particular, major cell loss was observed in three HD cases (HC82, HC85, HC95) that showed 73-79% CB⁺ interneuron loss, one HD case (HC85) that showed 79% CR⁺ interneuron loss, and five HD cases (HC79, HC82, HC85, HC95, HC101) that showed 73-86% PV⁺ interneuron loss.
7.3. The Pattern of Interneuron Loss Correlated with the Dominant HD Symptom Profile in the Anterior Cingulate Cortex

We next investigated whether the pattern of interneuron cell loss and the variation between individual HD cases were related to the symptom profiles of HD in the anterior cingulate cortex. The 11 HD cases were categorised into three dominant symptom groups: three cases were classified as mainly “motor” (HC68, HC73, HC99); four cases were classified as mainly “mood” (HC82, HC85, HC95, HC101); and four were classified as “mixed” (motor/mood) (HC60, HC79, HC93, HC107) symptoms, and the extent of the interneuron loss in the anterior cingulate cortex between these groups was compared. The 11 HD cases for which we had detailed symptomatology records are listed in Table 3.2, Chapter 3. For more details on the assessment of HD symptomatology, see Chapter 3 and also Tippett and colleagues (2007), and Thu and colleagues (2010).

7.3.1. Stereological Analyses of the Total Number of Interneurons

The total interneuron loss in HD motor cases showed no significant change in all three interneuronal populations in the anterior cingulate cortex compared with normal controls. There was however, a significant loss of interneurons observed in the HD mixed cases in two interneuron populations (CR and PV). There was 23% loss of CB⁺ interneurons, which was not significant (p>0.05), but a significant 44% loss of CR⁺ (**p=0.0034), and 56% loss of PV⁻ (*p=0.013) interneurons was observed. The largest interneuron loss was observed in the HD mood subgroup with all three interneuronal populations showing major losses of 60 to 80%. The counting results showed a major significant 71% loss of CB⁺ (**p=0.00015), 60% loss of CR⁻ (**p=0.00024), and 80% loss of PV⁺ interneurons (**p=0.0009) compared to the normal controls in the mood symptom subgroup (Figure 7.3)

Most importantly, testing for significance of each type of interneuron between symptom subgroups showed that there was a significant difference between the number of interneurons lost in the HD mood and HD mixed subgroups which were severely affected, compared to the interneuron counts which were not affected in the HD motor subgroup. As detailed in Figure 7.4, the results showed that for the total number of CB⁺ interneurons, there was a significant difference between: (1) the HD mood and HD motor groups
(***p=0.00019); (2) between the HD mixed and HD motor cases (*p=0.042); and, (3) between the HD mood and HD mixed cases (*p=0.02). For the numbers of **CR^+ interneurons** there was a significant difference between (Figure 7.4): (1) the HD mood and HD motor cases (***p=0.0006); and, (2) between the HD mixed and HD motor cases (**p=0.0042). Also, the numbers of **PV^+ interneurons** showed a significant difference between (Figure 7.4): (1) the HD mood and HD motor cases (**p=0.0023); and, (2) between the HD mixed and HD motor cases (*p=0.016). These results clearly establish that the significant loss of CB, CR, PV interneurons in the HD mood and HD mixed subgroups contrasts markedly with the lack of any significant interneuron loss in the HD motor symptom subgroup.

The average coefficient of error (CE) for the total number of interneurons [i.e., CE(Nv) and CE(Vref)] was less than 0.10 for all the normal controls, and mostly less than 0.10 and always less than 0.14 for HD cases of the three symptom profiles for all three populations (Appendix III). These data indicate that the estimate of the total interneuron number was in general a reliable estimate (CE ≤0.10). In addition, for the normal controls and the three HD symptom groups, the observed mean variance of the individual total interneuronal number estimates (i.e., CE^2) was less than half the observed mean relative variance of the group (i.e., CV^2), or CE^2/CV^2 <0.5. This indicates that the variability is due to a true difference between cases in total interneuron number, and not the precision of the estimates made with the stereological counting techniques (Gundersen, et al., 1999; West, 2002; Glaser, et al., 2007).

### 7.3.2. Morphological Changes of Interneurons in the HD Anterior Cingulate Cortex

The pattern of degeneration of interneurons in the HD anterior cingulate cortex in symptom subgroups is further illustrated in Figure 7.5. In terms of cell morphology, the interneurons showed marked differences in cell morphology in the different HD cases. In the cases with major “motor” symptoms, all three interneuronal populations (CB^+, CR^+, and PV^+ interneurons) showed only a small variation in cell morphology and the size of the cell soma and dendritic branching closely resembled the normal control cases (Figure 7.5). In contrast,
the HD cases with mainly “mood” and “mixed” symptoms showed dramatic changes in all three interneuronal populations. As illustrated in Figure 7.5, in the **HD mood** cases, the cell density was markedly reduced and the cell soma appeared to be substantially shrunken, and a loss of dendritic processes was observed compared with normal control cases. These changes in the HD mixed cases were generally less advanced than those in the HD mood cases, but pronounced cell dystrophy and truncated proximal dendrites in all three interneuronal populations were observed. The shrinkage of cell bodies and extensive loss of dendrites in the remaining cingulate cortical interneurons suggest that there is an ongoing progressive neuronal dysfunction.

In summary, when the total number of interneurons were quantified and analysed for each of the three dominant HD symptom profiles, there was a major significant interneuron loss of all three populations (CB⁺, CR⁺, PV⁺ interneurons) in the anterior cingulate cortex of HD cases with major “mood” symptoms, and two populations (CR⁺ and PV⁺ interneurons) in the HD “mixed” cases. Furthermore, another important finding is the demonstration of major morphological changes of the surviving cells in the anterior cingulate cortex in HD in the cases showing “mood” and “mixed” symptoms, suggesting that the surviving cortical neurons show major neuronal dysfunction. However, there was no significant reduction of all three interneuronal populations observed in HD cases with mainly “motor” symptoms.
Figure 7.3 Total number of interneurons in the anterior cingulate cortex (BA 24) according to the dominant HD symptom profile

The graph shows the total number of interneurons defined by the three calcium-binding proteins (CB⁺, CR⁺, and PV⁺ interneurons) in the anterior cingulate cortex of normal and Huntington’s disease brains as determined by stereological cell counting methods, and grouped according to the dominant symptom profile. The HD cases with mainly “motor” symptoms showed no significant interneuron loss in all three populations. The HD cases with “mixed” symptoms showed a non-significant loss of CB⁺ interneurons. However, there was significant losses of CR⁺ (44% loss) and PV⁺ (56% loss) interneurons. Major significant loss of all three types of interneurons was observed in the HD “mood” cases. There was a major significant reduction of CB⁺ (71% loss), CR⁺ (60% loss), and PV⁺ (80% loss) interneurons in the HD anterior cingulate cortex specifically in these mood symptom cases.

Table 7.1 Variation in the mean total interneuron number between normal controls and dominant HD symptom subgroups in the anterior cingulate cortex

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HD Motor</th>
<th>HD Mixed</th>
<th>HD Mood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of CB⁺ interneurons (± SEM)</td>
<td>500927 ± 42284</td>
<td>612882 ± 46125</td>
<td>387246 ± 88868</td>
<td>143546 ± 30736</td>
</tr>
<tr>
<td>Total mean number of CR⁺ interneurons (± SEM)</td>
<td>1329161 ± 96473</td>
<td>1512260 ± 287661</td>
<td>738638 ± 102522</td>
<td>538871 ± 102529</td>
</tr>
<tr>
<td>Total mean number of PV⁺ interneurons (± SEM)</td>
<td>702545 ± 79534</td>
<td>821181 ± 250091</td>
<td>310278 ± 70670</td>
<td>143488 ± 22131</td>
</tr>
<tr>
<td>Number of cases (n)</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Percentage loss (%)</td>
<td>-</td>
<td>CB: ↑22%</td>
<td>CB: 23% loss</td>
<td>CB: 71% loss</td>
</tr>
<tr>
<td></td>
<td>CR: ↑14%</td>
<td>CR: 44% loss</td>
<td>CR: 60% loss</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PV: ↑17%</td>
<td>PV: 56% loss</td>
<td>PV: 80% loss</td>
<td></td>
</tr>
</tbody>
</table>

Note: Significant cell loss is indicated in red text.
Figure 7.4 Total number of interneurons in the anterior cingulate cortex (BA 24) according to the dominant HD symptom profile showing statistical difference between HD groups

The graph shows the statistical difference between three HD symptom subgroups and normal controls. The results show a significant difference in the total number of **CB+ interneurons** between: the normal and HD mood group (**p=0.00015**); the HD motor and HD mood groups (**p=0.0002**); the HD motor and HD mixed groups (**p=0.042**); and the HD mixed and HD mood groups (**p=0.02**). There was a significant difference in the total number of **CR+ interneurons** between: the normal and HD mood group (**p=0.00024**); the normal and HD mixed group (**p=0.0034**); the HD motor and HD mood groups (**p=0.0006**); and the HD motor and HD mixed groups (**p=0.0042**). Also, there was a significant difference in the total number of **PV+ interneurons** between: the normal and HD mood group (**p=0.0009**); the normal and HD mixed group (**p=0.013**); the HD motor and HD mood groups (**p=0.0023**); and the HD motor and HD mixed groups (**p=0.016**).
Figure 7.5 Representative photomicrographs showing the morphology of GABAergic interneurons in the anterior cingulate cortex (BA 24) of normal and HD cases of varying symptom profile

The figure shows the morphological changes of the three interneuronal populations (CB, CR, PV) in the anterior cingulate cortex of representative normal and HD cases with different symptom profiles (“motor”, “mixed”, and “mood” symptoms). Note the severe degeneration of the interneurons in the HD mood and HD mixed cases.

A-D: Area 24 immunohistochemically stained for calbindin-D28k (CB) cells in layers II/III in the (A) normal and HD cases of dominant symptom profiles: (B) HD Motor, (C) HD Mixed, and (D) HD Mood cases. (C and D) Shows clear degeneration of CB+ cells in the HD mixed and HD mood cases.

E-H: Area 24 immunohistochemically stained for calretinin (CR) cells in layers II/III in the (E) normal and HD cases of dominant symptom profiles: (F) HD Motor, (G) HD Mixed, and (H) HD Mood cases. (G and H) Shows clear degeneration of CR+ cells in the HD mixed and HD mood cases.

I-L: Area 24 immunohistochemically stained for parvalbumin (PV) cells in layer V in the (I) normal and HD cases of dominant symptom profiles: (J) HD Motor, (K) HD Mixed, and (L) HD Mood cases. (K and L) Shows clear degeneration of PV+ cells in the HD mixed and HD mood cases.

Scale bar: A-L = 50 μm
Figure 7.5

<table>
<thead>
<tr>
<th>Normal</th>
<th>HD Motor</th>
<th>HD Mixed</th>
<th>HD Mood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin-D28k</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>H118 CAG 15/16</td>
<td>HC73 CAG 19/49</td>
<td>HC86 CAG 17/37</td>
<td>HC82 CAG 15/42</td>
</tr>
<tr>
<td>Calretinin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
</tr>
<tr>
<td>H127 CAG 15/17</td>
<td>HC68 CAG 17/42</td>
<td>HC86 CAG 17/37</td>
<td>HC101 CAG 17/44</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>J</td>
<td>K</td>
<td>L</td>
</tr>
<tr>
<td>H120 CAG 18/22</td>
<td>HC73 CAG 19/49</td>
<td>HC83 CAG 20/43</td>
<td>HC85 CAG 24/44</td>
</tr>
</tbody>
</table>

CAG: Cysteine in the CAG Repeat Region
7.4. The Pattern of Interneuron Loss Correlated with HD Striatal Neuropathological Grade in the Anterior Cingulate Cortex

In order to investigate the relationship between the pathology in the striatum and the anterior cingulate cortex, the 11 HD cases were also independently examined by a neuropathologist (Dr. B. Synek) and each case were graded according to the striatal neuropathology using the Vonsattel grading criteria (Vonsattel, et al., 1985; Vonsattel & DiFiglia, 1998). The grades of the 11 HD cases ranged from 1 to 3. There were three HD grade 1 cases (HC68, HC79, HC101), four HD grade 2 cases (HC73, HC82, HC95, HC99), and four HD grade 3 cases (HC60, HC85, HC93, HC107) (case details are listed on Table 3.2, Chapter 3). There was a general trend towards a greater loss of interneurons with increasing neuropathological grade in the anterior cingulate cortex. These results are ranked by HD grade as follows: The HD grade 1 brains showed a non-significant loss of 21% CB⁺, 20% CR⁺, and 33% of PV⁺ interneurons (p>0.05 for all markers) compared to the normal controls. In the HD grade 2 subgroup, there was 30% CB⁺, 24% CR⁺, and 41% PV⁺ interneuron reduction (p>0.05 for all markers). The greatest interneuron reduction was demonstrated in HD grade 3 cases, with 32% loss of CB⁺ (not significant; p>0.05), and significant losses of 54% CR⁺ (**p=0.0046) and 57% PV⁺ (*p=0.038) interneurons (Figure 7.6). In summary, the pattern of average interneuron loss appeared to parallel the overall increasing striatal neuropathological grades and this was most severe in the HD grade 3 compared to the normal control brains in all three interneuronal populations.

The average coefficient of error (CE) for the total number of neurons [i.e., CE(Nv) and CE(Vref)] was mostly less than 0.10, but always less than 0.11 for all normal controls, and for all HD grade subgroups (grade 1-3) in all three populations of interneurons (see Appendix III). These data indicate that the estimate of the total interneuron number was generally reliable (CE ≤0.10). In addition, for the normal and the three HD grade groups, the observed mean variance of the individual total interneuron number estimates (i.e., CE²) was less than half the observed mean relative variance of the group (i.e., CV²), or CE²/CV² <0.5. This indicates that the variability is due to a true difference between cases in total interneuron number, and not due to any lack of precision in the stereological counting methodology employed (Gundersen, et al., 1999; West, 2002; Glaser, et al., 2007).
The graph shows the total number of interneurons defined by the three calcium-binding proteins (CB⁺, CR⁺, and PV⁺ interneurons) in the anterior cingulate cortex of normal and Huntington’s disease brains as determined using stereological counting methods, and grouped according to the different striatal neuropathological grade. In general terms, there is a trend towards a decrease in interneuron number with higher striatal neuropathological grades.

**Table 7.2 Variation in the mean total interneuron number between normal controls and striatal pathological grade subgroups in the anterior cingulate cortex**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HD Grade 1</th>
<th>HD Grade 2</th>
<th>HD Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of CB⁺ interneurons (± SEM)</td>
<td>500927 ± 42284</td>
<td>395186 ± 174702</td>
<td>352450 ± 152874</td>
<td>341614 ± 121383</td>
</tr>
<tr>
<td>Total mean number of CR⁺ interneurons (± SEM)</td>
<td>1329161 ± 96473</td>
<td>1058800 ± 411999</td>
<td>1010282 ± 314436</td>
<td>607322 ± 161539</td>
</tr>
<tr>
<td>Total mean number of PV⁺ interneurons (± SEM)</td>
<td>702545 ± 79534</td>
<td>472722 ± 387590</td>
<td>413368 ± 215123</td>
<td>301742 ± 77822</td>
</tr>
<tr>
<td>Number of cases (n)</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Percentage loss (%)</td>
<td>-</td>
<td>CB: 21% loss</td>
<td>CB: 30% loss</td>
<td>CB: 32% loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CR: 20% loss</td>
<td>CR: 24% loss</td>
<td>CR: 54% loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PV: 33% loss</td>
<td>PV: 41% loss</td>
<td>PV: 57% loss</td>
</tr>
</tbody>
</table>

*Note: Significant cell loss is indicated in red text.*
7.5. The Pattern of Interneuron Loss Correlated with CAG Repeat Length, PMI, and Age in the HD Anterior Cingulate Cortex

We next investigated whether the total interneuron loss was correlated with the CAG repeat lengths in the *HD* gene, post-mortem interval, and age of the three interneuronal populations (CB, CR, PV) in 11 HD cases in the anterior cingulate cortex (Figure 7.7).

*CAG repeat length*

As shown in Figure 7.7 (panel A, D, G), there were no significant correlations between the total number of interneurons and the CAG repeat length in HD, although there appeared to be a general trend towards a decrease in interneuron number with increasing number of CAG repeats. A Pearson regression statistical analysis demonstrated that there was no significant deviation in the CB+ (r²=0.1035, p=0.3346) and CR+ (r²=0.1686, p=0.2097), and PV+ (r²=0.182, p=0.1908) interneurons.

