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Neural Progenitor Cells in the Huntington's Disease Human Brain

Maurice A. Curtis

A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy in Anatomy and Pharmacology at The University of Auckland, May, 2004
“... the functional specialization of the brain imposes on the neurones two great lacunae; proliferation inability and irreversability of intraprotoplasmic differentiation. It is for this reason that, once the development was ended, the founts of growth and regeneration of axons and dendrites dried up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.”

Santiago Ramon Y. Cajal (1913)
Nobel Prize in Medicine 1906
(Ramon y Cajal, 1991)
ABSTRACT

The recent demonstration of endogenous progenitor cells in the adult mammalian brain raises the exciting possibility that these undifferentiated cells may be able to generate new neurons for cell replacement in diseases such as Huntington’s disease (HD). Previous studies have shown that neural stem cells in the rodent brain subependymal layer (SEL), adjacent to the caudate nucleus, proliferate and differentiate into neurons and glial cells but no previous study has characterised the human SEL or shown neurogenesis in the diseased human brain. In this study, histochemical and immunohistochemical techniques were used to demonstrate the regional anatomy and staining characteristics of the normal and HD brain SEL using light and laser scanning confocal microscopy. The results demonstrated that the normal and HD SEL contained migrating neuroblasts, glial cells and precursor cells but there were more of each cell type present in the HD brain, and that the increase in cell numbers correlated with HD neuropathological grade. The normal and HD SEL was stained with a proliferative marker, proliferating cell nuclear antigen (PCNA), to label dividing cells. The results showed a significant increase in the number of dividing cells in the HD brain that correlated with HD grade and with CAG repeat length. Furthermore, the results showed that neurogenesis had occurred in the SEL as evidenced by co-localisation of PCNA and the neuronal marker βIII-tubulin. Also, gliogenesis had occurred in the SEL as evidenced by the co-localisation of PCNA with the glial marker GFAP. These studies also revealed a 2.6 fold increase in the number of new neurons in the HD SEL. PCNA positive cells were distributed throughout the SEL overlying the caudate nucleus but most notably the ventral and central regions of the SEL adjacent to the caudate nucleus contained the highest number of proliferating cells. I examined the SEL for mature cell markers and demonstrated many of the same cell types that are present in the normal striatum. With the exception of neuropeptide Y (NPY) neurons, there was a reduction in the number of mature neurons in the HD SEL. The NPY neurons were more abundant in the HD SEL suggesting they play a role in progenitor cell proliferation. The results in this thesis provide evidence of increased progenitor cell proliferation and neurogenesis in the diseased adult human brain and indicate the regenerative potential of the human brain. These findings may be of major relevance to the development of therapeutic approaches in the treatment of neurodegenerative diseases.
ACKNOWLEDGEMENTS

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I would like to thank Professor Mike Dragunow who, as my co-supervisor, has provided me with new ideas and fresh ways of looking at old problems. Mike has such an infectious enthusiasm for science that it is virtually impossible to leave Mike’s office without being so fired up that you want to cancel your holidays and spend the time doing exciting experiments. Thank you for all your ideas and enthusiasm.

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represented in chapter 5 and has weathered some of my fairly inclement questions regarding statistics. Thank you for all your assistance. Thanks also to Jocelyn Bullock for your work cutting sections to obtain Western blotting tissues, discussion about interesting destinations and for lending/giving me quality literature of the adventure variety.

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<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>Ara-C</td>
<td>cytosine-β-D-arabinofuranoside</td>
</tr>
<tr>
<td>bcl-2</td>
<td>B-cell lymphoma protein-2</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<tr>
<td>BLBP</td>
<td>brain lipid binding protein</td>
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<tr>
<td>BrdU</td>
<td>2-5 bromodeoxyuridine</td>
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<tr>
<td>Calr</td>
<td>calretinin</td>
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<td>ChAT</td>
<td>choline-acetyl transferase</td>
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<td>CN</td>
<td>caudate nucleus</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic adenosine monophosphate response element binding protein</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>DAB</td>
<td>3, 3 diamino benzidine</td>
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<td>DNA</td>
<td>deoxy-ribose nucleic acid</td>
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<td>EGF</td>
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<td>enkephalin</td>
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<td>glutamic acid decarboxylase</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>HD</td>
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<td>middle cerebral artery occlusion</td>
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<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
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<td>NeuN</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<td>neuropeptide Y</td>
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<td>NSE</td>
<td>neuron-specific enolase</td>
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<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<td>polysialylated-neural cell adhesion molecule</td>
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<td>Ts</td>
<td>time spent in the S-phase of the cell cycle</td>
</tr>
<tr>
<td>TUNEL</td>
<td>transferase mediated 14-deoxyuridine triphosphate nick end labelling</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>VIM</td>
<td>vimentin</td>
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Chapter 1

GENERAL INTRODUCTION

Until very recently it was a common belief that the mature brain was incapable of producing new neurons. Thus when a neuron died due to disease or injury it was never replaced. Recent studies have demonstrated otherwise. Detailed studies on the mammalian (Lewis, 1968b; Morshead et al., 1994; Reynolds and Weiss, 1992; Weiss et al., 1996a) and avian (Goldman and Nottebohm, 1983; Goldman et al., 1992) brain have revealed the presence of progenitor cells that reside in the germinal zone of the forebrain called the subependymal layer (SEL). These progenitor cells are capable of extensive self-renewal and can differentiate into a wide range of mature cell types, in particular neurons and glial cells (Craig et al., 1996; Eriksson et al., 1998; Kempermann et al., 2003; Kukekov et al., 1999; Nait-Oumesmar et al., 1999; Reynolds and Weiss, 1992; Weiss et al., 1996a; Zigova et al., 1998a). Progenitor cells possess the ability to last the life of the organism and can differentiate down any cell lineage, despite a slight reduction in proliferating cells in the SEL very late in the life of the animal. They have been identified in the SEL, especially in the region of the SEL that overlies the basal ganglia and also in the granule cell layer of the dentate gyrus in rodents (Altman and Das, 1965; Altman and Das, 1966; Lewis, 1968b; Luskin, 1993; Morshead et al., 1994; Reynolds and Weiss, 1992; Weiss et al., 1996a) and non-human primates (Brand and Rakic, 1979; Lewis, 1968b; Rakic, 1978). In the human brain however, the only structure found to contain progenitor cells has been the granule cell layer of the dentate gyrus (Eriksson et al., 1998; Reynolds and Weiss, 1992). In animal models of stroke (Arvidsson et al., 2002) and epilepsy (Ferland et al., 2002; Parent, 2003; Parent et al., 1998; Scott et al., 1998), increased numbers of progenitor cells have been identified in the SEL and dentate gyrus in response to the death of neurons in the injured brain. In particular, in animal models of stroke the progenitor cells in the SEL are induced to divide. These cells then migrate to the site of injury, in this case the striatum, where they differentiate into neurons in
response to the stroke-induced neurodegeneration (Arvidsson et al., 2002). Progenitor cell proliferation in the SEL was demonstrated by Parent et al (2002) who administered a chemoconvulsant to obtain a rodent model of epilepsy (Parent et al., 2002a). Progenitor cell proliferation and differentiation in the SEL in response to neurodegeneration in any area of the human brain has not yet been demonstrated. A number of cell proliferation and growth inducing agents, such as epidermal growth factor (Craig et al., 1996; Fallon et al., 1984; Kuhn et al., 1997; Reynolds et al., 1992; Tirassa et al., 2003), fibroblast growth factor-2 (Ciccolini and Svendsen, 1998; Kuhn et al., 1997; Martens et al., 2000; Vescovi et al., 1993) and brain-derived growth factor (Benraiss et al., 2001; Pencea et al., 2001; Tirassa et al., 2003; Zigova et al., 1998b), have also been identified that stimulate the production of new daughter cells from progenitor cells in the SEL.

The anatomical location of the region of the SEL that has been studied overlies the caudate nucleus. The caudate nucleus is one of the principal components of the basal ganglia, a group of subcortical nuclei that play a major role in movement control. The caudate nucleus is the primary site of neurodegeneration in Huntington’s disease (HD) (Aylward et al., 1996; Ferrante et al., 1991; Hedreen and Folstein, 1995; Vonsattel and DiFiglia, 1998). HD is an autosomal dominant neurodegenerative disorder that has motor, cognitive and behavioural symptoms with an onset in midlife (Vonsattel and DiFiglia, 1998; Vonsattel et al., 1997; Vonsattel et al., 1985). The motor dysfunction in HD comprises an involuntary choreiform (dance-like) movement of the limbs, which progresses until the person is unable to walk and becomes bedridden (Sharp and Ross, 1996). The person’s ability to speak and swallow is often affected thus leaving a susceptibility to aspiration pneumonia, which is a common cause of death for patients with advanced HD. Although the motor symptoms often signal the onset of HD, cognitive, psychological and behavioural changes can precede motor symptoms. The symptoms include depression and dementia, which may be related to the high incidence of suicide amongst HD sufferers (Deckel et al., 1998; Nance, 1997). The major alterations that occur in HD are changes in the neurochemistry, anatomy and cellular morphology of the basal ganglia in the brain. In particular the striatum (caudate nucleus and putamen), a major nuclear complex of the basal ganglia, demonstrates a marked loss of medium spiny efferent projection neurons (Kowall et al., 1987; Vonsattel and DiFiglia,
Medium spiny efferent projection neurons project to the globus pallidus and substantia nigra.

The most common methods used in the treatment of HD have been drugs to alleviate the movement and psychological disorder; more recently cell transplantation methods have been trialed to replace the cells that die in the striatum (Kopyov et al., 1998). To date drugs have failed to significantly improve the symptoms of HD because cell death continues and the disease progresses. Also, the recent transplantation of foetal striatal cells into the diseased striatum, in the hope that they will differentiate and replace the dying cells by establishing new connections between the striatum and the globus pallidus or substantia nigra, has thus far had mixed results and is still very much under development and therefore has not been suitable for the routine treatment of HD (Kopyov et al., 1998; Lindvall, 1995).

Since the rodent SEL contains progenitor cells and produces replacement neurons that migrate from the SEL to the stroke affected areas, the adult human brain may contain progenitor cells that produce new neurons in the SEL in the normal adult human brain. Furthermore, in response to degeneration of the caudate nucleus, the overlying SEL may produce increased numbers of progenitor cells that can replace the cells that die in HD. If the HD brain produces new cells in response to degeneration of the caudate nucleus, then the new cells may just need some form of manipulation to encourage them to replace the dying cells in the caudate nucleus. The research in this thesis therefore addresses the question of the potential of progenitor cells in the SEL overlying the caudate nucleus in the normal and HD human brain. In particular 4 aspects are specifically addressed: (1) The general organisation of cell types and cytoarchitecture in the normal and HD adult human SEL with an attempt to identify progenitor cell populations; (2) The proliferative capacity of progenitor cells in the normal and HD adult human SEL to determine whether proliferating cells are involved in neurogenesis or gliogenesis: (3) The distribution of proliferating progenitor cells in the normal and HD SEL; and (4) The range of mature cell types (neuronal and glial subtypes) that are present in the human and HD adult human SEL.
Chapter 2

LITERATURE REVIEW

2.1 Progenitor cells

In the development of the mammalian body from an embryo, extensive cell division gives rise to the vast array of differentiated, specialised cells that eventually constitute the adult animal. In order to maintain a constant number of differentiated cells in the different organs of the body, ongoing cell division and differentiation occur to compensate for cell death. The rate at which cell division occurs is dependent on the extent of cell death and injury (Weiss et al., 1996b). Many mammalian tissues contain immature cells that can self-renew to provide replacement cells in response to death resulting from injury or disease. These replacement cells are generally termed 'stem cells', a term that encompasses the first phases through which developing cells must pass. Stem cells are derived from germ cells (see figure 2.1A); the most primitive and undifferentiated of all stem cells is the totipotent fertilised oocyte that has the greatest ability to produce all the cell types necessary to form the adult animal (see figure 2.1B). These totipotent stem cells produce not only the inner cell mass of the blastocyst, which later differentiate to form all the tissue types of the animal, but also the placenta that sustains the developing organism throughout prenatal life (Clarke et al., 2000) (see figure 2.1C). The inner cell mass is comprised of cells called pluripotent cells (see figure 2.1) that are more differentiated than totipotent stem cells (see figure 2.1C). Pluripotent progenitor cells give rise to the cells of the 3 primary germ layers; the mesoderm, endoderm and ectoderm. Pluripotent progenitor cells are capable of extensive self-renewal (Craig et al., 1996; Gage et al., 1998; Haydar et al., 2000; Johansson et al., 1999; Nait-Oumesmar et al., 1999; Zigova et al., 1998a) (see figure 2.1C, D); they undergo symmetric or asymmetric division in order to maintain a population of immature cells and to provide some cells that terminally differentiate (see figure 2.2A, B):
Figure 2.1

Stem and progenitor cells during development and in the adult brain

Diagrammatic representation of the two germ cells (sperm and oocyte) that fuse to form the zygote which subsequently divides to give rise to totipotent stem cells. Totipotent stem cells have the capacity to differentiate into any cell type. The totipotent stem cells form the blastocyst which contains cells of the inner cell mass. Cells of the inner cell mass are pluripotent progenitor cells that ultimately give rise to the neural tube and other tissues in the body. During development of the foetal brain there is a supply of pluripotent progenitor cells that are responsible for the production of mature neurons and glial cells that comprise the adult mammalian brain. In the adult mammalian brain, the subependymal layer (SEL) maintains a population of multipotent progenitor cells that can divide throughout life to produce new neurons and glial cells.
Stem and progenitor cells during development and in the adult brain

A. Germ cells
B. Totipotent stem cells
C. Pluripotent progenitor cells
D. Multipotent progenitor cells
E. Adult brain

- Blastocyst
- Inner cell mass
- Foetus
- Ganglionic eminence
- Subependymal layer
Figure 2.2

Progenitor cells during *in vitro* and *in vivo* expansion, and during symmetric or asymmetric division

*In vitro* the inner cell mass, foetal and adult SEL cells can be expanded in culture and differentiated into either neurons or glial cells (A). Likewise, progenitor cells from these regions can divide *in vivo* (B) to form either two self-renewing progenitor cells by symmetric division or one progenitor cell and one potential neuron/glial cell by asymmetric division (B).
Progenitor cells

A

in vitro progenitor cell expansion

B

Asymmetric division

Symmetric division

Neurons/glia

Progenitors

Progenitor

in vivo progenitor cell expansion
1. Symmetric division occurs when a stem/progenitor cell divides into two daughter cells that each retain their ability to self-renew and are no more differentiated than the parent cell.

2. Asymmetric division occurs when a stem/progenitor cell divides and gives rise to one daughter cell that is more differentiated than its parent and another daughter cell that has stem cell characteristics.

Another level of cellular differentiation are the multipotent progenitor cells. Multipotent progenitor cells possess a limited capacity for self-renewal and are often unipotent (see figure 2.1E) (Weiss et al., 1996b), this means that the cell is limited to differentiate within only one predetermined germ layer. The daughter cells of multipotent progenitors also undergo symmetric or asymmetric division. The term 'precursor cell' is often used to encompass both stem cells and progenitor cells (Ciccolini, 2001; Weiss et al., 1996a; Weiss et al., 1996b).

In order to determine the self-renewal and multipotentiality (ability of a cell to differentiate down multiple lineages) of a particular cell, the cell must be removed from the animal and cultured. The clonal derivation of a single cell must be ensured in order to meet the criteria of self-renewal. A single cell is grown in culture and if it divides to produce two identical daughter cells, neither of which differentiates prior to the next division, then clonality has been established (see figure 2.2A). These cells must then be subject to extensive passaging to prove the capacity of the cell to self-renew followed by differentiation of a daughter cell into a mature cell type (Morshead et al., 1994; Morshead and van der Kooy, 1992). In vitro analysis of self-renewal and multipotentiality is performed routinely; however testing cells in vivo for self-renewal and multipotentiality has proven impossible at this stage due to the lack of specific stem cell markers. The best available technology currently in use are mitotic markers such as thymidine analogues that are incorporated into the DNA of dividing cells and retained within the cell. Mitotic markers are used routinely in animal studies to identify progenitor cells, but ethically cannot be used routinely in humans. In these situations, cell division marker proteins that are expressed during the cell cycle can be used to demonstrate the mitotic activity in the
tissue of interest. These markers demonstrate the cells that were dividing at the time of death.

2.2 Progenitor cells in the brain

2.2.1 Animal brain

Unlike other organs of the body, the adult brain was initially considered to contain a fixed number of neurons that were not replaced following cell death. However, in 1962 Altman demonstrated neural plasticity and neural replacement for the first time in the adult mammalian brain. The 1966 paper by Altman and Das detailed their extensive work looking for continued neurogenesis in the rodent brain. In these studies, tritiated thymidine was injected into the peritoneum of 6 hour, 2, 13, 30 day, and 4 month old rats. Thymidine is one of the base nucleoside constituents, a basic structural component of DNA and is incorporated into the nucleus of cells when the DNA is formed prior to cell division. If a thymidine analog, such as radiolabelled thymidine, is present it will be incorporated into the DNA instead of the endogenous thymidine (Dolbeare, 1996; Dolbeare and Selden, 1994). The thymidine is radiolabelled with tritium and the incorporated radioactivity can be localised using autoradiographic techniques to identify cells that were undergoing mitosis at the time of injection. Because the incorporated tritiated thymidine is diluted with each division it is possible to detect those cells that were mitotically active at the time of the radioisotope injection (Altman and Das, 1966; Goldman and Nottebohm, 1983; Goldman et al., 1992). Using this technique Altman and Das (1966) established, that in rats that were 6 hours old when the tritiated thymidine was injected and killed 6 hours post-injection, 23% of the cells in the ependymal layer (EPL) and SEL (see figure 2.3A) of the striatum had incorporated thymidine. The EPL and SEL are germinal zones during development but were believed to be dormant in the adult rat brain. In the EPL and SEL of animals that were killed 1 or 3 days after tritiated thymidine injection, 43% and 81% of the cells were labelled respectively. By 6 days post-injection, there was a reduction in the number of positively labelled cells in the EPL and SEL.
The subependymal layer and granule cell layer in the rodent brain

The adult rodent brain was not thought to be capable of producing new neurons however; the SEL and subgranular zone of the granule cell layer in the hippocampus in the adult rodent brain do remain neurogenic throughout the life of the animal.