*Post-mortem interval*

No significant correlation was found between the total interneuron number and PMI of the CB+ (r²=0.034, p=0.5875), CR+ (r²=0.126, p=0.2843), and PV+ (r²=0.0646, p=0.4508) interneurons in the 11 HD cases in the anterior cingulate cortex (Figure 7.7, panel B, E, H).

*Age*

There was no significant correlation between the total interneuron number and age at death of the CB+ (r²=0.000001, p=0.9976), CR+ (r²=0.024, p=0.647), and PV+ (r²=0.0021, p=0.8926) interneurons in the HD anterior cingulate cortex (Figure 7.7, panel C, F, I).
Figure 7.7 Correlation between the total number of interneurons with CAG repeat length, post-mortem interval and age in the anterior cingulate cortex (BA 24) of HD cases

The figure shows the correlation of the total interneuron number with CAG repeat length, post-mortem interval, and age of the 11 HD cases in the anterior cingulate cortex. There were no significant correlations between the total interneuron number and CAG repeat length, post-mortem interval and age in the HD cases used in this study.
7.6. Discussion

The present chapter provides a detailed account of the degeneration of the GABAergic interneurons in BA 24 of the anterior cingulate cortex in HD using immunohistochemical and unbiased stereological counting techniques. There have been no previous detailed studies on the GABAergic interneurons in BA 24 of the anterior cingulate cortex in HD. The results of this study have clearly demonstrated that there was an extensive interneuron cell loss in two interneuron populations (CR and PV) in the anterior cingulate cortex in all HD cases grouped together (n=11) compared to the neurologically normal controls (n=14). There was a non-significant loss of 28% CB+ interneurons (p>0.05), however, a significant loss of 34% CR+ interneurons (*p=0.012), and a significant loss of 45% PV+ interneurons (*p=0.019) was observed (Figure 7.1).

Most importantly, in addition to the overall interneuron cell loss, there was an observation of considerable variation in the extent of interneuron loss in the anterior cingulate cortex between HD cases (Figure 7.2). This finding prompted the hypothesis that the variable pattern of interneuron loss in the anterior cingulate cortex may be related to mood and behavioural symptoms of HD. Indeed, a previous study on the anterior cingulate cortex in HD in our laboratory has shown that there was a major cell loss of all neuronal types (NeuN+ cells) and a subpopulation of pyramidal neurons (SMI32+ pyramidal neurons) in the cingulate cortex in HD cases that showed major “mood” symptoms, while cases characterised by “motor” symptoms showed minimal cell loss in this region, even between cases of the same neuropathological grade (Thu, 2006; Thu, et al., 2010). This was the first type of study which demonstrated that phenotypic variability in HD is related to the variable neuronal degeneration in the anterior cingulate cortex. This suggests that a heterogeneous pattern of cortical cell loss is a key feature of the neuropathology of HD and that the variable neuronal loss in this cortical region is associated with the pattern of variable symptoms in HD. The results presented in this chapter also showed a differential pattern of GABAergic interneuron loss which correlates with the symptom profile of HD in the anterior cingulate cortex. In particular, there was a major loss of all three GABAergic interneurons (CB, CR, PV) in the HD cases with a major mood phenotype.
In addition, an association between the pattern of interneuron loss with striatal neuropathological grades, CAG repeat length, post-mortem interval, and age was also investigated for each HD case. These novel findings are of major importance in providing a better understanding on the pathophysiological basis of the variable clinical symptomatology of HD and may also provide a better understanding of the structural-functional relationships in the human cerebral cortex. These various findings are discussed in detail below.

7.6.1. The Relation between the Extent of Interneuron Loss and the Dominant Symptom Profile of HD in the Anterior Cingulate Cortex

Huntington’s disease patients suffer from a wide range of mood and behavioural disturbances. The anterior cingulate cortex forms a major component of the limbic system as well as having extensive connections with the amygdala, ventral striatum, autonomic brainstem motor nuclei and the hypothalamus. These direct relationships indicate that this region is responsible for regulating emotion, mood, autonomic and endocrine control (MacLean, 1993; Devinsky, et al., 1995; Mega, et al., 1997; Morecraft & Van Hoesen, 1998; Bush, et al., 2000; Paus, 2001; Vogt, et al., 2004; Vogt, et al., 2005). Thus, it was hypothesised that the greatest cell loss in the anterior cingulate cortex may occur in cases with predominant mood disorder. To test whether the pattern of interneuron loss in the anterior cingulate cortex was related to the variable symptom expression in HD, the blinding of the clinical and anatomical assessments was removed, and the average total interneuron loss of the cases in each of the three phenotypic HD subgroups (motor, mood and mixed groups) was compared. These 11 HD cases were grouped according to their symptoms regardless of their “striatal” neuropathological grade and were compared to the normal controls (a statistical inference of symptom vs. grade is outlined under methodological considerations in Section 8.2 in the General Discussion, Chapter 8).

The results of this study have demonstrated a major significant reduction of interneurons in the anterior cingulate cortex in the HD brain (Figure 7.1). Comparison of the total interneuron loss in the groups comprising the three symptom profiles in the anterior cingulate cortex showed an association between the pattern of interneuron loss with the
variable symptom phenotype in all three interneuron populations. The most significant changes were observed in the HD cases classified as “mood” symptoms, and the results showed that there was a major significant interneuron loss in all three populations, with an average total loss of 71% CB⁻ interneurons (***p=0.00012), 60% loss of CR⁺ interneurons (***p=0.00024), and 80% loss of PV⁺ interneurons (***p=0.0009). The total interneuron loss in the HD mixed cases showed a non-significant 23% loss of CB⁻ interneurons, however, a significant loss of 44% CR⁺ (**p=0.0034), and a significant loss of 56% PV⁺ interneurons (*p=0.013) was observed. It was most interesting that the results showed no significant interneuron loss (in all three populations) in HD cases in the “motor” symptom group in the anterior cingulate cortex (Figure 7.3).

In addition, there was a significant difference between the numbers of interneurons between different symptom subgroups which further established the major reductions of interneurons in the HD mood and HD mixed subgroups (Figure 7.4). For the total number of CB⁺ interneurons, there was a significant difference between: (1) the HD mood and HD motor cases (***p=0.0002); (2) between HD mood and HD mixed cases (*p=0.02); and, (3) between HD mixed and HD motor cases (*p=0.042). In addition, the total number of CR⁺ interneurons showed significance between: (1) the HD mood and HD motor cases (***p=0.0006); and, (2) between HD mixed and HD motor cases (**p=0.004). The total number of PV⁺ interneurons also showed a significant difference between: (1) the HD mood and HD motor cases (**p=0.002); and, (2) between HD mixed and HD motor cases (*p=0.016) (Figure 7.4). These results represent a major loss of all three CB⁺, CR⁺, and PV⁺ interneurons in the HD mood and HD mixed cases in the anterior cingulate cortex, which clearly establishes that the loss of these interneurons contrasts significantly with no interneuron reduction observed in the HD motor cases.

The results of the present study clearly demonstrate that neurodegeneration occurs in the anterior cingulate cortex in HD. This is further supported by evidence from previously reported functional imaging, transgenic HD mice, and post-mortem studies in the cingulate cortex (Sotrel, et al., 1991; Mayberg et al., 1992; Jackson, et al., 1995; Davies et al., 1999; Turmaine et al., 2000; Thu, et al., 2010). In particular, this is the first study to show that there is a major cell loss of all three interneuronal subtypes (CB, CR, PV), especially in
cases with major mood dysfunction implying that the GABAergic interneurons in the anterior cingulate cortex play an important role in the cortical pathogenesis of HD. Interestingly, detailed stereological cellular analyses in our laboratory (Thu, 2006; Thu, et al., 2010) on the same HD cases to the present study have shown that there was a clear correlation between the loss of SMI32⁺ pyramidal neurons (40% loss) in the anterior cingulate cortex with mood symptomatology, with no loss in the HD motor cases. This suggests that mood symptomatology which characterises HD is associated with the extent of pyramidal cell loss as well as CB⁺, CR⁺, and PV⁺ interneurons in the anterior cingulate cortex (Figure 7.8).

Figure 7.8 Schematic diagram showing the association between the patterns of pyramidal and interneuron cell loss in the HD anterior cingulate cortex according to the dominant symptom profiles

The heterogeneity in clinical symptomatology that characterises HD is associated with the pattern of SMI32⁺ (pyramidal cell loss (40% loss) (Thu et al., 2010) and loss of CB⁺ (71% loss), CR⁺ (60% loss) and PV⁺ (80% loss) interneurons only in the HD mood cases in the anterior cingulate cortex, but not in the HD motor cases.
As shown in Chapter 6, the synaptic connectivity of the various morphological forms of inhibitory local-circuit interneurons are known to contact distinct regions of the cortical pyramidal neurons, i.e., soma, dendrites, and axons in layer-specific spatial characteristics (Reyes et al., 1998; Larkum et al., 1999b; Thomson & Bannister, 2003). In addition, these actions can be time-dependent thus resulting in differential activation of neurons in the cortex (Thomson & Deuchars, 1994; Galarreta & Hestrin, 1998). The calcium-binding proteins CB and CR are mainly expressed by axo-dendritic targeting nonpyramidal neurons which are located to influence dendritic processing of pyramidal neurons such as generation and propagation of calcium transients (Traub, 1995; Larkum et al., 1999a) and integration of synaptic inputs (Markram, et al., 2004). These cells are found throughout layers II-VI, but are mainly occupied in the upper cortical layers II and III and therefore thought to be involved in cortico-cortical processing of pyramidal neuron output. The calcium-binding protein PV is expressed in nonpyramidal neurons that make axo-somatic contacts with pyramidal neurons (Jones & Hendry, 1984; Kawaguchi & Kubota, 1997; Kawaguchi & Kondo, 2002; Okhotin & Kalinichenko, 2002) which exert a powerful inhibitory action to shape the action potential discharge of pyramidal neurons found in layers III and V (Buhl et al., 1995; Miles et al., 1996; Wang, et al., 2002). These cells are also involved in temporal phasing and synchronisation of neuronal activity (Cobb et al., 1995; Pouille & Scanziani, 2001). PV also labels cells which form unique axo-axonic synapses in the axon initial segment (AIS) region of pyramidal neurons (Conde, et al., 1994; del Rio & DeFelipe, 1997a; Kawaguchi & Kubota, 1997; Zhu, et al., 2004). Thus, PV+ cells are strategically important for the control of pyramidal neuron excitability and impart powerful inhibitory action.

Hence, inhibition of pyramidal cells by various types of interneurons is a critical determinant in shaping the output activity of the cerebral cortex (Figure 7.8). Specifically, the loss of the CB+, CR+, and PV+ interneurons in the HD mood cases in the anterior cingulate cortex suggests that the inhibitory actions of these interneurons are severely affected and this may disrupt the complex pattern of connectivity between cortico-cortical processing as well as in the cortico-striatal pathway. The loss of inhibition by these interneurons may lead to hyperexcitability of the pyramidal neurons which may contribute to the striatal excitotoxic processes in HD (Beal, 1994; Sieradzan & Mann, 2001; Cepeda et al., 2007).
It is also interesting to note in a pathological study conducted by Tippett and colleagues (2007) in the human striatum of HD, where the authors found that HD cases with greater levels of mood disturbance showed greater damage to the striosome compartment. The striosome compartment has been shown to receive inputs from the “limbic” related cortical areas in rodent (Donoghue & Herkenham, 1986; Gerfen, 1989) and primate studies (Eblen & Graybiel, 1995). This suggests that the variable pattern of symptomatology in HD is associated with the degeneration in different compartments of the striatum. Since the anterior cingulate cortex is closely associated with limbic functions, and has extensive connections with the striosome compartment of the striatum (Eblen & Graybiel, 1995; Holt, et al., 1997), degeneration in the anterior cingulate cortico-striate projection may serve to induce “trans-neuronal” degeneration in the striosome compartment and so contribute to the pathogenic mechanisms underlying the neural basis of symptom heterogeneity in HD.

Interestingly, alterations in the expression of the three calcium-binding proteins (CB, CR, PV) have been implicated in mood-related disorders such as epilepsy, schizophrenia, bipolar disorder and depression as well as other neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease (Baimbridge, et al., 1992; Heizmann & Braun, 1992; Andressen, et al., 1993; Schwaller, et al., 2002; Schwaller, 2009). In particular, parvalbumin (PV) expressing GABAergic interneurons have been reported to be decreased in the anterior cingulate and prefrontal cortices of patients with schizophrenia and bipolar disorder (Beasley & Reynolds, 1997; Benes & Berretta, 2001; Beasley et al., 2002; Hashimoto et al., 2003). In addition, the chandelier cells which mainly express PV have been observed to be particularly reduced at epileptic foci in both animal models and epileptic patients and thus are proposed to form important components in the establishment of human temporal lobe epilepsy (DeFelipe, 1999). It is interesting to note that the present study also found that the PV⁺ interneurons were those most affected in HD cases with major mood disorder in the cingulate cortex, which suggests that the cortical GABAergic PV⁺ interneurons may especially contribute to abnormalities in the function of limbic-related cortical circuits in HD and neuropsychiatric diseases.

In conclusion, this is the first study to demonstrate that there is a major significant loss of all three interneuron populations (CB, CR, PV) in the anterior cingulate cortex and that the
extent of cell death of GABAergic interneurons correlates with the mood symptomatology of HD, suggesting that interneuron loss in this region may play a major role in the cortical pathogenesis which leads to mood abnormalities in HD.

### 7.6.2. The Relation between the Extent of Interneuron Loss and the Striatal Neuropathological Grades of HD in the Anterior Cingulate Cortex

In this study, the 11 cases of HD were also categorised into three groups based on the Vonsattel neuropathological grades - grade 1 (three cases), grade 2 (four cases) and grade 3 (four cases) for the data analysis. The results of our detailed study using stereological counting methods demonstrate that there was a greater loss of interneurons with increasing striatal neuropathological grade (Figure 7.6). On average, in HD grade 1 cases, there was 21% loss of CB+, 20% loss of CR+, and 33% loss of PV+ interneurons. In HD grade 2 cases, there was reductions of 30% CB+, 24% CR+, and 41% PV+ interneurons. The greatest reduction was observed in HD grade 3 cases, with 32% loss of CB+, and, significant losses of 54% CR+ and 57% PV+ interneurons. This suggests that neuronal degeneration in the anterior cingulate cortex parallels degeneration in the striatum and that the neuropathology in these regions may be closely linked (see later under Chapter 8 General Discussion). These findings are generally consistent with previous studies on the human anterior cingulate cortex in our laboratory, where neuronal loss was associated with increasing striatal neuropathological grades (Thu, 2006; Thu, et al., 2010). In particular, the number of SMI32+ pyramidal neurons decreased with higher striatal HD grades which may contribute to dysfunction in other cortical and subcortical brain regions. In conclusion, the finding in the present study shows an overall reduction of GABAergic interneurons in the anterior cingulate cortex with greater loss of cells in higher HD striatal grades which suggests that there may be a link between cortical and striatal degeneration.

### 7.6.3. Correlation between Interneuron Loss and the CAG Repeat Length, Post-Mortem Interval, and Age in the HD Anterior Cingulate Cortex

The results from the stereological counting analysis was also investigated with regard to the CAG repeat length, post-mortem interval, and age of HD cases which may account for the
variable differences observed in the human brain. Striatal pathology has been shown to be closely associated with the number of CAG repeats in HD (Penney, et al., 1997; Vonsattel, et al., 2008), however, it is not yet clear if there is a link between CAG repeat length and cortical pathology, especially with interneuron loss. The correlation between the length of **CAG repeat** and the total number interneurons in HD cases showed no significant correlation using a Pearson regression analysis at the 5% level for all three interneuronal populations (Figure 7.7). However, there was a general trend towards a decreasing number of interneurons with increasing CAG repeat length. The analysis of **post-mortem interval (PMI)** showed no overall correlation between the total interneuron number and PMI in the anterior cingulate cortex in HD cases. This suggests that the differences between the interval between death and time of tissue processing, and the possible modifications in neuronal immunoreactivity was not accountable in this study. Pakkenburg and Gundersen (1997) have found an association between the decreasing number of cortical neurons with increasing age. However, the analysis of **age at death** in this study showed no overall correlation between the total interneuron number and age in the anterior cingulate cortex in HD cases. In line with the present study, a previous study on the age-related changes of the three calcium-binding proteins (CB, CR, PV) in the human anterior cingulate cortex also showed no significant reductions in the number of interneurons immunoreactive for CB, CR, and PV compared to normal controls, although a trend towards a decrease in the number of these neurons with increasing age was observed (Bu, et al., 2003). These outcomes indicate that in general, the results between the total interneuron number with the symptom profile and striatal disease grade described above can be regarded as separate from the influence of CAG repeat length, post-mortem interval, and age of the HD cases used in this study.

### 7.7. Conclusion

The results of the present study demonstrated a significant overall reduction of GABAergic interneurons that express calcium-binding proteins in the anterior cingulate cortex in HD. The major findings of this study is the unique and striking correlation between the variable pattern of GABAergic interneuron loss in HD cases with major mood disorder in the anterior cingulate cortex, a region that is involved in mood and behaviour. The results demonstrated a significant loss of all three interneuronal populations (CB, CR, PV) in HD
mood cases in the anterior cingulate cortex in HD. This observation suggests that the differential pattern of interneuron loss in the cingulate cortex may influence variable pathways of cortical pathogenesis which contribute greatly to the symptom heterogeneity in HD. More importantly, there was lack of any significant loss of GABAergic interneurons in HD cases with major motor disorder, which may well correlate to lack of any dominant mood and behavioural abnormalities in these particular cases, suggesting that GABAergic interneurons are critical determinants in the generation of mood phenotype in HD.
CHAPTER 8. GENERAL DISCUSSION

8.1. Introduction

The results of the present study provide the first comprehensive and detailed research on the deficits of GABAergic interneurons in the cerebral cortex in Huntington’s disease (HD). The human cerebral cortex is a large and complex structure. This study focused on two functionally distinct regions of the cortex, comprising the primary motor cortex which is involved in the regulation of movement, and the anterior cingulate cortex which is a region involved in mood and behaviour. The overall goal of this study was to investigate the variable pattern of interneuron cell loss in the two cortical regions in HD and to compare these findings with: (1) the pattern of symptomatology in HD; (2) the striatal neuropathological grade; and (3) the CAG repeat length, post-mortem interval and age. To carry out this study, immunohistochemistry and unbiased stereological cell counting techniques were employed. The results in Chapters 4 and 6 investigated the immunostaining patterns of the GABAergic interneurons defined by calcium-binding proteins (calbindin-D28k, calretinin, parvalbumin) in the normal human brain of the primary motor and anterior cingulate cortices, respectively; and Chapters 5 and 7 investigated the cellular changes of the GABAergic interneurons in the primary motor and anterior cingulate cortices of the HD human brain compared to the normal control brain using stereological counting methods. The pattern of interneuron cell loss in each case was then correlated to the variable symptom profiles of HD determined by retrospective analysis of clinical symptom data collected from patients, family members and clinical records, and striatal neuropathological grade and CAG repeat length determined by neuropathologists. The overall findings and the key results are discussed in this chapter detailed below.

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 (Huntington's Disease Collaborative Research
The disease is clinically characterised by involuntary movements, cognitive deficits, and mood and behavioural changes. Despite the single gene aetiology of HD, there is clear and considerable phenotypic variation in the pattern of symptoms exhibited by each individual during the course of the disease. 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). The major neuronal degeneration in HD occurs principally in the striatum of the basal ganglia and the cerebral cortex (Vonsattel & DiFiglia, 1998; Vonsattel, et al., 2008). Recent evidence suggests that the variation in clinical symptoms in HD is strongly associated with the variable neurodegeneration in the striatum and in different functional regions of the cerebral cortex. For example, in the cerebral cortex, recent neuroimaging studies showed discrete patterns of cortical grey matter thinning and dysfunction which were associated with different clinical symptoms of HD such as variable cognitive and motor deficits (Sax, et al., 1996; Kassubek, et al., 2004; Rosas, et al., 2005; Montoya, et al., 2006; Rosas, et al., 2008). In addition, extensive cortical pathological studies in our laboratory have demonstrated an association between the variable pattern of cell loss in the motor and cingulate cortices to the dominant motor and mood symptom profile of HD, respectively (Thu, 2006; Thu, et al., 2010). In particular, the number of SMI32+ pyramidal neurons was most affected in the primary motor cortex (BA 4) in patients with major motor disorder, while the number of SMI32+ pyramidal neurons was significantly reduced in the anterior cingulate cortex (BA 24) in patients with major mood disorder (Thu, et al., 2010). These studies show that the different patterns of degeneration in the cerebral cortex may contribute significantly to the symptom heterogeneity in HD.

The neuronal population in the cerebral cortex is principally made up of two main functional classes: the pyramidal neurons; and the local-circuit interneurons (DeFelipe & Farinas, 1992; DeFelipe, 2002). The cortical pyramidal neurons are projection neurons that use excitatory glutamate transmitter while local-circuit interneurons use the inhibitory GABA neurotransmitter and act locally to modulate the activity of pyramidal neurons. The question arises then, whether the cell loss in the cerebral cortex in HD comprises a selective or differential loss of pyramidal neurons, inhibitory interneurons, or both neuronal types? Previous studies in our laboratory have shown a major significant loss of pyramidal neurons
in the cerebral cortex (Nana, 2009; Thu, et al., 2010). However, detailed cellular changes of the GABAergic interneurons in the cortex have not been undertaken in HD. This study is based on the hypothesis that HD may involve an abnormality in one of the key modulators of cortical output, the GABAergic interneurons. Therefore, the present study has extended the cortical pathological studies to the cortical interneurons to investigate the cellular changes of the total number of GABAergic interneurons defined by calcium-binding proteins in the primary motor (BA 4) and anterior cingulate cortices (BA 24) of the human brain in 13 HD brains compared to the 14 normal control brains for our specific cohort of neurologically defined brains from the Neurological Foundation of New Zealand Human Brain Bank. This project forms an important part of a larger, systematic study on the pathological changes in various cell populations across the entire cerebral cortex in HD which is being carried out in our laboratory at the Centre for Brain Research, The University of Auckland. The results showed a heterogeneous pattern of GABAergic interneuron loss in two functionally diverse cortical regions, the primary motor cortex and the anterior cingulate cortex. Most importantly, there was a differential pattern of interneuron loss which correlated with the symptom variation in HD cases. In addition, an association between the patterns of interneuron loss was also compared with the striatal neuropathological grade to determine the pathological relationship between the cortex and the striatum, as well as with the CAG repeat length in the HD gene, post-mortem interval, and age of each HD case. These findings extend our understanding of the neural basis of cortical degeneration which greatly contributes to the symptom heterogeneity in HD.

8.2. Methodological Considerations

8.2.1. Human Brain Tissue

There were limits on the number of cases used in this study. Due to the availability of human tissue and age of stored tissue, there were in total 13 HD and 14 normal control cases analysed in this study. The HD cases were further subdivided into the dominant symptom profiles (four cases were categorised as the “HD motor” symptom group; four cases in the “HD mood” symptom group; and five cases in the “HD mixed” (motor/mood) symptom group). The HD cases were also subdivided separately according to the different striatal neuropathological grade (four cases in the HD grade 0-1 group; five cases in the HD grade 2
group; and four cases in the HD grade 3 group). A greater sample size would increase the validity and the statistical power of this study, although significant changes in the interneuronal populations were observed in the two cortical regions even with the limited number of cases. In addition, it was not feasible to sample and perform immunohistochemistry on the entire cortical block of interest and therefore only the first 100 sections were sampled for the counting analysis. In order to determine the total cell number \( (N) \) in the two cortical regions, it was necessary to use Nissl-stained series encompassing the entire cortical block to calculate the total reference volume \( (V_{\text{ref}}) \) of the SM1 and CG1 blocks of the primary motor and anterior cingulate cortices, respectively (see Chapter 3 for more details). This approach is not optimal for stereology (Oorschot, 1994), and therefore a valid assumption was made that the counting analysis of a quarter to a fifth of the two cortical regions in a subvolume (or per length of cerebral cortex) would yield data that was representative of the entire cortical area. The previous results in our laboratory on the pyramidal cell loss in the same subregion of the primary motor cortex used as in the present study in HD (Thu, et al., 2010) was consistent with the findings from another stereological analysis of the entire primary motor cortex in HD (Macdonald & Halliday, 2002).

### 8.2.2. Immunohistochemistry

The immunohistochemical staining for calbindin-D28k (CB) labels nonpyramidal interneurons as well as smaller, lightly stained populations of pyramidal neurons (Chapters 4 and 6). These cells were present in both primary motor and anterior cingulate cortices. Every effort has been made to identify and exclude these cells from the counting analysis in the HD brains (Chapters 5 and 7) based on their morphological features such as conical shaped cell bodies and a prominent apical dendrite that were directed towards layer I by the staining intensity and by the laminar position of these cells. The proportion of these CB\(^+\) pyramidal neurons is not available at this stage, but a colocalisation study using CB with pyramidal neuron markers such as SMI32 (antibody against a non-phosphorylated neurofilament epitope) and MAP2 (antibody raised against epitope of microtubule associated proteins) warrants further study. A preliminary confocal double-labelling experiment using CB and SMI32 showed no colocalisation of these two markers in the human primary sensory cortex (BA 3).
Also, the colocalisation study using double immunofluorescent labelling of the three calcium-binding proteins (CB, CR, PV) were investigated in the primary motor and cingulate cortices of the normal human brain in Chapters 4 and 6. In these analyses, only cells with clearly visible nuclear, cytoplasmic and dendritic staining were included in the immunofluorescent counting analysis. However, it should be noted that there were cellular profiles that could not be clearly identified as neurons due to morphological artefacts caused by distortion, cell capping or the edge of the cells that were encountered in the plane of z-axis during image acquisition. These cellular structures or profiles were carefully assessed and were excluded from the immunofluorescent counting analysis. In addition, the autofluorescent pigment lipofuscin was observed in both cortical regions in the double immunofluorescent studies. The presence of lipofuscin can complicate the identification of a real labelling in cells due to its broad excitation and emission spectra which can overlap with commonly used fluorophores. Lipofuscin is characterised by round, punctate, globular formations that reside in the cytoplasm of neurons. However, the calcium-binding proteins distinctly label cells with clear nuclear, cytoplasmic, and proximal dendritic staining which greatly aided in the identification of interneurons in the two cortical regions, and was largely unaffected by the lipofuscin autofluorescence. There have been several methods to overcome autofluorescence of lipofuscin in neurons such as treatment with copper sulphate or sudan black, or λ-scan online fingerprinting method to exclude the collection of lipofuscin emission spectral profile during image acquisition on the Zeiss 710 confocal laser scanning microscope. However, these treatments can also reduce the intensity of immunofluorescent labelling, therefore a careful compromise between these treatments and the specific fluorescent labelling must be maintained (Schnell et al., 1999). Sudan black and online fingerprinting methods have been examined for the double immunofluorescent labelling in the present study but no significant difference was observed with regard to the colocalisation results.

8.2.3. Confounding Issue between the Analyses of HD Cases Grouped by Symptoms and Striatal Pathological Disease Grades

This study has grouped the HD cases into either dominant clinical symptom profiles or by striatal neuropathological disease grades. Thus, it was important to first consider whether these factors were related (i.e., confounding) and would possibly affect the analysis of
results. There was no difference between the two groups when the median of the symptom groups and striatal grade ratings were compared in both cortical regions (Figure 8.1). For 13 HD cases used in the primary motor cortex (Figure 8.1A) and 11 HD cases used in the anterior cingulate cortex (Figure 8.1B), there was a p-value of 0.7136 and 0.3705, respectively (One-way ANOVA; Kruskal-Wallis analysis), which indicates that there were no differences between the HD symptom groups and average pathological grades used in this study. Therefore, it was acceptable for these factors to be investigated separately without the results being confounded.

**Figure 8.1** Graph showing the relationship between striatal pathological grades and symptom subgroups of the HD cases used in this study

There is no significant difference at the 5% level observed between the median pathological grades and the three symptom subgroups in HD cases used for analyses in the (A) primary motor cortex (p=0.7136) and the (B) anterior cingulate cortex (p=0.3705).

### 8.2.4. Stereological Cell Counting Methods

In this study, the Nv:Vref method of stereological quantification was used to determine the total number of interneurons in the two regions of the cortex. The estimate of precision or reproducibility in stereology is expressed as the coefficient of error (CE) (Slomianka & West, 2005). This value needs to be kept within an acceptable range to ensure that any variation observed between different cases is a true reflection of any biological changes and not introduced by the experimental procedures, and also without compromising the time taken for cellular quantification. A limitation of the Nv:Vref method is the lack of a statistically valid formula for calculating coefficient of error (CE) for the total number of
cells, \( N \) (Schmitz & Hof, 2005). This is because both components of the \( N_v \) and \( V_{\text{ref}} \) procedures, i.e., optical dissector and Cavalieri estimator, respectively, have individual CE values. Hence, the CE values have been evaluated separately for each method employed, e.g., CE(\( N_v \)) and CE(\( V_{\text{ref}} \)) (see Appendix II). The average CE values for both \( N_v \) and \( V_{\text{ref}} \) were mostly less than 0.10 and always less than 0.14 in all cases in the two cortical regions. This indicates that the difference between different cases in the diseased and control cases represent a real biological difference rather than contributed by methodological counting procedures in this study. The problem of two values for CE, however, has been subsequently resolved by the use of the optimal fractionator method in which only one total CE value exists (West, et al., 1991; Gundersen, et al., 1999). Due to the human tissue constraints and the previously employed counting techniques in HD cortical studies in our laboratory (Thu, 2006; Thu, et al., 2010), the \( N_v:V_{\text{ref}} \) method has been continuously used for this study in order to make valid comparisons. However, additional counting analyses using the optical fractionator method has been conducted in part to validate the cellular quantification of the \( N_v:V_{\text{ref}} \) method. Comparisons of the \( N_v:V_{\text{ref}} \) method and the optical fractionator method displayed comparable results and showed only small percentage differences in the total number of interneurons counted in the same region of the cortex of the same cases (see Appendix IV).

8.3. The Overall Immunohistochemical Staining Patterns in the Primary Motor Cortex and the Anterior Cingulate Cortex in the Normal Human Brain

The GABAergic interneurons in this study were immunohistochemically identified by their expression of calcium-binding proteins, calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) in the primary motor and anterior cingulate cortices of the normal human brain (Chapters 4 and 6). The three calcium-binding proteins were expressed in distinct, non-overlapping subpopulations of GABAergic interneurons, and showed comparatively similar distribution patterns and morphological characteristics in both cortical areas. In both cortices (see schematic diagram below, Figure 8.2), CB preferentially labelled bipolar or multipolar shaped cells that were most prominent in the upper cortical layers II and III, with a smaller population of cells in the deeper cortical layers V and VI. These cells are likely to represent double bouquet, neurogliaform and Martinotti morphological cell
types previously identified in Golgi-preparations (Somogyi & Cowey, 1981; Fairén, et al., 1984; Jones, 1984b; Somogyi & Cowey, 1984; Kubota, et al., 1994; Gabbott & Bacon, 1996; Markram, et al., 2004; Kalinichenko, et al., 2006). These cells exert inhibitory action via axo-dendritic contacts with the pyramidal projection neurons. Also, a small population of lightly stained pyramidal neurons were labelled with CB in layers III and V, and these cells were exclusively excluded from the stereological cell counting studies in the HD cortex (Chapters 5 and 7) based on cell morphology and immunostaining patterns (see above, Section 8.2.2). The immunohistochemical staining results showed that CR labelled cells throughout all layers of the cortex, with higher abundance in the upper layers. The morphological cell types identified with CR are likely to represent Cajal-Retzius cells of layer I, double bouquet and bipolar cells that also provide inhibitory action via axo-dendritic contacts with pyramidal neurons. The PV immunostaining was found in layers II-VI, with the predominance of cells in the middle cortical layers. The neuropil was stained as a distinctive band which extended from lower part of layer III to layer V. The morphological cell types labelled with PV are likely to represent basket cells that are specialised in targeting the cell soma and proximal dendrites of pyramidal neurons and chandelier cells that provide powerful inhibitory action on the axon initial segment of pyramidal neurons (Fairén & Valverde, 1980; Peters, 1984b; DeFelipe, 1997; Somogyi, et al., 1998; Markram, et al., 2004; Zhu, et al., 2004). Besides classifying interneurons on the basis of morphological and biochemical features, different subpopulations also reveal various physiological properties. For example, low threshold spike (LTS) cells are bitufted or multipolar cells with sparsely spiny dendrites that show CB and CR immunoreactivity (Kawaguchi & Kubota, 1997; Kubota, et al., 2011), while fast spiking (FS) cells are mainly multipolar cells with smooth dendrites that show PV immunoreactivity (Buhl et al., 1994; Buhl, et al., 1995; Cauli, et al., 1997; Kawaguchi & Kubota, 1997; Kawaguchi & Kondo, 2002; Toledo-Rodriguez, et al., 2004). In particular, the PV expressing FS cells have been suggested to be involved in γ-frequency oscillations (30-80 Hz), which are postulated to improve information processing in the cortex (Sohal et al., 2009). These network rhythmic activity can modulate the firing patterns of pyramidal neurons and can also maintain oscillations even in the absence of excitatory inputs (Whittington et al., 1995). These studies show that cortical interneurons are morphologically, biochemically, and physiologically diverse and this suggests that different interneuronal subpopulations may play different functional roles in shaping cortical circuitry.
Also, the confocal double-labelling study of the three types of interneurons in Chapters 4 and 6 showed no colocalisation between the cell types identified with the three interneuronal markers, except for a small population of cells (0.8%) that showed coexpression of CB and PV markers in the anterior cingulate cortex (Chapter 6). The relative proportions of the interneurons were CB (11.6%), CR (41.6%), and PV (46.8%) in the primary motor cortex, and CB (14.4%), CR (45.2%), and PV (40.4%) in the anterior cingulate cortex (Figure 8.2). These findings show clear parcellations of CB, CR, and PV immunoreactivity and suggest that each of these calcium-binding proteins form distinct, chemically heterogeneous populations of GABAergic interneurons in the primary motor and anterior cingulate cortices of the human brain.