A. The SEL that overlies the striatum in the rodent brain (red line) contains progenitor cells that are capable of becoming neurons or glial cells. SEL = subependymal layer, STR = striatum, LV = lateral ventricle, CC = corpus callosum.

B. The granule cell layer (red line) also contains a population of progenitor cells that are capable of becoming neurons and glial cells in the adult rodent brain. HP = hippocampus, GCL = granule cell layer, V = ventricle.
indicating either that the cells had divided sufficiently to serially dilute the injected label below detection level, or that the cells that contained the label had migrated and were no longer resident in the EPL or SEL. In the previous experiment, rat brain sections were taken from the lateral ventricle and olfactory bulb. Using quantitative autoradiography to detect the incorporation of tritiated thymidine they examined the olfactory bulb and SEL (see figure 2.3A) for the presence of radiolabelled thymidine and found that in the animals killed 1 or 3 days after the tritiated thymidine injections, the SEL contained large numbers of radiolabelled cells and the olfactory bulb contained none. In animals that were killed up to 6 months after the thymidine injection, labelled cells were present in the olfactory bulb and fewer cells were seen in the EPL and SEL. This led the investigators to conclude that cells of the EPL and SEL had divided in the SEL and had subsequently migrated to the olfactory bulb. Their subsequent studies showed that the tritiated thymidine labelled cells that had migrated to the internal and external granular layer of the olfactory bulb had the morphology of mature granule cells (Altman, 1962; Altman and Das, 1965; Altman and Das, 1966). In the same studies, Altman and Das also demonstrated thymidine incorporation and the proliferation of cells in the EPL and SEL of the striatum and in the granule cell layer of the dentate gyrus (see figure 2.3B). However, they could not rule out that some of these cells arose from the dentate gyrus itself and not the SEL. To assess the effect of arrested mitosis on cell proliferation and migration, they performed studies where colchicine, which arrests cell division, was injected into some animals. Within the dentate gyrus they saw cells with the characteristic morphology of arrested mitosis, but it was still not clear if these cells originated in the dentate gyrus or the SEL. The finding of neurogenesis in the SEL in the rat brain was not immediately accepted by the scientific community, as the majority of the tritiated thymidine cell labelling demonstrated was from the first few postnatal weeks and may have represented late postnatal developmental events, since the rat brain is poorly developed at birth. Furthermore, it was unclear at the time how much DNA repair was contributing to the extensive uptake of the tritiated thymidine. To demonstrate that postnatal neurogenesis also occurs in animals born with developmentally mature brains Altman and Das (1967) repeated their experiments on guinea pigs, whose brains are developmentally complete at birth (Altman and Das, 1967). The results demonstrated
uptake of thymidine and evidence for neurogenesis, in the sub-granular zone of the hippocampal granule cell layer (see figure 2.3B), up to postnatal day 36 (Altman and Das, 1967).

Some years later Goldman et al (1983) showed findings of neurogenesis in avian brains and that the higher vocal centre (HVC) in the canary brain was larger in males, correlating with the observation that males sing more than females. However when testosterone was administered systemically to females they sang more, which increased the size of the HVC (Goldman and Nottebohm, 1983). The question was asked ‘does this increase in size represent new neurons?’ Goldman and colleagues specifically addressed this crucial question by subcutaneously injecting one-year-old female canaries with testosterone followed by systemic injection of tritiated thymidine (Goldman and Nottebohm, 1983). At early time points post-injection no cells in the HVC were labelled but cells within the SEL were. As the time lapse after injection increased, more labelled cells were seen in the HVC and less in the SEL. Using axonal tracing techniques, investigators were able to determine the morphology of the new HVC cells to be neuronal. Most new neurons were described as “local circuit microneurons” but a small proportion were projection neurons (Goldman and Nottebohm, 1983). Goldman and colleagues suggested that because the size and number of cells in the HVC remains approximately constant, new HVC neurons would appear to be replacement cells (Goldman and Nottebohm, 1983).

The problem with these results in the rodent and avian brains was that they constituted indirect evidence for neuronal replacement because it was difficult to be certain the new cells were neurons. Also, the use of thick sections made it difficult to unequivocably say that a histologically stained cell was the same cell that contained the radiolabelled thymidine.

These concerns were addressed by experiments using the same paradigm of the tritiated thymidine injections, except that prior to killing the animal, recording electrodes were used to impale and record from HVC neurons (Paton and Nottebohm, 1984). Each whole cell from which recordings were taken was back filled with horseradish peroxidase
(HRP) for microscopic visualisation. Subsequent autoradiography revealed that 7 out of 74 neurons that were filled with HRP also contained tritiated thymidine; thus the cell had undergone division and subsequently developed a neuronal phenotype. This was the first direct evidence that new neurons were being produced in the adult vertebrate brain.

To further characterise one population of proliferating cells in the murine brain SEL, Morshead and van der Kooy (1992) performed cell kinetic studies to determine the cell cycle time, cell fate and characteristics of proliferating cell migration. The developmental subventricular zone persists in the adult brain as the SEL and is several cell layers thick comprising many mitotically active cells (Lewis, 1968a; Lewis, 1968b; Lewis, 1968c; Morshead and van der Kooy, 1992; Privat and Leblond, 1972). To determine the cell cycle time of the most proliferative population of SEL cells, Morshead and van der Kooy (1992) injected adult mice with the thymidine analog 2-5 BromodeoxyUridine (BrdU) every hour for 14 hours. BrdU, like thymidine, is incorporated into newly synthesised DNA in the S-phase of cell division and can be detected using immunohistochemistry. BrdU is useful as a cell kinetics tool for determining the number of cells in the S-phase of cell division when chromosomes are duplicated by DNA synthesis and the thymidine base is incorporated into DNA (Dolbeare, 1996; Dolbeare and Selden, 1994). Animals were sacrificed and perfused each hour, half an hour after the last BrdU injection and the brains were processed for immunohistochemistry. To heavily label cells throughout the S-phase to detect either continued division or quiescence, another group of mice were given BrdU each hour for 5 hours (the approximate length of S-phase in these cells) and were sacrificed 2 or 8 days later. Furthermore, in order to determine what proportion of cells are in the S-phase of cell division at any one time, another group of mice each received a single BrdU injection and were sacrificed half an hour later. Since each BrdU injection labels only cells in the S-phase of division “The fraction of the proliferating population labelled at the instant of the first BrdU injection is equal to the percentage of the total cell cycle time (Tc) spent in S-phase (Ts), or Ts/Tc. This is equal to the labelling index (LI), the proportion of labelled cells in the total population at any given time (t)” (Morshead and van der Kooy, 1992). The data showed Tc labelling to be greatest at 12.7 hours post initial BrdU injection. There was a linear increase in the number of BrdU
labelled cells to 8.5 hours but the number of labelled cells rose only slightly from the 8.5 hour time point to a maximum labelled cell number at 12.7 hours. By assuming that maximum labelling occurred when each dividing cell had undergone a full S-phase in the presence of BrdU and that it took 8.5 hours to go through the other parts of the cell cycle, the calculated S-phase time length was 4.2 hours. At 12.7 hours the maximum percentage of the SEL cell population that labelled with BrdU was 33%. Furthermore, with a single injection of BrdU approximately 11% of the SEL cells were labelled (Morshead and van der Kooy, 1992). These experiments were the first to examine the cell cycle kinetics of a major population of dividing cells in the SEL but did not take into consideration other cell populations that may have a slower cell cycle time or were in a quiescent phase at the time of BrdU labelling.

In the same study Morshead and van der Kooy (1992) performed cumulative BrdU injections over 5 hours in mice and sacrificed the animal either 2 or 8 days later to determine the fate of this rapidly dividing cell population. They found that 2 days post BrdU injection there were many BrdU labelled cells in the SEL (comparison with earlier time points using densitometry was not performed); however, at the 8 day time point there were no cells present in the SEL. This suggests either: (1) that these proliferating cells were either dividing rapidly, diluting the amount of BrdU in the cells by 50% each division, making the cells difficult to detect; or, (2) that these rapidly dividing cells in the SEL are dying shortly after division. Because the size of the SEL and the number of cells in it remain approximately the same throughout life and because BrdU labelled progeny were not detected outside the SEL they concluded that the BrdU labelled cells were dying, but in times of brain injury these cells may migrate to the site of injury and replace dying glial cells or microglial cells as was indicated in Altman’s findings (Altman, 1962; Morshead and van der Kooy, 1992). To demonstrate that dividing cells were not migrating out of the SEL, injections of LacZ-containing retrovirus were made into the lateral ventricle at a concentration that would label only a very small number of SEL cells per animal. The progeny of the initially infected cell would be detected by staining for the LacZ gene product β-galactosidase. In this experiment infected cells were only demonstrated within the SEL after 8 days. Taken together, with the results from the BrdU
labelling studies, the conclusion drawn was that the fate of rapidly proliferating cells in the SEL is cell death in the normal rodent brain. More recent studies have, however, demonstrated co-localisation of BrdU with neuronal and glial specific markers. This indicates that some of the proliferating cells in the SEL are becoming mature cells and do not succumb immediately to post mitotic cell death as previously described (Goldman et al., 1992; Wei et al., 2002; Weickert et al., 2000).

2.2.2 Human brain

Human neurogenesis or the presence of neural progenitor cells in the adult human brain has long been deemed impossible because of the very specialised function of the neurons within the human nervous system. A statement made by Santiago Ramon y Cajal in 1913 summarises the common thread of belief held by many up until this decade.