Figure 8.2 Diverse populations of cortical GABAergic interneurons and the expression of calcium-binding proteins

Schematic diagram to illustrate morphological and neurochemical diversity of GABAergic interneurons as identified by the expression of calcium-binding proteins (calbindin-D28k, calretinin, parvalbumin) and their relative proportions in this group of interneurons in the normal human brain of the primary motor and anterior cingulate cortices.
8.4. The Overall Interneuron Loss in the Cerebral Cortex in All HD cases

Our study showed that on analysis of all HD cases grouped together there was no overall significant interneuron cell loss in all three populations (9% of loss of CB\(^+\), 19% loss of CR\(^-\), and 4% loss of PV\(^+\) interneurons) in the **primary motor cortex** in the 13 HD cases compared to the 14 normal control cases (Figure 5.1; Chapter 5). These results in the motor cortex were comparable to a study by Macdonald and Halliday (2002) who have also shown no significant loss in all three GABAergic interneurons (CB, CR, PV) in the motor cortex of post-mortem HD brain. However, in contrast, the major finding of the present study was the observation of a major reduction in two interneuronal populations (CR and PV) in the **anterior cingulate cortex** (Figure 7.1; Chapter 7). The results showed that there was an overall non-significant 28% loss of CB\(^-\) interneurons (p>0.05), but a significant loss of 34% CR\(^+\) (*p=0.012), and a significant 45% loss of PV\(^+\) interneurons (*p=0.019) was observed in the 11 HD cases compared to the 14 normal control cases. The present study showing a major loss of PV\(^+\) interneurons in the anterior cingulate cortex is in contrast to a previous study in the cingulate gyrus, and frontal and occipital cortices (Cudkowicz & Kowall, 1990b), where the authors found no significant loss of PV\(^+\) interneurons in 20 HD and 15 control brains suggesting relative sparing of the PV\(^+\) cortical interneurons in these cortical regions. However, another study by Ferrer and colleagues (1994) found a significant decrease in PV\(^+\) interneurons in the frontal cortex but not in the occipital and temporal lobes. These discrepancies suggest that there is a differential mode of interneuron loss in the different functional regions of the human cerebral cortex and perhaps that this heterogeneous pattern of loss may account for the variable cortical pathogenesis in HD.

In addition to these results, it was most interesting to note that the results in our study demonstrated a **prominent variation** in the number of interneurons **between individual HD cases** which were not highlighted in previous reports. Comparison between HD cases in our study showed a marked variation in the degree of the interneuron loss in the primary motor cortex (CB\(^+\) interneurons varied from 0-59% loss; CR\(^-\) interneurons varied from 0-61% loss; PV\(^+\) interneurons varied from 0-64% loss) (Figure 5.2; Chapter 5) and also in the anterior cingulate cortex (CB\(^+\) interneurons varied from 0-79% loss; CR\(^+\) interneurons varied from 0-79% loss; PV\(^+\) interneurons varied from 0-86% loss) (Figure 7.2; Chapter 7). This between-case variation in the two cortical regions in HD is a novel and characteristic
feature of our results. This suggests that heterogeneity in the pattern of cortical interneuron loss is a key feature of the neuropathology in HD. Hence, this observation has prompted the hypothesis that the substantial heterogeneity of the number of cortical interneurons may be related to the symptom variation in HD, as well as to the severity of degeneration in the striatum of the basal ganglia as indicated by striatal neuropathological grade. Thus, these investigations were carried out next and the results are discussed in the sections below.

8.5. Relation of the Pattern of Interneuron Loss to the Pattern of Symptomatology in HD

The symptoms of HD vary greatly, both in the disease onset and the type of symptoms exhibited by individual HD patients during the course of the disease. This has been observed in monozygotic twins where remarkable symptom differences occur with the same genetic mutation and environmental factors (Georgiou, et al., 1999; Anca, et al., 2004; Friedman, et al., 2005; Gomez-Esteban, et al., 2007). Although the number of CAG repeats in the HD gene has been correlated with the age of symptom onset in HD (Andrew, et al., 1993; Duyao, et al., 1993; Wexler, et al., 2004), a clear association between the CAG repeat length and the symptom variation is yet to be determined. Consequently, there is much interest in whether there are any underlying pathological differences in HD brains which may account for symptom heterogeneity. Characteristic phenotypic manifestation is likely to represent neuronal degeneration and dysfunction in the brain regions responsible for specific functional roles. For example, alterations in the circuitry of primary motor cortex and anterior cingulate cortex may contribute respectively to impairments of certain motor and mood functions in HD. This is a particular interest in our lab, and neuronal loss in a number of regions across the brain has been continually investigated in extensively characterised HD cases with specific symptom profiles.

Major alterations in GABA neurotransmission and the inhibitory function of GABAergic interneurons which play a critical role in the modulation of cortical output, have been implicated in the striatum and cortex of HD (Faull, et al., 1993; Cha et al., 1998; Buzsaki et al., 2004; Markram, et al., 2004; Gu, et al., 2005; Spamanato, et al., 2008; Cummings, et al., 2009; Kubota, et al., 2011). Therefore, the present study is directed towards
investigating the cellular changes of the GABAergic interneurons in two functionally diverse regions of the cerebral cortex (primary motor cortex and anterior cingulate cortex) and an association between the patterns of interneuron loss was investigated to the dominant symptomatology of HD cases. Our results in Chapters 5 and 7 showed that there were major significant changes of the GABAergic interneurons in these two cortical regions. The most striking finding of this study is the heterogeneous pattern of specific types of GABAergic interneuron loss which correlates with the variable pattern of motor and mood disorder in the motor and cingulate cortices, respectively. This is the first study to show that in the primary motor cortex of the human brain in HD (Chapter 5), there was a selective loss of calbindin-D28k (CB) interneurons (57% loss) in HD cases with major motor disorder, while no interneuron loss was observed in the motor cortex in HD cases with major mood disorder. On the other hand, in the anterior cingulate cortex (Chapter 7), an area that is involved in mood and behaviour, there was a major significant loss in all three interneuronal types - 71% loss of calbindin-D28k (CB), 60% loss of calretinin (CR), and 80% loss of parvalbumin (PV) interneurons - in HD cases with major mood disorder, while no significant interneuron loss was observed in the anterior cingulate cortex in HD cases with major motor disorder. This suggests that motor and mood symptomatology which characterises HD is linked with the variable interneuron loss in the primary motor and anterior cingulate cortices, which reflects the established role in the regulation of movement and mood of these cortices, respectively.

The involvement of cortical interneurons in the pathogenesis of HD is supported by several studies in mouse models of HD. For example, electrophysiological studies in the Cre/LoxP conditional HD mice showed an early deficit of the GABAergic inhibitory input by the interneurons to the cortical pyramidal neurons (Gu, et al., 2005). Another study in BAC transgenic HD mice also observed specific synaptic pathology of interneurons and pyramidal neurons in the form of loss of inhibition of pyramidal neurons in the motor cortex (Spampanato, et al., 2008). In addition, other HD mouse models (R6/2, YAC128, CAG140 knock-in) displayed alterations in the IPSP patterns and frequency of the GABAergic interneurons in the somatosensory cortex (Cummings, et al., 2009). These animal model studies suggest that an early loss of inhibition by the interneurons in the cortex is a major contributing factor in the pathogenesis of HD and that altered cortical
excitability as a result of loss inhibition by the GABAergic interneurons could be a crucial early event in the HD pathogenesis.

The relationship between the pattern of interneuron cell loss and pyramidal cell loss in HD

The present study considerably extends our previous findings in our laboratory on the pattern of cell loss in the primary motor and anterior cingulate cortices. The detailed stereological cellular analyses in our laboratory of the same HD cases in the two cortical regions have shown that there was a clear association between the loss of all neuronal types (NeuN+ neurons) and SMI32+ pyramidal neurons with the dominant symptom profile in HD (Thu, 2006; Thu, et al., 2010). As shown in Figure 8.3, in the primary motor cortex, there was a clear association between the loss of all neuronal types (28% loss of NeuN+ neurons) and 45% loss of SMI32+ pyramidal neurons only in the HD motor cases, but no cell loss was observed in the motor cortex in the HD mood cases (Figure 8.3A). The results of the present study showed a major selective 57% loss of CB+ interneurons which were also specifically correlated with severe motor symptomatology in the primary motor cortex (Figure 8.3A). Interneurons labelled with CB preferentially innervate the dendritic domain of pyramidal neurons and are positioned to influence the dendritic processing and integration of synaptic inputs such as generation and propagation of dendritic calcium spikes (Traub, 1995; Larkum, et al., 1999a; Larkum, et al., 1999b). Therefore, the progressive loss of CB+ interneurons along with pyramidal cell loss in HD motor cases in the primary motor cortex, and perturbations in these complex cortico-cortical and cortico-striate connections are believed to contribute significantly to the generation of motor clinical symptoms in HD. Also, constitutive CB–/– null mutant mice demonstrated impairment in motor coordination (Airaksinen, et al., 1997), although these changes are likely to act mainly via altered transmission of cerebellar Purkinje cell signalling (Barski, et al., 2002; Barski, et al., 2003). This suggests that motor symptomatology in HD is characterised by loss of pyramidal neurons and a major selective CB+ interneuron loss in the primary motor cortex, which is an area that plays a major role in the control of kinematic and dynamic properties of voluntary movements (Naidich, et al., 2001; Binkofski, et al., 2002; Matelli, et al., 2004; Zilles, 2004; Chouinard & Paus, 2006). In addition, it is interesting to note that the studies in the striatum by Tippett and colleagues (2007) showed the loss of CB immunoreactivity in the matrix compartment (which is linked to sensorimotor areas) in HD
cases with severe motor impairment. These results together show that calcium-binding protein expressing cells, especially CB are differentially affected in the cortex and striatum, which is suggestive of possible similar mechanisms of cell death and consequent motor symptoms in both brain regions in HD.

On the other hand, as shown in Figure 8.4, in the anterior cingulate cortex, there was loss of all neuronal types (54% loss of NeuN+ neurons) and 40% loss of SMI32+ pyramidal neurons only in the HD mood cases, but no cell loss was observed in the anterior cingulate cortex in the HD motor cases (Figure 8.4A). Also, in the present study, there was a major significant loss of all three GABAergic interneurons in the HD mood cases (71% loss of CB+, 60% loss of CR+, 80% loss of PV+ interneurons), but not in the HD motor cases (Figure 8.4A). The anterior cingulate cortex forms one of the major components of the limbic system and is involved in a wide range of higher-level neural functions including emotion, mood, visceromotor control, and attention to action (MacLean, 1993; Devinsky, et al., 1995; Mega, et al., 1997; Morecraft & Van Hoesen, 1998; Bush, et al., 2000; Paus, 2001; Vogt, et al., 2004; Vogt, et al., 2005). As such, extensive cell loss in this region including pyramidal neurons and GABAergic interneurons (CB+, CR+, PV+ interneurons) would lead to disruption in the limbic circuits which are thought to be involved in mood and emotional disturbances observed in HD patients. Also, cortico-striatal and other limbic related connections may be affected. Evidence for this comes through a detailed pathological study in the human striatum of HD which demonstrated that patients with greater mood disturbance showed greater damage to the striosome compartment (Tippett, et al., 2007). The striosome compartment has been shown to receive inputs from the “limbic” related cortical areas in rodents (Donoghue & Herkenham, 1986; Gerfen, 1989) and primates (Elen & Graybiel, 1995). The limbic structures including the anterior cingulate cortex, hippocampus, and amygdala send massive projections to the ventral striatum and striosome compartment of the dorsal striatum which in turn sends projections to the ventral pallidum and the rostrodorsal SNr in the rat and monkey. These in turn project to a paramedian portion of mediiodorsal (MD) nucleus of the thalamus and back to the limbic cortex. It was previously suggested that symptoms of agitation, irritation and euphoria in HD are suggested to be secondary to the underactivity of the indirect motor pathways which arise from the ventromedial caudate and ventral striatum connecting the limbic circuit (Litvan, et al., 1998; Joel, 2001). Since the anterior cingulate cortex has extensive
connections with the striosome compartment of the striatum, variable degeneration in both of these regions may serve as possible pathogenic mechanisms underlying the neural basis of emotional and mood disturbances in HD.

In addition, it was most interesting to note that the pattern of interneuron cell loss paralleled pyramidal cell loss, i.e., CB\(^+\) interneuron loss was only observed in the HD motor cases in the primary motor cortex where there was also major pyramidal cell loss. In the anterior cingulate cortex there was loss in all interneuron types (CB, CR, PV) where there was also major pyramidal cell loss in the HD mood cases (Figures 8.3 and 8.4). This suggests that the perturbation between the pyramidal-interneuron interactions is a critical factor in the establishment of pathophysiology of the cortex in HD. Indeed, a study in Cre/LoxP conditional HD mice showed that the interaction between interneurons and pyramidal neurons was necessary to produce motor deficits and cortical pathology (Gu, et al., 2005). In their study, restricting the expression of toxic mutant huntingtin fragment only to the pyramidal neurons was insufficient to cause widespread cortical neuropathology or motor deficits, however, the expression of mutant huntingtin in all neuronal types (pyramidal and interneurons) elicited both progressive motor deficits and characteristics of HD cortical pathology including neuronal degeneration and dysmorphic neurites. This suggests that the interaction between pyramidal neurons and local-circuit interneurons is critical in the cortical pathogenesis in HD.

Taken together with the present study, our combined overall studies suggest that the variable motor and mood symptomatology which characterises HD is associated with a variation in the extent of pyramidal cell loss and a differential loss of interneurons in the corresponding functional regions of the cerebral cortex (Figure 8.3 and 8.4); That is, in the primary motor cortex a loss of pyramidal cells and CB interneuron is associated with motor dysfunction in HD, whereas in the anterior cingulate cortex the loss of pyramidal cells and ALL three types of GABAergic interneurons is associated with the mood symptom profile in HD. These findings are very important in the formulation of the cortical cellular basis of the variable symptom profiles in HD.
Figure 8.3 The pattern of pyramidal neuron and interneuron cell loss in the primary motor cortex (BA 4) of normal and Huntington's disease brains with dominant "motor" and "mood" symptomatology

The graph in (A) shows the total number of neurons (NeuN\(^+\) neurons; SMI32\(^+\) pyramidal neurons; and interneurons labelled with CB, CR, PV) in the primary motor cortex (BA 4) of normal and Huntington's disease brains as determined using stereological counting methods and grouped according to dominant symptoms (motor and mood). The most significant cell reduction was observed in HD cases with mainly "motor" symptoms (reduction of 28% NeuN\(^+\) neurons, 45% SMI32\(^+\) pyramidal neurons, and 57% CB\(^+\) interneurons), while no significant cell loss in all types of neurons were observed in HD cases with mainly "mood" symptoms. The figure in (B) shows a schematic diagram of the pattern of neuronal loss in the primary motor cortex in HD cases with "motor" symptomatology.
Figure 8.4 The pattern of pyramidal neuron and interneuron cell loss in the anterior cingulate cortex (BA 24) of normal and Huntington's disease brains with dominant "motor" and "mood" symptomatology

The graph in (A) shows the total number of neurons (NeuN+ neurons; SMI32+ pyramidal neurons; and interneurons labelled with CB, CR, PV) in the anterior cingulate cortex (BA 24) of normal and HD brains as determined using stereological counting methods and grouped according to dominant symptoms (motor and mood). The most significant cell reduction was observed in HD cases with mainly "mood" symptoms (reduction of 54% NeuN+ neurons, 40% SMI32+ pyramidal neurons, and loss of 71% CB+, 60% CR+, 80% PV+ interneurons), while no significant cell loss in all types of neurons were observed in HD cases with mainly "motor" symptoms. The figure in (B) shows a schematic diagram of the pattern of neuronal loss in the anterior cingulate cortex in HD cases with "mood" symptomatology.


Alterations of GABAergic interneurons in mood-related diseases

Interestingly, alterations in the expression of the three calcium-binding proteins (CB, CR, PV) have been particularly implicated in mood-related disorders such as schizophrenia, bipolar disorder, epilepsy and depression (Baimbridge, et al., 1992; Heizmann & Braun, 1992; Andressen, et al., 1993; Schwaller, et al., 2002; Schwaller, 2009; Luscher et al., 2011). For example, a selective loss of calbindin-D28k (CB) interneurons has been observed in the prefrontal cortex in depression patients (Rajkowska et al., 2007). Also, parvalbumin (PV) GABAergic interneurons have been reported to be reduced principally in the anterior cingulate and prefrontal cortices in schizophrenia and bipolar disorder patients (Beasley & Reynolds, 1997; Benes & Berretta, 2001; Beasley, et al., 2002; Hashimoto, et al., 2003), and the PV+ chandelier cells were proposed to be important components in the establishment of human temporal lobe epilepsy (DeFelipe, 1999). These studies in conjunction with the findings of the present study (major loss of PV+ interneurons in HD cases with major mood disorder in the anterior cingulate cortex), suggests that the cortical GABAergic interneurons, especially PV+ interneurons may contribute to abnormalities in the function of limbic-related cortical circuits in HD and other neuropsychiatric diseases. Network synchronisation is a critical component of proper neural function within and between cortical and subcortical regions in the brain (Jones, 2001; Schnitzler & Gross, 2005; Bruno & Sakmann, 2006). The fast-spiking, perisomatic PV interneurons are known to be a critical component of synchronised neuronal activity (Cobb, et al., 1995; Gonchar & Burkhalter, 1997; Pouille & Scanziani, 2001; Freund, 2003; Klausberger, et al., 2003; Buzsaki & Draguhn, 2004; Hajos et al., 2004; Mann et al., 2005; Bartos, et al., 2007; Fuchs et al., 2007). It has been reported recently that reduced synchronisation between cortical areas was shown in presymptomatic HD patients (Thiruvady et al., 2007).

In conclusion, the findings of the present study provide a new and exciting perspective on the significance of the pattern of cortical interneuron degeneration in HD by demonstrating that symptom heterogeneity in HD is reflected by a corresponding variable pattern of degeneration in the different cortical regions of the cortex. These novel findings support the view that the variable pattern of cortical pathogenesis may be an underlying principle in symptom heterogeneity in HD.
8.6. Relation of the Pattern of Interneuron Loss to the HD Striatal Neuropathological Grade

Neuropathology in HD is primarily characterised by neuronal loss in the striatum and the cerebral cortex. In the striatum, medium spiny neurons (MSNs) are most affected and degeneration of these neurons occurs progressively. The extent of striatal degeneration has been used as a grading system to indicate the severity of the disease (Vonsattel, et al., 1985; Vonsattel & DiFiglia, 1998; Vonsattel, et al., 2008). One of the prevailing questions in the field of HD pathology is how and when neuronal loss occurs and more importantly, whether the progressive loss of neurons in the striatum is a primary process, or whether degeneration in the cortex leads to striatal cell death; and also how these changes initiate the development of symptomatic features. There is considerable evidence that early observed changes in HD are emotional and cognitive disturbances (Lawrence et al., 1996; Paulsen, et al., 2001a; Paulsen, et al., 2001b). Therefore it may be possible that areas associated with these early symptoms, such as the limbic system and cerebral cortex (Harrison, 2002; Reading et al., 2004; Wolf et al., 2008), are the principal site of degeneration.

In our study the pattern of interneuron cell loss in the primary motor and anterior cingulate cortices generally appeared to correlate with the striatal neuropathological grade, i.e., there was a trend towards a greater loss of interneurons with increasing striatal neuropathological grade, suggesting that the cortical degeneration may be linked with the striatal degeneration in HD (Figure 5.6, Chapter 5 and Figure 7.6, Chapter 7). These findings are generally consistent with the previous studies on the human primary motor and anterior cingulate cortices in our laboratory, where greater neuronal loss in the cortex was associated with increasing striatal neuropathological grades (Thu, 2006; Thu, et al., 2010). Also, a study by Halliday and colleagues (1998) showed that the degree of cortical atrophy was associated with the degree of striatal atrophy, suggesting that the disease processes in the cortex and the striatum are related. However, the association between cortical interneuron loss and striatal pathology in the present study was not a clear-cut trend. This indicates that the pattern of interneuron degeneration is non-uniform and suggests that other factors may be involved in the cortical pathology in HD.
The striatum and the cortex are linked via the “cortico striatal pathway” and share many functional roles including movement, behaviour and cognition. One of the proposed mechanisms of cell death in HD involves early changes in the cortex and major alterations in the “corticostriatal” pathway which are thought to be critical in the pathogenesis of HD (Cepeda, et al., 2007). It has been suggested that cortical changes are fundamental to the onset and progression of the HD phenotype in humans and in mouse models (Laforet et al., 2001). For example, the anterior cingulate cortex is the first cortical area to develop nuclear inclusions in the R6/2 HD mice (Davies, et al., 1999) and is the site of ubiquitin-reactive dystrophic neurites in HD patients (Cammarata et al., 1993). Also, the accumulation of mutant huntingtin were found more frequently in the cortex than in the striatum in post-mortem HD brains (DiFiglia, et al., 1995; DiFiglia, et al., 1997; Sapp, et al., 1999) and abundant neuropil aggregates have been detected in the cortex of presymptomatic HD cases (Gutekunst, et al., 1999; Sieradzan & Mann, 2001). The widespread changes in the cortex are further supported by a microarray study in the human primary motor cortex (which included cases used in the present study) where 3% (1482) of the genes were differentially expressed in the HD cases. Also, Hodges and colleagues (2006) showed greater abnormalities in mRNA expression in the motor cortex than in the prefrontal association cortex, suggesting a distinct regional pattern of transcriptional alteration in the cortex of HD. In addition, as described above, the early involvement of cortical interneurons in HD has been shown in studies in mouse models of HD. For example, an early deficit of inhibitory input by the GABAergic interneurons to the cortical pyramidal neurons has been observed in the Cre/LoxP conditional HD mice (Gu, et al., 2005). Similarly, loss of inhibitory action and synaptic pathology of interneurons has been shown in the BAC HD transgenic mice (Spampanato, et al., 2008). In addition, three other HD mouse models (R6/2, YAC128, CAG140 knock-in) displayed alterations in the IPSP patterns of the GABAergic interneurons in the somatosensory cortex (Cummings, et al., 2009). These studies suggest that the loss of inhibitory functions of the cortical interneurons is an early event in HD and may contribute significantly to the early pathophysiological changes in HD.

Also, early dysfunction of the “cortico striatal” pathway is indicated by early motor dysfunction which preceded significant neuronal atrophy in both cortical and striatal regions in HD (Laforet, et al., 2001; Cepeda, et al., 2003; Li, et al., 2003; Andre et al., 2006;
Cepeda, et al., 2007; Milnerwood & Raymond, 2007; Spampanato, et al., 2008). The medium spiny neurons (MSNs) are projection neurons in the striatum that receive massive excitatory glutamatergic input from the pyramidal neurons from all regions of the cerebral cortex. Early changes in cortical pyramidal neurons in addition to the lack of inhibition due to the loss of GABAergic interneurons as shown in the present study may further exacerbate the disease process and alter the ability of pyramidal neurons to release glutamate. Excess glutamate in the striatum is also thought to make MSNs particularly vulnerable to NMDA receptor-mediated excitotoxic damage (Zeron et al., 2001; Zeron, et al., 2002). In addition, mutant huntingtin has been shown to reduce the production and transport of brain-derived neurotrophic factor (BDNF) - a growth factor necessary for striatal neuron survival (Strand, et al., 2007) - in the cortex via the corticostriate pathway (Altar et al., 1997; Conner et al., 1997; Ferrer et al., 2000; Cattaneo, et al., 2001; Zuccato et al., 2001; Fusco, et al., 2003; Baquet et al., 2004). Also, an early dysregulation of the BDNF gene due to mutant huntingtin has been suggested to disrupt microcircuitry in the cerebral cortex in cellular in vitro model of HD (Gambazzi et al., 2010). In separate studies, PV interneurons in different brain regions have been shown to specifically require BDNF for proper anatomical and physiological maturation, and maintain extensive dendritic arborisation and synaptic integrity (Gorski et al., 2003; Berghuis et al., 2006; Itami et al., 2007). These studies are highly suggestive that degeneration of the cortex and the corticostriatal pathway occur in the early stages of HD.

In summary, the cerebral cortex is anatomically and functionally closely connected with the basal ganglia through various functional circuits, and the generation of clinical symptoms in HD is likely to be caused by a combination of factors including pathological processes that occur in both the cortex and the striatum. In conclusion, the findings in the present study show that in general, there is a greater loss of interneurons in higher HD striatal grades in the primary motor and anterior cingulate cortices and this suggests that there is a close link between cortical and striatal degeneration.
8.7. Relation of the Pattern of Interneuron Loss to the CAG Repeat Length, Post-Mortem Interval, and Age in HD

This study also investigated the differences in the CAG repeat length, post-mortem interval, and age parameters which may account for the differences for the variable nature of the human brain in HD. The expansion of CAG repeats in HD is known to play an important role in relation to the age of onset, progression, and severity of the disease (Andrew, et al., 1993; Duyao, et al., 1993; Wexler, et al., 2004). The correlation between the age of onset and the length of CAG repeat for HD cases used in the present study showed a general trend towards an earlier age of symptom onset with longer CAG repeat length, although no significant correlation was observed using a Pearson regression analysis at the 5% level (p=0.1746; r²=0.1944) (Figure 8.5).

![Figure 8.5 Age of onset and CAG repeat length of HD cases used in this study](image)

The linear dependence of age of symptomatic onset on expanded CAG repeat length was analysed for the Huntington's disease cases used in this study. The correlation using a Pearson regression analysis at the 5% level showed no significant differences (p=0.1746; r²=0.1944). However, there is a general trend towards an earlier age of onset with longer CAG repeats, although it is important to note that there is a large variability of age of onset values, even between cases of the same repeat length.

The correlation between the extent of cortical atrophy and the number of CAG repeats has been demonstrated in HD, i.e., pronounced cerebral volume were correlated with higher CAG repeat number (Halliday, et al., 1998; Ruocco et al., 2008; Gomez-Anson et al., 2009). However, it is not yet known if there is a clear link with cortical interneuron cell loss with the CAG repeat length. The correlation between the number of interneurons and the length
of CAG repeat in both regions of the cortex in the present study showed no significant correlation using a Pearson regression analysis at the 5% level. However, there appeared to be a general trend towards a decrease in interneuron number with increasing CAG repeat length in all three interneuronal populations in both cortices in HD (Figure 5.7 and 7.7). A possible explanation for the lack of clear correlation could be due to a small sample size used in this study. On the other hand, it could also suggest that the cortical degeneration (unlike the striatum) may not be solely and strongly dependent on the CAG repeat length. There was, however, a significant correlation between the CAG repeat length with the number of PV⁺ interneurons in the primary motor cortex ($r^2=0.3412$, *p=0.0361) (Figure 5.7; Chapter 5).

An exact mechanism of selective striatal and cortical neurodegeneration is not yet fully understood, but CAG repeat instability has been implicated for a possible role in selective neuropathogenesis in HD. Although fairly minor, somatic and germline repeat instability (i.e., inter and intra-generational variability) has been shown in various HD mouse models and human HD tissue (Georgiou, et al., 1999; Kennedy & Shelbourne, 2000; Ishiguro et al., 2001; Kennedy et al., 2003; Squitieri, et al., 2003; Anca, et al., 2004; Shelbourne et al., 2007). Somatic repeat expansion may be modified by mismatch repair systems (Manley et al., 1999; Wheeler et al., 2003; Wheeler, et al., 2007), and also initiated by an age-dependent manner as shown in the striatum and cortex of the HD knock-in mice (Kennedy & Shelbourne, 2000; Ishiguro, et al., 2001). The age-dependent instability has been associated with single base excision DNA repair of oxidised nucleotides (Kovtun et al., 2007). Together, these studies promote that increased instability of CAG repeat expansion in mutant huntingtin may play a crucial role in marked neurodegeneration in HD, although some studies displayed an inconsistent relationship between somatic CAG expansion mosaicism and the increased vulnerability of striatal neurons in BACHD mice (Gray et al., 2008) and other polyglutamine expansion diseases (Lopes-Cendes et al., 1996; Watase et al., 2003).

In addition, HD gene mutation and its effects have been shown to be modulated by other factors such as modifier genes and environmental/epigenetic changes. For example, significant genetic modifiers have been identified in HD patients (Djousse, et al., 2004).
Also, studies in transgenic HD models showed that environmental enrichment delayed the onset and rate of symptom progression (van Dellen et al., 2000; Glass et al., 2004; Spires et al., 2004a; Spires et al., 2004b) and significantly increased the survival of these transgenic mice (Carter et al., 2000b). In addition, it has been suggested that correlation between symptom onset and CAG repeat length was attributed to 44% of the variance in a study of 443 HD patients (Wexler, et al., 2004). Of the remaining variance, about 40% was accounted for by other modifier genes and 60% accounting for environmental influences. These collective findings and the results of the present study indicate that although the CAG expansion in the HD gene is a critical risk factor, it is not the sole determinant of cortical pathology especially with interneuron loss and that numerous other factors such as genetic and environmental modifiers which play a role in determining the phenotypic variability of HD.

The analysis of post-mortem interval (PMI) showed no overall correlation between interneuron number and PMI in the two cortical regions in HD cases used in this study. This suggests that the differences in the time interval between death and tissue processing, and the possible resultant modifications in neuronal immunoreactivity was not accountable in this study. In addition, previous studies have found an association between the decrease in the number of cortical neurons with increasing age (Pakkenberg & Gundersen, 1997). The analysis of age at death in the present study showed no overall correlation between interneuron number and age in the two cortical regions in HD. Indeed, a previous study of age-related changes of the interneurons that express the calcium-binding proteins (CB, CR, PV) in the primary motor and anterior cingulate cortices also showed no significant reductions in the number of interneurons with age, although a trend towards a decreased number of these interneurons was observed with increasing age (Bu, et al., 2003). The other cortical areas examined in their study showed the largest age-related CB interneuron decrease in the primary and associative visual cortices (42% and 46% loss in comparison to controls, respectively), posterior cingulate cortex (45% loss) and the parahippocampal gyrus (21% loss). The number of CR interneurons was most affected in the auditory association cortex (37% loss), and middle and inferior temporal cortices (34% and 26% loss, respectively). PV interneurons displayed no significant age-related changes in the all cortical areas examined in their study.
These outcomes indicate that in general, the results between the total interneuron number with the symptom profile and striatal disease grade described above can be regarded as separate from the influence of CAG repeat length, post-mortem interval, and age of the HD cases used in this study. However, where evidence of a correlation was found, possible influence of these factors must be taken into consideration.