"... the functional specialization of the brain imposes on the neurones two great lacunae; proliferation inability and irreversibility of intraprotoplasmic differentiation. It is for this reason that, once the development was ended, the founts of growth and regeneration of axons and dendrites dried up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."
(Ramon y Cajal, 1991)

However a recent study performed by Eriksson and colleagues (1998) provided the first evidence that the adult human brain contained neuronal progenitor cells that undergo neurogenesis in the hippocampus. In their study, the brains of 5 patients, who had a previous diagnosis of oropharyngeal squamous cell carcinoma, were examined post-mortem. Each patient had previously been treated with BrdU (250 mg in 100 ml saline) intravenously to assess the proliferative activity of the tumour cells. The brain of one patient who also had oropharyngeal cancer but had not received BrdU treatment served as a control in this study. When the patients died the brains were removed and processed for single and double-label immunohistochemical analysis. The use of BrdU in these patients
enabled the spread of the tumour to be traced by the detection of proliferating tumour cells that had incorporated the BrdU into their DNA; not only in the tumour but in other proliferative areas of the body also. The use of the brain tissues for these experiments in no way influenced the treatment of these patients whilst they were alive. BrdU staining was evident in all the cases in which BrdU was administered. The time period between the BrdU injection and death ranged from 16 to 781 days. As the interval between BrdU injection and time of death increased the number of BrdU immunopositive cells that were located in the subgranular zone of the dentate gyrus decreased. There were approximately 220 positively labelled BrdU cells per mm$^3$ at the 16 day post-injection interval time compared with 15-30 BrdU positive cells per mm$^3$ at the longer injection to post-mortem intervals. In the hilus of the dentate gyrus, at longer post-injection time points, the number of cells ranged from approximately 45 to 10 cells per mm$^3$. In the granule cell layer the number of BrdU positive cells ranged from approximately 160 BrdU positive cells per mm$^3$ at shorter intervals to 20 BrdU positive cells per mm$^3$ at longer intervals. The decline in the number of BrdU positive cells may be due to death of the cells or may indicate that the cell has undergone repeated divisions such that the BrdU was diluted and could no longer be detected. To determine that neurogenesis had taken place, and to determine the cell fates, Eriksson and colleagues (1998) used fluorescent triple-labelling techniques to determine what cell type the BrdU positive cells had differentiated into. In this study the mature neuron markers neuronal nuclei (NeuN), neuron specific enolase (NSE) and calbindin, as well as the mature glial marker glial fibrillary acidic protein (GFAP) were used to detect neurons and glial cells respectively (Eriksson et al., 1998). The results demonstrated that glial cells closely surrounded the neurons. The presence of BrdU/NeuN double-labelled small oval and round cells in the dentate gyrus demonstrated that new neurons had been formed in the adult human brain that were remarkably similar to granular neurons and 22.0% ± 2.4% of all BrdU positive cells in the granule cell layer were also NeuN positive. BrdU/NSE double-labelled cells could be detected in all cases, except in the unlabelled control brain, and were present in approximately the same numbers as BrdU/NeuN double-labelled cells. BrdU/calbindin double-labelled cells were also demonstrated in the dentate gyrus but there were less than the numbers of NeuN or NSE positive cells; 7.9% ± 2.2% of all BrdU positive cells also expressed calbindin.
Calbindin is a marker of a specific subset of neurons, which explains this lower percentage of calbindin positive cells; thus, NeuN and NSE are better markers for labelling neurons in general. Within the dentate gyrus 18.1% ± 1.8% of all BrdU positive cells were also GFAP positive. These cells were typically star shaped and resembled astrocytes with long, thin GFAP positive processes. In all of the immunostaining experiments that were performed, co-localisation of neuronal and glial markers in the same cell was never detected. Taken together these results demonstrate unequivocally the presence of both new neurons and glial cells in the normal adult human dentate gyrus.

Using the same cases as above, Eriksson and colleagues (1998) examined the SEL in the region of the caudate nucleus for the presence of BrdU positive cells. A small number of BrdU positive cells were detected in this region, however none were double-labelled with glial or neuron specific markers. Thus it was concluded that newly generated cells in the SEL must first migrate from the SEL before terminal differentiation could take place (Eriksson et al., 1998).

Experiments carried out by Kukekov and colleagues in 1999 demonstrated that the SEL contained cells that could be extracted from the post-mortem human brain and be cultured in vitro to form multicellular clumps of mitotically active cells termed 'spheres' or 'neurospheres' (Kukekov et al., 1999). For these studies 10 temporal lobe, hippocampal and SEL specimens were used from patients undergoing a temporal lobectomy for intractable epilepsy. The temporal lobe, hippocampus and SEL of each brain sample were separated and processed in separate vials; each of the 10 cases were also processed separately but concurrently under identical culture conditions. Each sample was collected into and dissociated in serum free culture media. The samples were trypsinised, tritutated, mechanically filtered through nylon gauze and centrifugated for 5 minutes at 1200 revolutions per minute (rpm) before being resuspended in nutrient rich medium on a non-adhesive substrate and were incubated at 37°C with 5% CO2. The cells had their nutrient media changed every 3-4 days, which not only replenished essential nutrients but also helped remove debris that remained from the initial dissection. After 7-10 days small
spheres of cells appeared that contained a self-renewing cell population, much like those seen in rodents (Chiasson et al., 1999; Morshead et al., 1994; Reynolds and Weiss, 1996; Tropepe et al., 1999; Vescovi et al., 1993; Weiss et al., 1996a). Because the coating on the culture plates prevented the cells from adhering, the cells could not migrate or differentiate and thus remained as neurospheres. The neurospheres could be broken up by trituration into single cells and would again form neurosphere-like structures indicating that the dissected cells could clonally give rise to new cells that would further divide to produce secondary neurospheres. Approximately 100-150 neurospheres were generated per 200,000 cells originally dissected. In these experiments Kukekov and colleagues were unable to generate secondary neurospheres from cortical tissue, but were able to generate secondary neurospheres by clonal regeneration from hippocampal and SEL primary neurosphere preparations. Immunohistochemical analysis of the fixed neurosphere cultures, and of cultures which had been differentiated on a membrane that enabled the neurospheres to adhere, flatten and differentiate, demonstrated the presence of proliferative, neuronal and glial cell types within the neurospheres. In particular, some of the cells within the neurospheres were immunoreactive for the immature, neuron specific marker βIII-tubulin. Others were immunoreactive for the glial markers vimentin and GFAP (Kukekov et al., 1999). To further test that the cells demonstrated in these cultures were in fact new neurons and glial cells, and not mature cells that remained from the primary cultures, BrdU was added to the secondary neurosphere cultures before the cells were differentiated and fixed. Further double immunohistochemical analysis demonstrated the presence of BrdU and βIII-tubulin immunoreactivity in the same cell; some of the BrdU/βIII-tubulin positive cells had a pyramidal cell morphology. Many BrdU and GFAP double-labelled cells were also demonstrated within and at the periphery of the neurospheres. In the study performed by Kukekov and colleagues (1999), SEL tissue was also paraffin embedded and processed for immunohistochemical staining and analysis. An antibody to one of the proliferating cell nuclear antigens, Ki67, was used to detect dividing cells that were between G1 and M phases of cell division. In this study Ki67 positive cells were demonstrated surrounded by numerous Nissl counterstained cells in the SEL in the dentate gyrus. No mention was made of the number of Ki67 positive
cell bodies present or whether GFAP, βIII-tubulin or other cell specific markers were present in the SEL sections.

Taken together, these experiments demonstrate that stem/progenitor cells can be dissected from the adult human hippocampus and SVZ, but not from the temporal cortex, and be cultured to produce neurospheres in which single cells divide that give rise to both neurons and glial cells (Kukekov et al., 1999). Experiments carried out by Palmer and colleagues have demonstrated that neuron and glial forming SEL progenitor cells can be extracted from post-mortem brains with post-mortem delays of more than 20 hours (Palmer et al., 2001).

2.3 General organisation of the subependymal layer

2.3.1 Anatomy of the rodent and bovine subependymal layer

Within the SEL the cells have a unique pattern of immunostaining characteristics, morphology, and cytoarchitecture. A small number of studies have been carried out to demonstrate the anatomy of the SEL. In the rodent, Lois et al (1996) and Doetsch et al (1997) have extensively characterised the cytoarchitecture of the SEL (Doetsch et al., 1997; Lois et al., 1996). In the bovine, one study by Rodriguez-Perez et al (2003) has characterised the cytoarchitecture of the SEL and the lateral ventricle wall. In the human brain Bernier et al (2002) have performed one preliminary study aimed at demonstrating some of the cell types in the SEL.

The light microscopic and ultrastructural analysis performed by Doetsch and colleagues (1997) in the adult mouse brain is the only extensive characterisation of the major cell types in the SEL (Doetsch et al., 1997); a remarkably similar study has demonstrated the cell types in the rostral migratory stream which is an extension of the SEL (Lois et al., 1996). Doetsch and colleagues (1997) used immunohistochemistry to look at PSA-NCAM, βIII-tubulin, GFAP, vimentin and nestin (see table 2.1 detailing the various
Table 2.1  
Table of antibodies and the cell or cellular component detected by the antibody

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Abbreviation</th>
<th>Cell or cellular component detected by antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD17</td>
<td>BD17</td>
<td>β2, β3 subunits of the GABA&lt;sub&gt;A&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>BD24</td>
<td>BD24</td>
<td>α1 subunit of the GABA&lt;sub&gt;A&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>Beta III-Tubulin</td>
<td>βIII-tubulin</td>
<td>Immature neurons</td>
</tr>
<tr>
<td>Brain Lipid Binding Protein</td>
<td>BLBP</td>
<td>Radial glial cell marker</td>
</tr>
<tr>
<td>BromodeoxyUridine</td>
<td>BrdU</td>
<td>Cells that have divided and incorporated BrdU into their DNA</td>
</tr>
<tr>
<td>Calbindin</td>
<td>Calb</td>
<td>Calcium binding protein that labels GABAergic medium spiny neurons in the striatum</td>
</tr>
<tr>
<td>Choline-Acetyl Transferase</td>
<td>ChAT</td>
<td>Cholinergic interneurons in the striatum</td>
</tr>
<tr>
<td>DARP-32</td>
<td></td>
<td>Mature medium spiny neurons</td>
</tr>
<tr>
<td>Enkephalin</td>
<td>ENK</td>
<td>GABAergic neurons that project from the striatum to the globus pallidus (externus)</td>
</tr>
<tr>
<td>Ferritin</td>
<td></td>
<td>Microglial cells</td>
</tr>
<tr>
<td>Gamma 2</td>
<td>γ2</td>
<td>γ2 subunit of the GABA&lt;sub&gt;A&lt;/sub&gt; receptor</td>
</tr>
<tr>
<td>Glial Fibrillary Acidic Protein</td>
<td>GFAP</td>
<td>Glial cells (astrocytes)</td>
</tr>
<tr>
<td>Glutamic Acid Decarboxlase</td>
<td>GAD</td>
<td>GABAergic cells (predominantly striatal GABAergic interneurons)</td>
</tr>
<tr>
<td>LFA Lectin</td>
<td></td>
<td>Cell surface marker for sialylation</td>
</tr>
<tr>
<td>Meis-2</td>
<td></td>
<td>Transcription factor that is a marker for developing medium spiny neurons</td>
</tr>
<tr>
<td>Nestin</td>
<td></td>
<td>Precursor cell marker/immature glial cell marker</td>
</tr>
<tr>
<td>Neuron-Specific Enolase</td>
<td>NSE</td>
<td>Neurons</td>
</tr>
<tr>
<td>NeuroPeptide Y</td>
<td>NPY</td>
<td>Subset of striatal interneurons</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Parv</td>
<td>Calcium binding protein located on a subset of striatal interneurons</td>
</tr>
<tr>
<td>Polysialylated-Neural Cell Adhesion Molecule</td>
<td>PSA-NCAM</td>
<td>Labels migrating neuroblasts</td>
</tr>
<tr>
<td>Proliferating Cell Nuclear Antigen</td>
<td>PCNA</td>
<td>Cell cycle marker that labels cells undergoing cell division</td>
</tr>
<tr>
<td>S 100 beta</td>
<td></td>
<td>Immature glial cell marker</td>
</tr>
<tr>
<td>Substance P</td>
<td>SP</td>
<td>GABAergic neurons that project from the striatum to the globus pallidus (internus) &amp; substantia nigra</td>
</tr>
<tr>
<td>Vimentin</td>
<td>VIM</td>
<td>Immature glial cells</td>
</tr>
</tbody>
</table>
antibody labels used in these studies) antibodies on adult mice brain sections to detect specific cell types in the SEL. They also used tritiated thymidine and various histological markers for mitotic cells and their cellular morphology (Doetsch et al., 1997). From ultrastructural studies 5 different cell types were identified in the rodent (see figure 2.4):

**Type A** - The type A cells were characterised by an elongated cell body with one or two processes and a cytoplasm that contained free ribosomes and a few short cisternae of rough endoplasmic reticulum. These cells had a small Golgi apparatus and many microtubules oriented along the axis of the cell (Doetsch et al., 1997). In coronal sections the type A cells had the smallest cross sectional area of any of the SEL cells. The type A cells in the SEL greatly resembled, or were identical to, the type A cells in the murine rostral migratory stream (Lois et al., 1996). In immunohistochemical light microscopy studies, Doetsch and co-workers demonstrated that type A cells were strongly immunoreactive for both PSA-NCAM and for βIII-tubulin. Immunoreactivity for PSA-NCAM was evident along the entire cell membrane irrespective of which cell type they contacted. βIII-tubulin mainly stained the cytoplasm of type A cells. Type A cells formed chains of cells that were most often oriented tangentially to the SEL as if migrating in the direction of the olfactory bulb. Lois et al (1996) and Doetsch et al (1997) suggest that these cells are migrating neuroblasts/immature neurons that are migrating toward the olfactory bulb (Doetsch et al., 1997; Lois et al., 1996)(see figure 2.4).

**Type B** - Type B cells have had irregular contours that often filled the spaces around neighbouring cells (Doetsch et al., 1997). Most of the type B cells were abundant in intermediate filaments and had the appearance of astrocytes. Two types of type B cell astrocytes were identified, type B₁ and type B₂. Type B₁ cells were larger, had more cytoplasm and were less electron dense compared with type B₂ cells. Type B₁ cells were usually located adjacent to the ependymal cells where they extended many processes that covered the ependymal cells. Type B₂ cells were located adjacent to the striatal parenchyma. Both type B₁ and B₂ cells had the ultrastructural and immunocytochemical characteristics of astrocytes (Doetsch et al., 1997; Lois et al., 1996) (see figure 2.4).
Figure 2.4

Diagrammatic representation of the mouse subependymal layer
(as described by Doetsch et al (1997))

This figure demonstrates the anatomy of the mouse SEL. There are 5 major cell types present in the rodent SEL (Types A-E).

**Type A** neuroblasts become the mature neurons for the olfactory bulb during adulthood and form the cerebral cortex during development.

**Type B** glial cells support the migration of neurons from the SEL and are predominantly large sized cells in the SEL.

**Type C** possible precursor cells may be capable of forming any of the cell types of the brain.

**Type D** tanycytes are rare cells that are seen in the SEL.

**Type E** ependymal cells are the cells that form the EPL. The EPL is the barrier between the cerebrospinal fluid of the lateral ventricle and the SEL.

Beneath the EPL and SEL is a myelin layer that separates the SEL from the caudate nucleus.
Diagrammatic representation of the mouse subependymal layer (as described by Doetsch et al., 1997)
Type C - Type C cells were described as being “larger, more spherical (less elongated) and more electron lucent than type A cells but more electron dense than type B cells.” (Doetsch et al., 1997). The type C cell contours were smoother than other cell types and were often demonstrated to contact the type A cells. Type C cells had fewer processes than the type B cells. In detailed immunohistochemical studies it was demonstrated that type C cells were negative for GFAP, vimentin, PSA-NCAM and βIII-tubulin (see table 2.1 for a description of what these antibodies label). Nestin immunostaining was present in clumps in the cytoplasm. Because of their staining characteristics, type C cells were thought to be immature cells. Type C cells had the greatest proliferative activity with 50% of the triitated thymidine positive cells fitting the description of cell type C. Type C cells were often found to be juxtaposed to the chains of type A cells and electron microscopic analysis demonstrated contacts between them. A distinguishing feature of this type of cell was that its processes contacted both the EPL and the striatal parenchyma (Doetsch et al., 1997). Doetsch and colleagues suggested that these cells might be the precursors of migrating neuroblasts, although the glial precursor is also believed to reside within the SEL; a definitive precursor cell type or location has yet to be identified (see figure 2.4).

The ratio of type A:B:C cells was expressed as a 3:2:1 respectively; type A, B and C cells accounted for approximately 30%, 20% and 10% respectively, of the SEL in the lateral ventricle wall. Type C cells were not present in the rostral migratory stream and the type C cell number declined at about 2 mm anterior to bregma near the rostral striatum (Doetsch et al., 1997).

Type D - Type D cells contained microvilli, had irregularly shaped nuclei, and often contacted the lateral ventricle. They occurred infrequently and were located amongst the ependymal cells that are in contact with the cerebrospinal fluid (see figure 2.4).

Type E - Type E cells are ependymal cells and comprise the second most abundant cell type in the lateral ventricular wall. They form a single layer that separates the SEL from the ventricle. They are large cells that have many microvilli that protrude into the lateral
ventricle and are often ciliated. Type E cells are tightly packed together apart from when a type D cell separates them. Type E cells are usually moderately nestin immunoreactive (Doetsch et al., 1997) (see figure 2.4).

In a further immunohistochemical cytoarchitectural characterisation of the SEL, Rodriguez-Perez and colleagues (2003) undertook to divide the bovine SEL into its respective cell layers. The lateral wall, medial wall, dorsal roof and ventral floor of the lateral ventricle were examined. For this study standard immunohistochemical staining techniques were used for paraffin embedded and cryoprotected tissue. Sections were cut at either 10 or 50 μm to look at the cellular structure throughout the rostral-dorsal aspect of the lateral ventricle. The antibodies that were used included βIII-tubulin, PCNA, S100β (immature glial marker), vimentin, nestin, PSA-NCAM, LFA lectin (cell surface marker for sialylation), brain lipid binding protein (BLBP) and NSE (Rodríguez-Pérez, 2003). Upon examination of the different regions of the lateral ventricle wall Rodriguez-Perez and colleagues identified type A, B, C and E cells that resembled those seen by Doetsch et al (1997) and Lois et al (1996), and also noted that the distribution and orientation of the type A, B and C cells differed throughout the ventricle. They divided the lateral ventricle wall into 3 distinct wall types, type 1-3.