8.8. Other Possible Mechanisms of Cortical Degeneration and Implications for Cortical GABAergic Interneurons

8.8.1. Calcium-Binding Proteins and Their Neuroprotective Role in Neurological Diseases

Calcium (Ca$^{2+}$) signalling plays a vital role in neurons, including neurotransmitter release, membrane excitability, gene expression, neuronal plasticity, and cellular changes underlying learning and memory (Berridge, et al., 2003; Rusakov, 2006; Clapham, 2007). Impaired neuronal Ca$^{2+}$ homeostasis and increased intracellular Ca$^{2+}$ concentration have been suggested to be involved in brain aging and in a number of neurodegenerative diseases, including HD (Ferrer, et al., 1994; Kirischuk & Verkhratsky, 1996; Muller & Gispen, 1996; Verkhratsky & Toescu, 1998; Thibault et al., 2007; Wojda, et al., 2008). Prolonged increase in Ca$^{2+}$ over sustained periods is toxic to neurons and therefore the intracellular Ca$^{2+}$ levels must be tightly regulated. In particular, the three calcium-binding proteins, calbindin-D28k (CB), calretinin (CR), and parvalbumin (PV) are high-affinity cytosolic binding proteins that belong to the EF-hand family which are thought to play an important role in buffering intracellular Ca$^{2+}$ (van Brederode, et al., 1991; Miller, 1995; Schwaller, et al., 2002; Capozzi, et al., 2006; Grabarek, 2006; Gifford, et al., 2007; Schwaller, 2009). The calcium-binding proteins are widely found throughout the brain, and interestingly, are expressed in subpopulations of neurons in the cortex, specifically by the GABAergic interneurons (Baimbridge, et al., 1992; Heizmann & Braun, 1992; Andressen, et al., 1993; DeFelipe, 1997, 2002). Although these proteins have the capacity to buffer intracellular Ca$^{2+}$, their precise functional significance in neurons remains speculative. It has been suggested that these calcium-binding proteins protect against Ca$^{2+}$ overload, thus rendering neurons more resistant against cellular insults such as excitotoxicity (Iacopino et al., 1992; Lukas & Jones,
1994; Van Den Bosch et al., 2002), in aging (Idrizbegovic et al., 2001), and in neurodegenerative diseases including Alzheimer’s disease (Ferrer et al., 1991; Hof et al., 1991; Fonseca & Soriano, 1995; Leuba et al., 1998), Parkinson’s disease (German et al., 1992), and Huntington’s disease (Cudkowicz & Kowall, 1990b; Macdonald & Halliday, 2002). However, the neuroprotective role of calcium-binding proteins is controversial, as various reports also show vulnerability of the calcium-binding proteins to excitotoxicity in cultured neurons (Isaacs et al., 2000; D’Orlando, et al., 2002), motor neuron disease (Ince, et al., 1993), multiple sclerosis (Clements et al., 2008), schizophrenia and bipolar disease (Cotter et al., 2002), dementia and Alzheimer’s disease (Ichimiya et al., 1988; Hof & Morrison, 1991; Ferrer et al., 1993), Parkinson’s and Huntington’s diseases (Iacopino & Christakos, 1990; Yamada et al., 1990; Heizmann & Braun, 1992).

The findings of the present study on the GABAergic interneurons that express these calcium-binding proteins also show differential pattern of vulnerability in the primary motor and anterior cingulate cortices in HD. As shown in Chapter 5, when all the HD cases were grouped together, there were no significant loss of all three types of interneurons (CB, CR, PV) in the primary motor cortex. However, in the anterior cingulate cortex (Chapter 7), there was a non-significant reduction of CB⁺ interneurons (28% loss), but significant reductions of CR⁺ interneurons (34% loss) and PV⁺ interneurons (45% loss).

In summary, many studies have observed changes in the three calcium-binding proteins in various regions of the brain in different neurological diseases. These findings suggest that there is a differential pattern of interneuron degeneration in the different disease states, and this is also governed by regional and species differences. The precise mechanisms underlying these disturbances are largely unknown and further studies on the physiological functions of these proteins and the neuronal systems in which they are expressed may become important therapeutic targets for preventing neuronal death in an array of neurological diseases.
8.8.2. GABA\(_A\) Receptor Changes in the Cerebral Cortex in HD

The findings of the present study showed cellular deficits of specific subpopulations of GABAergic interneurons in the primary motor and anterior cingulate cortices in HD. In addition, alterations in GABAergic neurotransmission in the HD cerebral cortex have been indicated in several reports in animal models of HD (Cha, et al., 1998; Gu, et al., 2005; Spampanato, et al., 2008; Cummings, et al., 2009). Thus, cellular abnormalities of the GABAergic system in the cortex play an important role in the pathophysiology of HD. Importantly, the cortical GABAergic interneurons exert their presumed inhibitory roles via differential expression of various types of GABA receptors. The GABA\(_A\) receptors are fast-activating ionotropic receptors that belong to the Cys-loop family of ligand-gated ion channels (Pritchett et al., 1989; Macdonald & Olsen, 1994; Macdonald & Botzolakis, 2009). These receptors are heterogeneously found throughout the different layers of the cortex and are expressed in both pyramidal cells and interneurons, and cause membrane hyperpolarisation by allowing Cl\(^-\) ion influx upon activation (Fritschy & Mohler, 1995; Whiting et al., 1995; Jentsch et al., 2002; Macdonald & Botzolakis, 2009). Alterations in GABA\(_A\) receptor subunit composition are known to affect kinetics (Okada et al., 2000; Vicini et al., 2001), and decreased composition of \(\alpha5\) and \(\gamma2\) subunits have been demonstrated in the motor cortex of HD patients (Hodges et al., 2006). Also, more recently, a study in our laboratory has investigated the changes in the pattern of GABA\(_A\) receptor subunit expression (\(\alpha1\), \(\beta2/3\), \(\alpha3\) subunits) of the same HD cases in the same two cortical regions used in the present study (Sharma, 2009). Interestingly, the heterogeneous pattern of GABA\(_A\) receptor expression was associated with the dominant symptomatology of HD. That is, in the primary motor cortex, the HD cases with predominant motor symptoms (but not in HD cases with major mood disorder) showed a significant increase in protein and mRNA levels in all three GABA\(_A\) receptor subunits normalised per cell. Conversely, in the anterior cingulate cortex, there was a significant increase in protein and mRNA levels of all GABA\(_A\) receptor subunits in HD cases with major mood disorder (but not in the HD cases with predominant motor symptoms) (Sharma, 2009). This major finding shows that the variable pattern of GABA\(_A\) receptor levels correlates with the symptom profiles in HD in two functionally diverse cortical regions.
This is consistent with the pattern of interneuron cell loss observed in the present study (Chapters 5 and 7). These findings together suggest that there is a corresponding increase in the expression of GABA<sub>A</sub> receptors where there was a major interneuron loss (i.e., significant reduction of CB<sup>+</sup> interneurons in HD motor cases in the primary motor cortex, and significant reduction of all interneurons in HD mood cases in the anterior cingulate cortex).

The increase in GABA<sub>A</sub> receptor profile can be interpreted as reflecting a compensatory upregulation of postsynaptic receptors, due to losses of GABAergic interneurons in the two cortical regions, which are associated with specific symptom profiles in HD. A similar compensatory upregulation and alterations of GABA<sub>A</sub> receptors due to selective deficits of GABAergic interneurons have been reported in the prefrontal and anterior cingulate cortices in patients with schizophrenia, bipolar disorder, and major depression (Benes et al., 1992; Benes et al., 1996; Benes et al., 2000; Benes & Berretta, 2001; Beasley et al., 2002; Blum & Mann, 2002), and epilepsies associated with GABA<sub>A</sub> receptor mutations (Olsen et al., 1999; Macdonald & Kang, 2009). This suggests that deficits of specific GABAergic neurons are present in HD and a number of neuropsychiatric disorders and that there is a pattern of related upregulation of GABA<sub>A</sub> receptors in these diseases.

### 8.8.3. Association with Huntingtin Neuronal Inclusions in the Cerebral Cortex

The role of huntingtin inclusions as a pathogenic or protective mediator for neuronal cell death is still a controversial issue. Huntingtin inclusions were investigated in the different neuronal populations in the same cortical regions and the same HD cases used in the present study in our laboratory (Mitchell, 2009). The presence of inclusions using 1C2 (marker for polyglutamine expansion; Millipore) was greatest in the anterior cingulate cortex (ACC), followed by primary motor (M1), primary sensory (S1), and primary and secondary visual cortices (V1 and V2). Double immunofluorescent labelling showed that 1C2 was colocalised with SMI32<sup>+</sup> pyramidal neurons, but the inclusions were not readily found in CB<sup>+</sup> and PV<sup>+</sup> interneurons, although these inclusions were found in close proximity to cell bodies (Mitchell, 2009). A small amount of inclusions were observed in the cortical interneurons in other studies (Becher et al., 1998; Meade et al., 2002), and previous reports
have indicated that the neuronal inclusions developed more rapidly in pyramidal neurons compared to cortical CB and PV interneurons in the R6/2 transgenic HD mice (Meade, et al., 2002).

Interestingly, when the quantitative inclusion data was compared to the dominant symptom profile of HD cases, some correlation was found between different cortical regions and the different symptom profiles, although no clear correlation was observed with regard to the pattern of related cortical cell loss. Therefore, these results indicate that the amount of inclusions in the different HD symptom groups is not associated with neuronal cell loss and support the hypothesis that inclusions are not directly involved in the cortical pathogenesis of HD. Other findings supporting this idea include the observation that nuclear localisation of mutant huntingtin can induce pathological changes without the formation of aggregates (Klement et al., 1998), and no observable neuronal dysfunction or loss with widespread huntingtin inclusion expression was shown in several different mouse models of HD (Davies, et al., 1997; Slow, et al., 2005). In addition, inclusions have also been shown to appear after the symptom onset (Wheeler, et al., 2000; Menalled, et al., 2002; Menalled, et al., 2003; Slow, et al., 2003; Slow, et al., 2005; Nguyen, et al., 2006). These findings together indicate that these intranuclear inclusions are mainly associated with the pyramidal neurons in the cortex and are not good indicators of cellular dysfunction in GABAergic interneurons.

8.9. Future Directions

The present study has shown an important relationship between symptomatology and variable loss of GABAergic interneurons in two functionally diverse regions of the cerebral cortex in HD. To gain a more detailed insight of neuronal changes and to further investigate current promising findings in the human cerebral cortex in HD, it will also be important to investigate the following prospective studies:

- **Other cortical regions:** Previous pathological studies in our laboratory have examined the pattern of cell loss (NeuN and SMI32 pyramidal neurons) across 8 regions of the cortex which included two cortical regions examined in the present study (Thu, 2006;
Nana, 2009). The other six regions include representative cortical regions from the frontal, parietal, occipital and temporal lobes (superior frontal cortex, primary sensory cortex, superior parietal cortex, primary and secondary visual cortices, and middle temporal cortex). Therefore, it would be interesting to further investigate the pattern of interneuron cell loss and its relation to symptomatology in these different functional areas of the cortex to further expand the understanding of the variable cortical pathogenesis in HD.

- **Morphological changes of interneurons:** Significant atrophy of the brain in HD is a characteristic feature of the disease in both human and animal models. However, although cell loss is observed in the human, a significant reduction of neuron number is not readily observed in animal models of HD. Hence, it has been postulated that reduced size of cell soma, and the loss or thinning of dendrites are major contributors of neuronal degeneration (Klapstein et al., 2001; Laforet, et al., 2001). For example, neuronal death may underlie many symptoms in late stage of HD, however, early deficits are more likely associated with cellular and synaptic dysfunction in the cortex which are apparent years before cell death or neurological symptoms (Backman & Farde, 2001; Berrios et al., 2002; Rosas, et al., 2005; Paulsen et al., 2008; Rosas, et al., 2008). Hence, it is important to investigate whether there are any underlying morphological changes in the GABAergic interneurons examined in the present study. Although not included as part of the thesis, a preliminary morphometric analysis was carried out to quantify the changes in neuronal processes of the GABAergic interneurons investigated in this study. A total of 25 images for each case were sampled and analysed using the neurite outgrowth and integrated morphometry analysis (IMA) neurite assay on MetaMorph™ 6.2.6 imaging software (Molecular Devices). The preliminary data have shown considerable morphological changes in the remaining interneurons in the two cortices, and these dystrophic changes followed the pattern of interneuron cell loss, i.e., major dendritic shrinkage was observed where there was major interneuron cell loss. Further investigations are currently being carried out with increased sample size and other regions of the cerebral cortex.

- **Neuropeptides:** Different interneuron subpopulations in the cortex can be reliably identified by their expression of calcium-binding proteins. However, there are other neurochemical markers such as neuropeptides that are used to label specific subsets of interneurons. These include somatostatin (SMT), cholecystokinin (CCK), neuropeptide
Y (NPY), vasoactive intestinal protein (VIP), corticotrophin-releasing factor (CRF), substance P receptor (SPR), and nitric oxide synthase (NOS) (Hendry et al., 1984; Rogers, 1992; Kawaguchi & Kubota, 1996, 1997; Markram, et al., 2004; Monyer & Markram, 2004; Petilla Interneuron Nomenclature Group, 2008; Kubota, et al., 2011). As with calcium-binding proteins, no single neuropeptide correlates with a single anatomical or electrophysiological type of interneuron. Expression patterns using combinations of calcium-binding proteins and neuropeptides may form important indicators for anatomical and physiological differences of interneurons in the HD cortex. Interneurons are therefore diverse in terms of their molecular properties and there are multiple biochemically definable subgroups of interneurons.

**Glial cells:** Glial cell changes have been shown in HD (Lieveens et al., 2001; Sapp et al., 2001; Shin et al., 2005). Glial cells protect against excitotoxicity by removing excess excitatory neurotransmitters from the extracellular space (Rothstein et al., 1996; Fields & Stevens-Graham, 2002). This protective function may be particularly relevant to the neuropathology of HD, since excitotoxicity has been a long-standing theory to account for the pathogenesis of HD (DiFiglia, 1990; Beal, 1994). For example, the striatal MSNs that receive massive excitatory input from the cortex are particularly vulnerable and are significantly lost in HD. Transgenic HD mice showed increased NMDA receptor activity in these neurons (Zeron, et al., 2002; Cepeda, et al., 2003). This may be due to an increase in cortical glutamatergic input or a decrease in clearance of extracellular glutamate. Clearance of extracellular glutamate is performed by GLT-1 (glutamate transporter-1) and GLAST (glutamate aspartate transporter) receptors that are expressed in surrounding astrocytes and transport extracellular glutamate into the cytoplasm, where glutamate is subsequently metabolised by glutamine synthase (Maragakis & Rothstein, 2001). It has been shown that GLT-1 expression level is decreased in transgenic mouse models of HD (Lieveens, et al., 2001; Behrens et al., 2002). This decrease in GLT-1 activity may lead to increased extracellular glutamate which may be the cause of increased vulnerability of the MSNs of the striatum to excitotoxicity. Thus, these studies suggest that glial cells and the glia-neuron interactions also play important roles in the pathogenesis of HD (Shin, et al., 2005).

**Gene, protein and functional studies:** Previous studies have demonstrated different gene expression in the striatum and cortex in HD (Luthi-Carter et al., 2000; Hodges, et al., 2006). For example, a total number of 9763 gene probe sets showed alterations in
the caudate nucleus in HD, and 1482 gene probe sets showed a change in the motor cortex (BA 4) in HD. Therefore, future studies should be directed at investigating the molecular basis of the differential pattern of interneuron cell loss in the HD cerebral cortex, and its relation to the variable symptom profile of each HD case. Genome-wide profiling of interneurons can be investigated using techniques such as microarray and laser capture microdissection (LCM) analyses where single cells can be dissected out of the tissue for genetic and proteomic analyses, hence these combined methods can be used to determine the gene expression profile of interneurons in the motor and cingulate cortices in the different HD symptom cases used in this study. Such a study would extend the current finding by revealing the regional cortical gene expression profile at the cellular level which could help to explain the molecular processes underpinning the variable symptom expression seen in HD. In addition, a recent study has shown changes in microRNA (miRNA) in the motor cortex in the HD human brain, suggesting that transcriptional changes may be regulated at the miRNA level (Packer et al., 2008). Also, the observations in the present study cannot precisely distinguish between the loss of interneurons as opposed to a down-regulation of calcium-binding proteins in the surviving cells in the cortex. Therefore, further analysis using western blotting, ELISA (enzyme-linked immunosorbent assay), in situ hybridisation, real-time PCR (polymerase chain reaction) and reverse transcriptase PCR techniques may be necessary to understand whether there is a variability in gene expression, antigenecity or specific reductions in protein concentrations in the HD cortex. Lastly, electrophysiological studies in mouse models of HD have shown an alteration in the inhibitory transmission of GABAergic interneurons (Gu, et al., 2005; Spampanato, et al., 2008; Cummings, et al., 2009). Although, this is not feasible on human tissue, further studies to indicate the electrophysiological roles of GABAergic interneurons in the cortex in HD would provide an additional knowledge of the significance of these neurons in the cortical circuitry. These studies would contribute to our understanding of the molecular, protein, and functional basis of the symptom heterogeneity in HD, and hence major implications for a better understanding of the correlation between cellular dysfunction and symptom profile in HD and other neurodegenerative diseases as Parkinson’s and Alzheimer’s diseases which are also characterised by a heterogeneous symptomatology.
8.10. Conclusion

This is the first study to demonstrate that there is a heterogeneous pattern of specific types of cortical GABAergic interneuron loss in HD cases that exhibit different clinical phenotypes. Our results suggest that in HD, interneuron cell loss in two functionally diverse regions of the cerebral cortex - the primary motor cortex and anterior cingulate cortex - is associated with motor and mood symptomatology, respectively. The general implication of our results is that the expanded CAG sequence in the HD gene can produce variable topographical patterns of cortical neuronal degeneration that contribute to specific symptoms. This may well have major implications for other single gene neurological diseases. The relationship demonstrated between symptom profiles and cortical interneuronal degeneration provides a novel perspective on understanding the neural basis of clinical heterogeneity in the cerebral cortex in HD.