Type 1 lateral ventricle wall cytoarchitecture consisted of an EPL that overlaid a very thin SEL that was only one or two cells thick and consisted of subependymal neuroblasts and some PCNA immunoreactive cells. The neuroblasts were oriented parallel with the ependymal cells in coronal section. Immediately beneath the subependymal layer was a glial network layer with astrocytes that were aligned along the dorsal – ventral axis (see figure 2.5). The medial septum and dorsal roof aspect of the lateral ventricle beneath the corpus callosum were comprised of type 1 cytoarchitecture, as was the entire posterior and entire inferior horn of the lateral ventricle both medial and lateral. The bulk of the ventricle wall was made up of type 1 cytoarchitecture (see figure 2.5).
Figure 2.5

Diagrammatic representation of the 3 types of lateral ventricle wall cytoarchitecture
(as described by Rodriguez-Perez et al (2003))

This figure diagrammatically illustrates the 3 different types of lateral ventricle walls that have been identified in the bovine brain by Rodriguez-Perez et al (2003). The coronal brain slice diagrams demonstrate the location of each ventricle wall type. The diagrams below demonstrate the cell types and orientation of the cells within each type of lateral ventricle wall.

**Type 1** lateral ventricle wall consisted mostly of astrocytes, a few subependymal neuroblasts and the occasional PCNA positive cell. Type 1 wall was located throughout most of the septal and superior aspect of the ventricle and is the only ventricle wall type located in the posterior and inferior horns of the ventricle. Type 1 wall did not cover the striatum, rather was located mostly overlying white matter (Rodríguez-Pérez, 2003).

**Type 2** lateral ventricle wall consisted of many astrocytes, subependymal neuroblasts and many PCNA positive cells. The SEL is much thicker in these regions compared with type 1 lateral ventricle wall. The type 2 lateral ventricle wall was much thicker compared with type 1 ventricle wall. Type 2 lateral ventricle wall was located overlying the striatum (STR) on the lateral part of the ventricle wall. Also, a short segment of type 2 lateral ventricle wall was present on the ventral part of the septum but only in the rostral region at levels where type 3 lateral ventricle wall was present.

**Type 3** lateral ventricle wall was comprised of astrocytes that were aligned in a radial orientation. Also in the type 3 locations there were many subependymal neuroblasts and PCNA positive cells. Type 3 lateral ventricle wall was only located in the ventral part of the rostral horn of the lateral ventricle and was reminiscent of the rostral migratory stream described by Doetsch et al (1997). Type 3 lateral ventricle wall was most distinguishable by the orientation of the astrocytes, perpendicular to the ependymal layer.
Type 1
Type 2
Type 3

Ependymal cells
Astrocytes
PCNA positive cells
Subependymal neuroblasts

EPL
SEL
Type 2 lateral ventricular wall was made up of an EPL. Unlike type 1 ventricle wall, some ependymal cells had processes that extended through the SEL. The SEL was densely packed with neuroblasts that were located close to the ependymal cells. Beneath the SEL was the glial network layer that contained astrocytes and other glial cells. Scattered throughout the glial cell layer and SEL were neuroblasts and PCNA positive cells (see figure 2.5). The subependymal layer and glial network layer reported by Rodriguez-Perez et al in the bovine brain together form the subependymal layer described by Doetsch et al (1997) (Doetsch et al., 1997; Rodríguez-Pérez, 2003). The type 2 cytoarchitecture was demonstrated only in the lateral wall of the lateral ventricle that overlay the body of the caudate nucleus and in the ventral fifth of the medial septum at the rostral-caudal level of the body of the caudate nucleus (see figure 2.5).

Type 3 cytoarchitecture consisted of a tightly packed EPL. The ependymal cells had long processes that projected down into the glial network layer. Beneath the EPL was the subependymal layer, which also comprised neuroblasts and the cell bodies of the occasional BLBP immunopositive cells, whose processes occasionally appeared between the ependymal cells. Cells immunoreactive for PCNA could also be seen scattered throughout the SEL and glial network layers; the separation between these two layers was indistinct. Beneath the SEL the astrocytes and neuroblasts in the glial network layer were oriented perpendicular to the EPL such that the cells appeared to be elongated and moving into or ventral to the striatum (see figure 2.5). The type 3 cytoarchitecture was only present in the ventral portion of the rostral horn of the lateral ventricle where the medial and lateral ventricular walls meet. This form of stratification appeared in only a small number of sections that were taken from the rostral aspect of the striatum. The location of the type 3 cytoarchitecture corresponded to the rostral migratory stream in the mouse, described by Lois (Lois et al., 1996; Rodríguez-Pérez, 2003). These cells could be identified up to the glomeruli of the olfactory bulb (see figure 2.5).

All 3 ventricular wall types demonstrated some positive staining for S100β, vimentin and nestin, which indicated that these cells were soon to divide, or had recently divided. In the SEL many βIII-tubulin immunoreactive strands were demonstrated and some cells
stained positively with antibodies to NSE and GFAP. Nuclei immunoreactive for PCNA were more numerous in type 2 and 3 ventricle walls than in type 1. This study was the first to demonstrate differences in the cytoarchitecture of the lateral ventricle wall along its length.

2.3.2 Anatomy of the human subependymal layer

The cytoarchitectural and precise cellular anatomy of the human SEL has not been fully characterised. However, there have been 2 reports of studies in humans reporting briefly on the anatomy of the human SEL. In the first study, Globus and Kuhlenbeck (1944) performed an analysis of the human SEL in order to classify the range of tumours in the subependymal region. They did not detail extensively the normal cytoarchitecture of the SEL but rather focussed on the neuropathology of subependymal tumours. This was a hallmark paper that identified the diversity of the pathology of the SEL. Their contribution to the understanding of the normal morphology of the SEL is detailed below.

In 1994 Globus and Kuhlenbeck examined post-mortem brains from patients who had died with ependymal, subependymal and choroid plexus tumours. In their classification of these tumours, Globus and Kuhlenbeck offered a clear but brief description of the wall of the lateral ventricle in the human brain.

"The well developed subependymal cell plate shows a more or less distinct stratification in three layers, 1) the ependymal lining, 2) a narrow clear layer of ependymal fibers and processes of spongioblasts and other elements described above. The subependymal cell plate is only moderately vascularised, the capillary loops reach occasionally the clear layer of ependymal and spongioblastic fibers and the row of ependymal cells. Where the subependymal cell plate becomes thinned out, the stratification into second and third layer is indistinct or lacking. Groups of cells may directly adjoin the ependymal lining. Individual variations in thickness and regional structural differences of the ependymal cell plate occur." (Globus and Kuhlenbeck, 1944)
Using histological stains to examine the post-mortem brain tissue they were able to identify neoplastic tumors that contained primitive cells likened to what had been described as bipotential mother cells and spongioblasts. Bipotential mother cells and spongioblasts were the products of cell division that occurred in the primitive medullary epithelium. The offspring of bipotential mother cells were neuroblasts, which become neurons or spongioblasts that become ependymal, choroid plexus, astrocytes and oligodendroglial cells. According to Globus and Kuhlenback (1944) spongioblasts could be formed as a direct daughter cell of the medullary epithelium or as a daughter cell of the bipotential mother cells (Globus and Kuhlenbeck, 1944). This approach is today considered simplistic and has in part been superseded. However, of note was the fact that the adult brain continued to produce primitive cells well into adulthood that, in these cases, had produced fatal neoplastic tumors.

Since this description numerous papers have described ependymal and subependymal pathology but there has been no detailed study of the normal anatomy and classification of the human SEL.

In the most comprehensive report on SEL anatomy to date Bernier and co-workers (2000) undertook an immunohistochemical study on 19 post-mortem human brains aimed at demonstrating some of the cell type markers present in the human SEL. In this study nestin, an intermediate filament protein which is present in precursor cells and immature glial progenitor cells (see table 2.1), was used to delineate the SEL which clearly labelled the wall of the lateral ventricle from as far rostral as the nucleus accumbens and remained strongly nestin positive as far caudal as the internal segment of the globus pallidus. Nestin positive staining was present in the wall of the third ventricle particularly in the hypothalamic ventral portion. There was a variation in the thickness of the nestin positive SEL between cases, a finding that did not correlate with age, sex, or cause of death. The most immunoreactive and thickest SEL sectors occurred at the level of the anterior commissure. To further study the immunohistochemical make up of the SEL, different antibodies were employed to demonstrate the presence of different cell types within the SEL (Bernier et al., 2000). βIII-tubulin (see table 2.1 for descriptions of antibodies used),
GFAP, PSA-NCAM and PCNA were each used to label different cell populations. Nestin was expressed in a dense polymorphic cell population, many of which displayed many short processes and resembled astrocytes, still others displayed only a few or no visible processes. βIII-tubulin immunostaining was present only in small round or oval cells with processes that stained strongly resembling neuroblasts or neurons. Bernier and colleagues often found βIII-tubulin positive cells that were linked together by their processes forming chains that were oriented in all directions. The intensity of βIII-tubulin immunostaining was greater in the lateral parenchyma away from the lateral ventricle. This, they speculated, was because many mature neurons outside the SEL also contain the βIII-tubulin antigen and it is expressed throughout the life of the neuron. Because the SEL mostly contains newly formed neurons it is less densely immunostained. This has also been reported in other studies that showed down regulation of βIII-tubulin in mature neurons (Lee et al., 1990; Menezes and Luskin, 1994). The most intense labelling in the SEL as demonstrated by Bernier et al. (2000) was for GFAP. GFAP stained a group of densely packed cells and processes that had a central circular area that was devoid of staining. The GFAP immunoreactivity was very dense in the SEL and became much less dense in the caudate nucleus. PSA-NCAM immunostaining was only demonstrated in small round cells that, when sectioned along the sagittal plane, were found to label small chains similar to that seen with βIII-tubulin staining. Using a double-label immunofluorescence technique, Bernier and co-workers (2000) studied the co-localisation of the various cell markers in the SEL with PCNA and nestin, nestin and GFAP, PSA-NCAM and βIII-tubulin. They found that a large proportion of SEL cells that were immunoreactive for PCNA were also nestin positive. However, many of the nestin positive cells were not PCNA positive. Nearly all the nestin positive cells expressed GFAP and a large proportion of GFAP positive cells were also immunoreactive for nestin. The migrating PSA-NCAM positive cells were all immunoreactive for βIII-tubulin, however, it was noted that many βIII-tubulin cells were not immunoreactive for PSA-NCAM. In this study the presence of PCNA and βIII-tubulin co-localisation was not reported, nor was it suggested as a means of detecting newly formed neurons (Bernier et al., 2000). This study reported the presence of migrating neuroblasts, immature neurons and cells capable of cell division, but did not
demonstrate neurogenesis in the human brain. Also, this study did not describe the stratification within the human SEL. Because of the close proximity of the caudate nucleus to the SEL, examining the SEL for increased activity would be most suitable in a disease that affects the caudate nucleus such as Huntington's disease.

2.4 Huntington's Disease

2.4.1 General pathology

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that has motor, cognitive and behavioural symptoms with a midlife onset. HD has an incidence of 5-7.5 per 100,000 population, with death occurring 12-15 years after symptomatic onset (Vonsattel and DiFiglia, 1998; Vonsattel et al., 1985). George Huntington first described HD in 1872. The disease was reported as having three cardinal features. Firstly, it is hereditary in nature but not all the offspring of an affected person become ill with the disease; furthermore, if the offspring are unaffected then they cannot pass the disease on to their offspring. Secondly, sufferers have a tendency to insanity and sometimes suicide and the mind becomes impaired. Thirdly, it has an adult onset with a chorea (dance-like movement) that becomes progressively worse (Chesselet and Delfs, 1996; The Huntington's disease collaborative research group, 1993; Gusella et al., 1983; Harris et al., 1999; Kowall et al., 1987; Sharp and Ross, 1996; Vonsattel and DiFiglia, 1998).

The motor dysfunction in HD comprises involuntary chorea and athetosis. Chorea is an involuntary rapid movement of the distal limbs and athetosis is an involuntary writhing movement of the proximal muscles of the trunk that can lead to sustained abnormalities of posture. These signs and symptoms progress with time until the person is unable to walk and becomes bedridden and incapable of voluntary movements (Sharp and Ross, 1996). The person's ability to speak and swallow is also often affected thus leaving the patient susceptible to aspiration pneumonia, which is the common cause of death in HD.
Although motor symptoms often signal the onset of HD, cognitive and psychological/behavioural changes can precede motor symptoms. The symptoms include depression and dementia that may be related to the high incidence of suicide amongst Huntington's sufferers (Deckel et al., 1998; Nance, 1997). Obsessive, impulsive behaviour and thought patterns can develop that may include gambling or eating disorders in persons otherwise asymptomatic at that point. The first sign of the onset of HD is often the slowing of intellectual ability with a loss of mental flexibility (Sharp and Ross, 1996). HD is caused by a genetic mutation comprised of an expanded trinucleotide repeat ('CAG') sequence that codes for the amino acid glutamine. This expansion is located on exon 1 of the short arm of chromosome 4 on a gene termed IT15 (Interesting Transcript 15) (The Huntington's disease collaborative research group, 1993; Gusella et al., 1983). The IT15 gene was discovered in 1993 by the combined efforts of a large international collaboration aimed at finding the gene responsible for HD. The IT15 gene codes for a protein called huntingtin; however it is unclear how the genetic mutation causes the pathology of HD. In the normal population there are up to 35 CAG trinucleotide repeats on this gene; however, in HD this is expanded to above 36 repeats. Persons with 36-39 CAG repeats may not develop HD whilst those with 40 or more will (The Huntington's disease collaborative research group, 1993; Nance, 1997; Snell et al., 1993; Vonsattel and DiFiglia, 1998). The number of repeat expansions is inversely proportional to the age of onset and proportional to the severity with which HD patients are affected (The Huntington's disease collaborative research group, 1993; Gusella et al., 1993; Nance, 1997; Snell et al., 1993). The HD gene length may vary in each successive generation and this is termed genetic anticipation. Anticipation in HD is more pronounced when the disease is of paternal origin. In studies performed by Gusella (1993) looking at repeat length variation amongst the sexes of HD origin, it was determined that the average range of repeat length variation when the gene was inherited paternally was 10 repeat lengths (3 repeats below and 7 above) in 62% of cases. The other 38% of cases examined demonstrated increases of between 9 and 42 repeats. The repeat length in the progeny is more stable if maternally inherited with an average variation range of 8 repeats (4 repeats above and 4 repeats below) (Gusella et al., 1993). Anticipation is due to the tendency for CAG repeats to vary during meiosis (The
Huntington's disease collaborative research group, 1993; Gusella et al., 1993; Hannan, 1996). The expanded repeat of the glutamine-coding region in huntingtin causes certain brain cells to dysfunction and die. The exact mechanism of this aspect of the pathology is unknown, although it is suggested that cleavage of the N-terminal region and its translocation to the nucleus causes intranuclear inclusions that may be an initiating factor in the HD neuropathology.

The major neurological alterations that occur in HD are changes in neurochemistry, anatomy and cellular morphology of the basal ganglia in the brain. The basal ganglia are a major group of extra-pyramidal nuclei that modulate mood and movement control. The basal ganglia consist of the caudate nucleus and putamen (collectively termed the striatum), globus pallidus (internal and external segments), substantia nigra (made up of pars compacta and pars reticulata) and the sub-thalamic nucleus. The striatum is most severely affected in HD and demonstrates a marked loss of medium spiny efferent projection neurons, which are the predominant cell type in the normal striatum (Kowall et al., 1987; Vonsattel and DiFiglia, 1998). In the normal striatum 95% of the cells are medium spiny neurons, but only about 5% of these cells remain in the late stages of HD (Ferrante et al., 1991). Medium spiny neurons project to both segments of the globus pallidus and to the substantia nigra. The first group of projection neurons to be lost in HD are the y-amino butyric acid (GABA) striatal neurons that project from the striatum to the external segment (externus) of the globus pallidus (striatopallidal fibres); followed secondly by loss of GABA striatal neurons that project from the striatum to the substantia nigra (striatonigral fibres); and thirdly the loss of GABA striatal neurons that project from the striatum to the internal segment (internus) of the globus pallidus (Glass et al., 2000; Reiner et al., 1988). All three of these projection neurons utilise GABA as their primary neurotransmitter, but the presence of secondary co-transmitters has also been identified. The striatopallidal externus projection neurons utilise the neuropeptide met-enkephalin and the striatopallidal internus and striatonigral projection neurons utilise the neuropeptide substance P as their co-transmitters (Glass et al., 2000; Glass et al., 1993; Reiner et al., 1988; Richfield and Herkenham, 1994). Interneurons are differentially susceptible in HD; some interneuron populations are affected early in the disease.
(parvalbumin and glutamic acid decarboxylase (GAD) interneurons) while others appear to be relatively less susceptible (choline-acetyl transferase (ChAT) and neuropeptide Y/somatostatin (NPY) interneurons) (Ferrante et al., 1987a; Ferrante et al., 1987b; Ferrante et al., 1991; Kowall et al., 1987). The medium spiny efferent projection neurons in the striatum also contain calbindin, a calcium binding protein that is often used as a marker of this population of striatal neurons (Ferrante et al., 1991). In HD there is a reduction in the levels of GABAergic medium spiny neurons and calbindin. Also, there is a dramatic reduction in the level of enkephalin and substance P in the globus pallidus and substantia nigra respectively (see table 2.1 for descriptions of the antibodies used) (Glass et al., 2000; Reiner et al., 1988).

2.4.2 Huntington’s disease grading scale

Huntington’s disease has been classified according to the clinical signs and symptoms and the extent of the neuropathological changes. There are many classifications that are used by psychiatrists and neurologists in assessing the cognitive, psychological and degenerative aspects of the disease. Anatomical and pathological grading is somewhat different because it is based solely on post-mortem examination of the HD patient’s brain. On a gross, external examination 80% of HD brains appear atrophic and in most cases the weight of the brain is approximately 200 grams lighter than a neurologically normal brain. Gross examination of coronal brain sections reveals bilateral atrophy of the striatum, enlarged lateral ventricles and a loss in white matter volume. In a study of more than 238 brains (158 brains had a definitive HD pathology) Vonsattel and colleagues (1985) classified the histopathological features of relative neuronal loss and gliosis in HD and derived a 5 point pathological grading scale that ranges from grade 0 to 4 (Vonsattel and DiFiglia, 1998; Vonsattel et al., 1985). The microscopic criteria for each grade will be discussed here.