The detailed pattern of interneuronal cell loss in the human cerebral cortex in HD has further implications for the understanding of the variability of disease progression in HD. It is likely that there are multiple pathways of degeneration affecting different cell types in different brain regions. Indeed our present findings in conjunction with previous studies have shown a major degeneration in the GABAergic interneurons as well as specific pyramidal cells across the cortical regions and this provides further support for the notion that the cortical degeneration is a major contributing factor to pathogenesis and symptom profile in HD. Lastly, future studies which document cognitive symptoms in addition to motor and mood symptomatology, will add to our understanding regarding the contribution of variable cortical pathology to symptom heterogeneity in HD.
**APPENDIX I**

Real sampling frame area $[a_{(frame)}]$ and disector height $[T_{(height)}]$ used for the estimate of average numerical density ($N_v$) by the optical disector method during stereological counting analysis in the two cortical regions

<table>
<thead>
<tr>
<th>Interneuron marker</th>
<th>Stereological parameters</th>
<th>Primary motor cortex (BA 4)</th>
<th>Anterior cingulate cortex (BA 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin-D28k</td>
<td>Counting frame area (XY) $[a_{(frame)}]$</td>
<td>0.0044 mm$^2$</td>
<td>0.0044 mm$^2$</td>
</tr>
<tr>
<td></td>
<td>Disector height (Z) $[T_{(height)}]$</td>
<td>0.01 mm</td>
<td>0.007 mm</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Counting frame area (XY) $[a_{(frame)}]$</td>
<td>0.0044 mm$^2$</td>
<td>0.0044 mm$^2$</td>
</tr>
<tr>
<td></td>
<td>Disector height (Z) $[T_{(height)}]$</td>
<td>0.008 mm</td>
<td>0.007 mm</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Counting frame area (XY) $[a_{(frame)}]$</td>
<td>0.0044 mm$^2$</td>
<td>0.0044 mm$^2$</td>
</tr>
<tr>
<td></td>
<td>Disector height (Z) $[T_{(height)}]$</td>
<td>0.008 mm</td>
<td>0.007 mm</td>
</tr>
</tbody>
</table>

Real grid size $[a_{(p)}]$ and sampling interval (d) used for the estimate of reference volume ($V_{ref}$) by the Cavalieri's estimator during stereological counting analysis in the two cortical regions

<table>
<thead>
<tr>
<th>Interneuron marker</th>
<th>Stereological parameters</th>
<th>Primary motor cortex (BA 4)</th>
<th>Anterior cingulate cortex (BA 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin-D28k</td>
<td>Grid size $[a_{(p)}]$</td>
<td>4 mm$^2$</td>
<td>2.56 mm$^2$</td>
</tr>
<tr>
<td></td>
<td>Sampling interval (d)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Grid size $[a_{(p)}]$</td>
<td>4 mm$^2$</td>
<td>2.56 mm$^2$</td>
</tr>
<tr>
<td></td>
<td>Sampling interval (d)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Grid size $[a_{(p)}]$</td>
<td>4 mm$^2$</td>
<td>2.56 mm$^2$</td>
</tr>
<tr>
<td></td>
<td>Sampling interval (d)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>
### APPENDIX II

CE values for the individual cases in the primary motor cortex (BA 4)

<table>
<thead>
<tr>
<th>Normal cases</th>
<th>Calbindin-D28k</th>
<th>Calretinin</th>
<th>Parvalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CE (Nv)</td>
<td>CE (Vref)</td>
<td>CE (Nv)</td>
</tr>
<tr>
<td>H108</td>
<td>0.09</td>
<td>0.022</td>
<td>0.05</td>
</tr>
<tr>
<td>H110</td>
<td>0.08</td>
<td>0.018</td>
<td>0.07</td>
</tr>
<tr>
<td>H111</td>
<td>0.06</td>
<td>0.016</td>
<td>0.05</td>
</tr>
<tr>
<td>H115</td>
<td>0.07</td>
<td>0.015</td>
<td>0.04</td>
</tr>
<tr>
<td>H118</td>
<td>0.07</td>
<td>0.015</td>
<td>0.05</td>
</tr>
<tr>
<td>H120</td>
<td>0.06</td>
<td>0.021</td>
<td>0.08</td>
</tr>
<tr>
<td>H121</td>
<td>0.09</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>H127</td>
<td>0.10</td>
<td>0.019</td>
<td>0.07</td>
</tr>
<tr>
<td>H129</td>
<td>0.09</td>
<td>0.021</td>
<td>0.05</td>
</tr>
<tr>
<td>H131</td>
<td>0.08</td>
<td>0.018</td>
<td>0.06</td>
</tr>
<tr>
<td>H132</td>
<td>0.08</td>
<td>0.014</td>
<td>0.05</td>
</tr>
<tr>
<td>H136</td>
<td>0.08</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>H139</td>
<td>0.08</td>
<td>0.018</td>
<td>0.05</td>
</tr>
<tr>
<td>H330</td>
<td>0.07</td>
<td>0.018</td>
<td>0.06</td>
</tr>
<tr>
<td>HD cases</td>
<td>Calbindin-D28k</td>
<td>Calretinin</td>
<td>Parvalbumin</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>CE (Nv)</td>
<td>CE (Vref)</td>
<td>CE (Nv)</td>
</tr>
<tr>
<td>HC60</td>
<td>0.07</td>
<td>0.018</td>
<td>0.07</td>
</tr>
<tr>
<td>HC68</td>
<td>0.10</td>
<td>0.018</td>
<td>0.05</td>
</tr>
<tr>
<td>HC72</td>
<td>0.08</td>
<td>0.016</td>
<td>0.05</td>
</tr>
<tr>
<td>HC73</td>
<td>0.09</td>
<td>0.015</td>
<td>0.07</td>
</tr>
<tr>
<td>HC79</td>
<td>0.09</td>
<td>0.015</td>
<td>0.05</td>
</tr>
<tr>
<td>HC82</td>
<td>0.07</td>
<td>0.019</td>
<td>0.08</td>
</tr>
<tr>
<td>HC85</td>
<td>0.07</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>HC86</td>
<td>0.07</td>
<td>0.015</td>
<td>0.07</td>
</tr>
<tr>
<td>HC93</td>
<td>0.06</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>HC95</td>
<td>0.08</td>
<td>0.019</td>
<td>0.06</td>
</tr>
<tr>
<td>HC99</td>
<td>0.10</td>
<td>0.015</td>
<td>0.04</td>
</tr>
<tr>
<td>HC101</td>
<td>0.08</td>
<td>0.017</td>
<td>0.06</td>
</tr>
<tr>
<td>HC107</td>
<td>0.06</td>
<td>0.019</td>
<td>0.06</td>
</tr>
</tbody>
</table>
CE values for the individual cases in the anterior cingulate cortex (BA 24)

<table>
<thead>
<tr>
<th>Normal cases</th>
<th>Calbindin-D28k</th>
<th>Calretinin</th>
<th>Parvalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CE (Nv)</td>
<td>CE (Vref)</td>
<td>CE (Nv)</td>
</tr>
<tr>
<td>H108</td>
<td>0.08</td>
<td>0.025</td>
<td>0.05</td>
</tr>
<tr>
<td>H110</td>
<td>0.08</td>
<td>0.018</td>
<td>0.04</td>
</tr>
<tr>
<td>H111</td>
<td>0.08</td>
<td>0.023</td>
<td>0.04</td>
</tr>
<tr>
<td>H112</td>
<td>0.08</td>
<td>0.027</td>
<td>0.06</td>
</tr>
<tr>
<td>H115</td>
<td>0.06</td>
<td>0.019</td>
<td>0.04</td>
</tr>
<tr>
<td>H118</td>
<td>0.07</td>
<td>0.017</td>
<td>0.05</td>
</tr>
<tr>
<td>H120</td>
<td>0.06</td>
<td>0.015</td>
<td>0.04</td>
</tr>
<tr>
<td>H121</td>
<td>0.07</td>
<td>0.019</td>
<td>0.04</td>
</tr>
<tr>
<td>H127</td>
<td>0.06</td>
<td>0.017</td>
<td>0.03</td>
</tr>
<tr>
<td>H131</td>
<td>0.09</td>
<td>0.019</td>
<td>0.05</td>
</tr>
<tr>
<td>H132</td>
<td>0.07</td>
<td>0.018</td>
<td>0.04</td>
</tr>
<tr>
<td>H136</td>
<td>0.07</td>
<td>0.018</td>
<td>0.05</td>
</tr>
<tr>
<td>H139</td>
<td>0.08</td>
<td>0.019</td>
<td>0.05</td>
</tr>
<tr>
<td>H330</td>
<td>0.07</td>
<td>0.017</td>
<td>0.04</td>
</tr>
<tr>
<td>HD cases</td>
<td>Calbindin-D28k</td>
<td>Calretinin</td>
<td>Parvalbumin</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>CE (Nv)</td>
<td>CE (Vref)</td>
<td>CE (Nv)</td>
</tr>
<tr>
<td>HC60</td>
<td>0.06</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>HC68</td>
<td>0.06</td>
<td>0.019</td>
<td>0.04</td>
</tr>
<tr>
<td>HC73</td>
<td>0.06</td>
<td>0.017</td>
<td>0.03</td>
</tr>
<tr>
<td>HC79</td>
<td>0.08</td>
<td>0.025</td>
<td>0.05</td>
</tr>
<tr>
<td>HC82</td>
<td>0.13</td>
<td>0.021</td>
<td>0.05</td>
</tr>
<tr>
<td>HC85</td>
<td>0.15</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>HC93</td>
<td>0.08</td>
<td>0.021</td>
<td>0.05</td>
</tr>
<tr>
<td>HC95</td>
<td>0.14</td>
<td>0.031</td>
<td>0.06</td>
</tr>
<tr>
<td>HC99</td>
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<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>HC101</td>
<td>0.12</td>
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<td>0.06</td>
</tr>
<tr>
<td>HC107</td>
<td>0.08</td>
<td>0.023</td>
<td>0.07</td>
</tr>
</tbody>
</table>
## APPENDIX III

Stereological parameters for the normal and HD cases grouped by dominant symptomatology in the primary motor cortex (BA 4)

<table>
<thead>
<tr>
<th>Stereological parameters</th>
<th>Normal</th>
<th>HD Motor</th>
<th>HD Mixed</th>
<th>HD Mood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB: 746532 ± 69281</td>
<td>CB: 322304 ± 7172</td>
<td>CB: 849246 ± 145411</td>
<td>CB: 834423 ± 99012</td>
</tr>
<tr>
<td></td>
<td>PV: 1555931 ± 128005</td>
<td>PV: 1364435 ± 393304</td>
<td>PV: 1445313 ± 132216</td>
<td>PV: 1694662 ± 254519</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>SD_{n,1}</td>
<td>CB: 24980</td>
<td>CB: 12423</td>
<td>CB: 290821</td>
<td>CB: 171494</td>
</tr>
<tr>
<td></td>
<td>PV: 461528</td>
<td>PV: 681222</td>
<td>PV: 264432</td>
<td>PV: 440840</td>
</tr>
<tr>
<td>CE (Nv) ≤ 0.10</td>
<td>CB: 0.0794: Yes</td>
<td>CB: 0.0929: Yes</td>
<td>CB: 0.0709: Yes</td>
<td>CB: 0.0752: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0567: Yes</td>
<td>CR: 0.0536: Yes</td>
<td>CR: 0.0624: Yes</td>
<td>CR: 0.068: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0653: Yes</td>
<td>PV: 0.0634: Yes</td>
<td>PV: 0.0642: Yes</td>
<td>PV: 0.063: Yes</td>
</tr>
<tr>
<td>CE (Vref) ≤ 0.10</td>
<td>CB: 0.0184: Yes</td>
<td>CB: 0.0161: Yes</td>
<td>CB: 0.0175: Yes</td>
<td>CB: 0.0188: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0173: Yes</td>
<td>CR: 0.0186: Yes</td>
<td>CR: 0.02: Yes</td>
<td>CR: 0.0186: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0175: Yes</td>
<td>PV: 0.0173: Yes</td>
<td>PV: 0.0187: Yes</td>
<td>PV: 0.0187: Yes</td>
</tr>
<tr>
<td>CV = SD_{n,1} / mean</td>
<td>CB: 0.3346</td>
<td>CB: 0.0385</td>
<td>CB: 0.3425</td>
<td>CB: 0.2055</td>
</tr>
<tr>
<td></td>
<td>CR: 0.4104</td>
<td>CR: 0.4917</td>
<td>CR: 0.3544</td>
<td>CR: 0.3029</td>
</tr>
<tr>
<td></td>
<td>PV: 0.2964</td>
<td>PV: 0.4993</td>
<td>PV: 0.183</td>
<td>PV: 0.2601</td>
</tr>
<tr>
<td>CE(Nv)^2 &lt; 0.5CV^2</td>
<td>CB: 0.0563: Yes</td>
<td>CB: 5.806: No</td>
<td>CB: 0.0428: Yes</td>
<td>CB: 0.1338: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0182: Yes</td>
<td>CR: 0.0119: Yes</td>
<td>CR: 0.0311: Yes</td>
<td>CR: 0.0504: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0485: Yes</td>
<td>PV: 0.0161: Yes</td>
<td>PV: 0.1231: Yes</td>
<td>PV: 0.0587: Yes</td>
</tr>
<tr>
<td>CE(Vref)^2 &lt; 0.5CV^2</td>
<td>CB: 0.003: Yes</td>
<td>CB: 0.1733: Yes</td>
<td>CB: 0.0026: Yes</td>
<td>CB: 0.0084: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0017: Yes</td>
<td>CR: 0.0014: Yes</td>
<td>CR: 0.0032: Yes</td>
<td>CR: 0.0038: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0035: Yes</td>
<td>PV: 0.0012: Yes</td>
<td>PV: 0.0010: Yes</td>
<td>PV: 0.0051: Yes</td>
</tr>
</tbody>
</table>

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Stereological parameters for the normal and HD cases grouped by striatal neuropathological grade in the primary motor cortex (BA 4)

<table>
<thead>
<tr>
<th>Stereological parameters</th>
<th>Normal</th>
<th>HD Grade 0-1</th>
<th>HD Grade 2</th>
<th>HD Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of interneurons (± SEM)</td>
<td>CB: 746532 ± 69281</td>
<td>CB: 708763 ± 210260</td>
<td>CB: 523023 ± 153682</td>
<td>CB: 855743 ± 129770</td>
</tr>
<tr>
<td></td>
<td>PV: 1556931 ± 128005</td>
<td>PV: 1744332 ± 131719</td>
<td>PV: 1517160 ± 329708</td>
<td>PV: 1224956 ± 79318</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>SD_{n-1}</td>
<td>CB: 24980</td>
<td>CB: 364181</td>
<td>CB: 307363</td>
<td>CB: 224768</td>
</tr>
<tr>
<td></td>
<td>CR: 880486</td>
<td>CR: 320998</td>
<td>CR: 824791</td>
<td>CR: 496053</td>
</tr>
<tr>
<td></td>
<td>PV: 461528</td>
<td>PV: 228144</td>
<td>PV: 659415</td>
<td>PV: 137382</td>
</tr>
<tr>
<td>CE (Nv) ≤ 0.10</td>
<td>CB: 0.0794: Yes</td>
<td>CB: 0.0857: Yes</td>
<td>CB: 0.0846: Yes</td>
<td>CB: 0.0652: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0567: Yes</td>
<td>CR: 0.0581: Yes</td>
<td>CR: 0.0616: Yes</td>
<td>CR: 0.0652: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0653: Yes</td>
<td>PV: 0.0604: Yes</td>
<td>PV: 0.0648: Yes</td>
<td>PV: 0.0652: Yes</td>
</tr>
<tr>
<td>CE (Vref) ≤ 0.10</td>
<td>CB: 0.0184: Yes</td>
<td>CB: 0.0163: Yes</td>
<td>CB: 0.0169: Yes</td>
<td>CB: 0.0193: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0173: Yes</td>
<td>CR: 0.0184: Yes</td>
<td>CR: 0.0181: Yes</td>
<td>CR: 0.0211: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0175: Yes</td>
<td>PV: 0.0168: Yes</td>
<td>PV: 0.0175: Yes</td>
<td>PV: 0.0206: Yes</td>
</tr>
<tr>
<td>CV = SD_{n-1} / mean</td>
<td>CB: 0.3346</td>
<td>CB: 0.5138</td>
<td>CB: 0.5877</td>
<td>CB: 0.2627</td>
</tr>
<tr>
<td></td>
<td>CR: 0.4104</td>
<td>CR: 0.159</td>
<td>CR: 0.472</td>
<td>CR: 0.3705</td>
</tr>
<tr>
<td></td>
<td>PV: 0.2964</td>
<td>PV: 0.1308</td>
<td>PV: 0.4346</td>
<td>PV: 0.1122</td>
</tr>
<tr>
<td>CE(Nv)^2 &lt; 0.5CV^2</td>
<td>CB: 0.0563: Yes</td>
<td>CB: 0.0278: Yes</td>
<td>CB: 0.0207: Yes</td>
<td>CB: 0.0616: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0182: Yes</td>
<td>CR: 0.1336: Yes</td>
<td>CR: 0.0171: Yes</td>
<td>CR: 0.031: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0485: Yes</td>
<td>PV: 0.2134: Yes</td>
<td>PV: 0.0222: Yes</td>
<td>PV: 0.3379: Yes</td>
</tr>
<tr>
<td>CE(Vref)^2 &lt; 0.5CV^2</td>
<td>CB: 0.003: Yes</td>
<td>CB: 0.001: Yes</td>
<td>CB: 0.0008: Yes</td>
<td>CB: 0.0054: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0017: Yes</td>
<td>CR: 0.0134: Yes</td>
<td>CR: 0.0015: Yes</td>
<td>CR: 0.0033: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0035: Yes</td>
<td>PV: 0.0165: Yes</td>
<td>PV: 0.0016: Yes</td>
<td>PV: 0.0336: Yes</td>
</tr>
</tbody>
</table>
Stereological parameters for the normal and HD cases grouped by dominant symptomatology in the anterior cingulate cortex (BA 24)

<table>
<thead>
<tr>
<th>Stereological parameters</th>
<th>Normal</th>
<th>HD Motor</th>
<th>HD Mixed</th>
<th>HD Mood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of interneurons (± SEM)</td>
<td>CB: 500927 ± 42284&lt;br&gt;CR: 1329161 ± 96473&lt;br&gt;PV: 702545 ± 79534</td>
<td>CB: 612882 ± 46125&lt;br&gt;CR: 1512260 ± 287661&lt;br&gt;PV: 821181 ± 250091</td>
<td>CB: 387246 ± 88868&lt;br&gt;CR: 738638 ± 102522&lt;br&gt;PV: 310278 ± 70670</td>
<td>CB: 143546 ± 30736&lt;br&gt;CR: 538871 ± 102529&lt;br&gt;PV: 143488 ± 22131</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
| CE ($N_v$) ≤ 0.10 | CB: 0.07339: Yes<br>CR: 0.04488: Yes<br>PV: 0.0609: Yes | CB: 0.0635: Yes<br>CR: 0.037: Yes<br>PV: 0.0574: Yes | CB: 0.0755: Yes<br>CR: 0.0536: Yes<br>PV: 0.0896: Yes | CB: **0.135: No**
| CE ($V_{ref}$) ≤ 0.10 | CB: 0.01962: Yes<br>CR: 0.0189: Yes<br>PV: 0.0176: Yes | CB: 0.0187: Yes<br>CR: 0.0178: Yes<br>PV: 0.0192: Yes | CB: 0.0223: Yes<br>CR: 0.0224: Yes<br>PV: 0.0216: Yes | CB: 0.0291: Yes<br>CR: 0.0279: Yes<br>PV: 0.0278: Yes |
| $CV = SD_{n,1} / mean$ | CB: 0.3044<br>CR: 0.2617<br>PV: 0.4082 | CB: 0.1064<br>CR: 0.269<br>PV: 0.4307 | CB: 0.3975<br>CR: 0.2404<br>PV: 0.3945 | CB: 0.3709: Yes<br>CR: 0.3296: Yes<br>PV: 0.2671: Yes |
| CE($N_v$)$^2$ < 0.5$CV^2$ | CB: 0.0581: Yes<br>CR: 0.0294: Yes<br>PV: 0.0223: Yes | CB: 0.3561: Yes<br>CR: 0.019: Yes<br>PV: 0.0178: Yes | CB: 0.0361: Yes<br>CR: 0.0497: Yes<br>PV: 0.0516: Yes | CB: 0.1334: Yes<br>CR: 0.041: Yes<br>PV: 0.2403: Yes |
| CE($V_{ref}$)$^2$ < 0.5$CV^2$ | CB: 0.0042: Yes<br>CR: 0.0052: Yes<br>PV: 0.0018: Yes | CB: 0.0309: Yes<br>CR: 0.0044: Yes<br>PV: 0.002: Yes | CB: 0.0032: Yes<br>CR: 0.0087: Yes<br>PV: 0.003: Yes | CB: 0.0062: Yes<br>CR: 0.0071: Yes<br>PV: 0.0108: Yes |
Stereological parameters for the normal and HD cases grouped by striatal neuropathological grade in the anterior cingulate cortex (BA 24)

<table>
<thead>
<tr>
<th>Stereological parameters</th>
<th>Normal</th>
<th>HD Grade 1</th>
<th>HD Grade 2</th>
<th>HD Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mean number of interneurons (± SEM)</td>
<td>CB: 500927 ± 42284</td>
<td>CB: 395186 ± 174702</td>
<td>CB: 352450 ± 152074</td>
<td>CB: 341614 ± 121383</td>
</tr>
<tr>
<td></td>
<td>PV: 702545 ± 79534</td>
<td>PV: 472722 ± 387590</td>
<td>PV: 413368 ± 215123</td>
<td>PV: 301742 ± 77822</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SDn&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CB: 152455</td>
<td>CB: 247066</td>
<td>CB: 264786</td>
<td>CB: 210241</td>
</tr>
<tr>
<td></td>
<td>PV: 286765</td>
<td>PV: 548135</td>
<td>PV: 372605</td>
<td>PV: 134792</td>
</tr>
<tr>
<td>CE (Nv) ≤ 0.10</td>
<td>CB: 0.07339: Yes</td>
<td>CB: 0.09: Yes</td>
<td>CB: 0.106: No</td>
<td>CB: 0.0986: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.04488: Yes</td>
<td>CR: 0.051: Yes</td>
<td>CR: 0.0464: Yes</td>
<td>CR: 0.0654: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0609: Yes</td>
<td>PV: 0.11: Yes</td>
<td>PV: 0.103: Yes</td>
<td>PV: 0.0896: Yes</td>
</tr>
<tr>
<td>CE (Vref) ≤ 0.10</td>
<td>CB: 0.01962: Yes</td>
<td>CB: 0.0263: Yes</td>
<td>CB: 0.0229: Yes</td>
<td>CB: 0.0238: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0189: Yes</td>
<td>CR: 0.0245: Yes</td>
<td>CR: 0.021: Yes</td>
<td>CR: 0.025: Yes</td>
</tr>
<tr>
<td></td>
<td>PV: 0.0176: Yes</td>
<td>PV: 0.0264: Yes</td>
<td>PV: 0.0218: Yes</td>
<td>PV: 0.0228: Yes</td>
</tr>
<tr>
<td>CV = SDn&lt;sub&gt;1&lt;/sub&gt; / mean</td>
<td>CB: 0.3044</td>
<td>CB: 0.6252</td>
<td>CB: 0.7513</td>
<td>CB: 0.6154</td>
</tr>
<tr>
<td></td>
<td>CR: 0.2617</td>
<td>CR: 0.5503</td>
<td>CR: 0.5391</td>
<td>CR: 0.4607</td>
</tr>
<tr>
<td></td>
<td>PV: 0.4082</td>
<td>PV: 1.16</td>
<td>PV: 0.9014</td>
<td>PV: 0.4467</td>
</tr>
<tr>
<td>CE(Nv)&lt;sup&gt;2&lt;/sup&gt; &lt; 0.5CV&lt;sup&gt;2&lt;/sup&gt;</td>
<td>CB: 0.0581: Yes</td>
<td>CB: 0.021: Yes</td>
<td>CB: 0.0199: Yes</td>
<td>CB: 0.0257: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0294: Yes</td>
<td>CR: 0.0085: Yes</td>
<td>CR: 0.0074: Yes</td>
<td>CR: 0.0201: Yes</td>
</tr>
<tr>
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<td>PV: 0.0223: Yes</td>
<td>PV: 0.009: Yes</td>
<td>PV: 0.0129: Yes</td>
<td>PV: 0.0402: Yes</td>
</tr>
<tr>
<td>CE(Vref)&lt;sup&gt;2&lt;/sup&gt; &lt; 0.5CV&lt;sup&gt;2&lt;/sup&gt;</td>
<td>CB: 0.0042: Yes</td>
<td>CB: 0.0018: Yes</td>
<td>CB: 0.0009: Yes</td>
<td>CB: 0.0015: Yes</td>
</tr>
<tr>
<td></td>
<td>CR: 0.0052: Yes</td>
<td>CR: 0.002: Yes</td>
<td>CR: 0.0015: Yes</td>
<td>CR: 0.0029: Yes</td>
</tr>
<tr>
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<td>PV: 0.0018: Yes</td>
<td>PV: 0.005: Yes</td>
<td>PV: 0.0006: Yes</td>
<td>PV: 0.0026: Yes</td>
</tr>
</tbody>
</table>


**APPENDIX IV**

Percentage difference between the values obtained from the Nv:Vref and optical fractionator methods for the first 100 sections in the primary motor cortex (BA 4)

<table>
<thead>
<tr>
<th>Normal cases</th>
<th>% difference CB</th>
<th>% difference CR</th>
<th>% difference PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>H108</td>
<td>6.78</td>
<td>1.90</td>
<td>4.00</td>
</tr>
<tr>
<td>H110</td>
<td>0.62</td>
<td>1.12</td>
<td>6.13</td>
</tr>
<tr>
<td>H111</td>
<td>1.31</td>
<td>5.10</td>
<td>0.22</td>
</tr>
<tr>
<td>H115</td>
<td>3.31</td>
<td>1.10</td>
<td>14.36</td>
</tr>
<tr>
<td>H118</td>
<td>3.21</td>
<td>5.02</td>
<td>7.62</td>
</tr>
<tr>
<td>H120</td>
<td>1.80</td>
<td>0.72</td>
<td>0.22</td>
</tr>
<tr>
<td>H121</td>
<td>0.08</td>
<td>0.30</td>
<td>3.35</td>
</tr>
<tr>
<td>H127</td>
<td>3.88</td>
<td>1.75</td>
<td>5.47</td>
</tr>
<tr>
<td>H129</td>
<td>4.74</td>
<td>1.84</td>
<td>2.60</td>
</tr>
<tr>
<td>H131</td>
<td>4.67</td>
<td>0.15</td>
<td>0.57</td>
</tr>
<tr>
<td>H132</td>
<td>2.57</td>
<td>0.80</td>
<td>2.05</td>
</tr>
<tr>
<td>H136</td>
<td>3.43</td>
<td>2.74</td>
<td>6.17</td>
</tr>
<tr>
<td>H139</td>
<td>3.64</td>
<td>4.69</td>
<td>3.79</td>
</tr>
<tr>
<td>H330</td>
<td>2.77</td>
<td>0.76</td>
<td>2.56</td>
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</table>

<table>
<thead>
<tr>
<th>HD cases</th>
<th>% difference CB</th>
<th>% difference CR</th>
<th>% difference PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC60</td>
<td>0.32</td>
<td>3.18</td>
<td>1.55</td>
</tr>
<tr>
<td>HC68</td>
<td>2.52</td>
<td>4.38</td>
<td>5.85</td>
</tr>
<tr>
<td>HC72</td>
<td>3.31</td>
<td>7.99</td>
<td>7.99</td>
</tr>
<tr>
<td>HC73</td>
<td>0.89</td>
<td>1.17</td>
<td>3.19</td>
</tr>
<tr>
<td>HC79</td>
<td>0.42</td>
<td>1.69</td>
<td>2.40</td>
</tr>
<tr>
<td>HC82</td>
<td>5.52</td>
<td>3.14</td>
<td>1.75</td>
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<tr>
<td>HC85</td>
<td>0.24</td>
<td>7.81</td>
<td>1.96</td>
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<td>HC86</td>
<td>0.57</td>
<td>3.48</td>
<td>3.82</td>
</tr>
<tr>
<td>HC93</td>
<td>6.90</td>
<td>6.82</td>
<td>4.09</td>
</tr>
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<td>HC95</td>
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<td>3.26</td>
<td>0.75</td>
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<td>HC99</td>
<td>1.23</td>
<td>3.73</td>
<td>4.26</td>
</tr>
<tr>
<td>HC101</td>
<td>0.95</td>
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<td>1.22</td>
</tr>
<tr>
<td>HC107</td>
<td>2.34</td>
<td>5.05</td>
<td>1.40</td>
</tr>
</tbody>
</table>
Percentage difference between the values obtained from the Nv:Vref and optical fractionator methods for the first 100 sections in the anterior cingulate cortex (BA 24)

<table>
<thead>
<tr>
<th>Normal cases</th>
<th>% difference CB</th>
<th>% difference CR</th>
<th>% difference PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>H108</td>
<td>8.64</td>
<td>4.80</td>
<td>0.26</td>
</tr>
<tr>
<td>H110</td>
<td>1.18</td>
<td>4.76</td>
<td>3.19</td>
</tr>
<tr>
<td>H111</td>
<td>1.66</td>
<td>3.65</td>
<td>5.17</td>
</tr>
<tr>
<td>H112</td>
<td>3.50</td>
<td>4.49</td>
<td>3.12</td>
</tr>
<tr>
<td>H115</td>
<td>1.45</td>
<td>3.28</td>
<td>4.05</td>
</tr>
<tr>
<td>H118</td>
<td>2.88</td>
<td>3.71</td>
<td>3.98</td>
</tr>
<tr>
<td>H120</td>
<td>5.09</td>
<td>0.05</td>
<td>3.23</td>
</tr>
<tr>
<td>H121</td>
<td>1.72</td>
<td>1.89</td>
<td>3.12</td>
</tr>
<tr>
<td>H127</td>
<td>3.73</td>
<td>4.80</td>
<td>3.08</td>
</tr>
<tr>
<td>H131</td>
<td>5.12</td>
<td>2.49</td>
<td>2.67</td>
</tr>
<tr>
<td>H132</td>
<td>4.03</td>
<td>4.85</td>
<td>3.42</td>
</tr>
<tr>
<td>H136</td>
<td>4.19</td>
<td>n/a</td>
<td>1.66</td>
</tr>
<tr>
<td>H139</td>
<td>2.14</td>
<td>4.43</td>
<td>2.50</td>
</tr>
<tr>
<td>H330</td>
<td>4.79</td>
<td>2.58</td>
<td>7.18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HD cases</th>
<th>% difference CB</th>
<th>% difference CR</th>
<th>% difference PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC60</td>
<td>3.49</td>
<td>3.16</td>
<td>2.33</td>
</tr>
<tr>
<td>HC68</td>
<td>1.60</td>
<td>5.98</td>
<td>6.03</td>
</tr>
<tr>
<td>HC73</td>
<td>6.86</td>
<td>5.97</td>
<td>2.26</td>
</tr>
<tr>
<td>HC79</td>
<td>4.34</td>
<td>4.53</td>
<td>2.39</td>
</tr>
<tr>
<td>HC82</td>
<td>4.58</td>
<td>5.49</td>
<td>2.14</td>
</tr>
<tr>
<td>HC85</td>
<td>1.64</td>
<td>0.71</td>
<td>0.57</td>
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