Grade 0 - Comprises 1% of all HD brains. Gross examination shows features indistinguishable from a neurologically normal brain, however up to a 30% neuron loss in the head of the caudate nucleus is often evident microscopically.
Grade 1 - Comprises 4% of all HD brains. Atrophy of the head and tail of the caudate nucleus is evident macroscopically; atrophy may also be demonstrated in the putamen. Cell counts of the head of the caudate nucleus demonstrate a 50% neuron loss. The entire tail of the caudate nucleus must be examined as the normal brain may have segments of narrowing in the tail but for only a short segment. Consistent atrophy of the tail of the caudate nucleus is necessary for a grade 1 to be assigned.

Grade 2 - Comprises 16% of all HD brains. Striatal atrophy is moderate. The body of the caudate nucleus is reduced to approximately half its normal size. The medial aspect of the head of the caudate nucleus shows some flattening but is still slightly convex and still bulges into the lateral ventricle.

Grade 3 - Comprises 54% of all HD brains. Striatal atrophy is severe. The body of the caudate nucleus is reduced to about 75% of its normal size. The medial aspect of the head of the caudate nucleus forms a straight line or is slightly concave relative to the normal caudate nucleus. The tail of the caudate nucleus is barely visible.

Grade 4 - Comprises 25% of all HD brains. In grade 4 the striatum is severely atrophic and 95% or more of the striatal neurons are lost. Astrogliosis is evident throughout the basal ganglia. The medial aspect of the head of the caudate nucleus is severely concave, as is the anterior limb of the internal capsule (Vonsattel and DiFiglia, 1998; Vonsattel et al., 1997; Vonsattel et al., 1985).

2.5 The striatum: Cell types and relationship to the subependymal layer

The striatum lies immediately adjacent to the subependymal layer (see figure 2.6). The importance of the SEL overlying the striatum is most evident during cortical and striatal development where the lateral and medial ganglionic eminence in the developing embryo
play a critical role in the development of the striatum, cortex and other telencephalic structures. To demonstrate the importance of the lateral ganglionic eminence in striatal development, Deacon and colleagues (1994) performed lesion and transplant studies on the foetal rat brain. In these studies, foetal rat brain medial and lateral ganglionic eminences were injected with the excitotoxin quinolinic acid (which produces a HD-like pathology by killing the striatal projection neurons) and subsequently striatal precursor grafts were placed into the lesioned area. To detect the transplanted cells, the cells were retrogradely labelled from the ipsilateral globus pallidus with either fluorogold or fast blue. Three months later, when the animals were sacrificed and processed for either electron microscopy or immunohistochemistry, it was demonstrated that the cells transplanted into the lateral ganglionic eminence displayed the marker DARp-32, which is a specific striatal projection neuron marker, and displayed striatal neuron morphology. The transplanted medial ganglionic eminence however, did not contain any neurons that resembled striatal neurons despite the transplant being of striatal origin (Deacon et al., 1994). During development the lateral ganglionic eminence becomes the ventricular and subventricular zone that in the adult become the EPL and SEL. Striatal neurons are born and migrate from the subventricular zone (developmental SEL) during development; however, by birth the SEL is significantly smaller than in the prenatal brain and by adulthood the SEL is just a few cell layers thick and is often described as inactive (Johnston et al., 1990; van der Kooy and Fishell, 1987).

The rodent and primate striatum lack the prominent cytoarchitectural arrangements like the laminations of the cerebral cortex. The neurons within the striatum, however, can be divided into striosomes and matrix. The striosomes are formed early on in embryonic development (E13 in rat) in the lateral ganglionic eminence. Further on in development, the matrix cells spill over from the embryonic SEL and divide the striosomes up; the matrix ultimately comprises approximately 80% of the striatum (Graybiel and Ragsdale, 1978; Johnston et al., 1990; van der Kooy and Fishell, 1987). The striatum is the closest anatomical structure to the SEL and is also the brain region most severely affected in HD (Kawaguchi et al., 1995; Vonsattel and DiFiglia, 1998; Vonsattel et al., 1985). The major neuronal cell types in the striatum are projection neurons that are medium spiny neurons,
and several different types of interneurons. These different cell types can be identified using immunohistochemical stains. Medium spiny neurons in the striatum can be identified by immunolabelling for calbindin. Calbindin is a calcium binding protein that is located specifically in GABAergic striatal efferent projection neurons (DiFiglia et al., 1989; Holt et al., 1997; Hontanilla et al., 1998; Waldvogel et al., 1991). Co-localised with striatal efferent projection neurons are the neurotransmitters enkephalin or substance P. Enkephalin is a marker for striato-pallidal (externus) GABAergic projection neurons and substance P is a marker of striato-pallidal (internus) and striato-nigral GABAergic projection neurons (see figure 2.6) (Cuello et al., 1981; Graybiel, 1990; Graybiel and Chesselet, 1984; Haber and Elde, 1981; Penny et al., 1986). Interneuron cell populations can also be labelled with antibodies to specific proteins within the cells structure. There are 4 major categories of interneurons and these can be labelled with the following antibodies: The large GABAergic interneurons immunostain for parvalbumin (Ferrer et al., 1994; Holt et al., 1997; Kita et al., 1990), a calcium binding protein. Cholinergic interneurons in the striatum are detected by using antibodies to choline-acetyl transferase; this is an enzyme involved in the synthesis of acetylcholine (Kawaguchi et al., 1995). Choline-acetyl transferase positive cells are large sized interneurons in the striatum. Also, a subset of small aspiny interneurons can be immunolabelled for the neurotransmitter neuropeptide Y/somatostatin. Another calcium binding protein present in interneurons of the striatum is calretinin, which is present in small aspiny GABAergic interneurons as well as large aspiny choline-acetyl transferase interneurons (see figure 2.6) (see table 2.1 for descriptions of the antibodies used here) (Cicchetti et al., 2000).

2.6 Therapeutic approaches to Huntington’s disease

HD is characterised by a specific, patterned degeneration of the striatal projection neurons with subsequent atrophy of the striatum. The disease is caused by a triplet repeat expansion on gene IT15, which encodes a novel protein called huntingtin. How the huntingtin gene brings about the neuropathology seen in HD is unclear. However, having a defined gene and a pattern of cell loss does give scientists some targets for the
Figure 2.6

Cell types in the striatum and the relationship of the striatum to the subependymal layer

The striatum is comprised of projection neurons and interneurons. Approximately 95% of the striatal neurons are medium-sized spiny projection neurons that use GABA as their primary neurotransmitter. The GABAergic projection neurons that project from the striatum to the globus pallidus externus also contain the co-transmitter enkephalin. The GABAergic projection neurons that project from the striatum to the globus pallidus internus and substantia nigra contain the co-transmitter substance P. Within the striatum there are 4 main types of interneuron: parvalbumin (Parv), calretinin (Calr), neuropeptide Y/somatostatin (NPY) and choline-acetyl transferase (ChAT) containing interneurons. Parv, Calr and NPY are all GABAergic interneurons, but the ChAT interneurons use acetylcholine as their neurotransmitter.

The SEL (demonstrated as a red line) lies immediately between the lateral ventricle and the caudate nucleus of the striatum. The SEL overlying the caudate nucleus is where adult progenitor cells are located. The EPL (demonstrated as a blue line) lines the lateral ventricle and overlays the SEL.
Cell types in the striatum and the relationship of the striatum to the subependymal layer

- Parv (GABAergic, enkephalin positive projection neurons)
- Calr (GABAergic, calretinin positive interneurons)
- NPY (GABAergic, neuropeptide Y positive interneurons)
- ChAT (Choline-acetyl transferase positive interneurons)
- SEL (Subependymal layer overlying the striatum)
- Ependymal layer overlying the subependymal layer
development of a treatment. Two therapies have been the main focus in HD research over the past decade. These have been: (1) drug therapy to replace the neurotransmitters lost as the striatal neurons die and also drug therapy aimed at preventing cellular degeneration. (2) Foetal cell transplantation therapy, which is aimed at replacing dying and dead striatal neurons with an exogenous source of neurons. Cell transplantation therapy has so far returned mixed results in the treatment of HD and is not available routinely to patients with HD. Thus far drug related therapies have failed to stop neuronal cell death and have not robustly relieved the symptoms of HD. Drug related therapies will not be discussed further in this review of the literature.

2.6.1 Neural transplantation

A recent interesting and novel approach to the treatment of HD has been the possibility that transplants containing young, immature neurons can be placed into the diseased or lesioned brain. The hope has been that by replacing the cells that are lost from the HD striatum, the transplanted cells will grow and develop in the striatum and resume the function of the depleted neuronal populations. The first neural transplant studies to be carried out on animals involved lesioning a brain region with an excitotoxin and then one to two weeks later transplanting foetal cells into the cell deprived area (Bjorklund et al., 1980b; Bjorklund et al., 1983; Isacson et al., 1985). The cells in the transplants were expected to extend projections and reinnervate the projection areas to the same extent as healthy cells would in the neurologically normal brain. In these studies, Bjorklund and colleagues (1980) demonstrated that animal-animal transplants were possible; the transplanted cells had the capability to grow and produce the neurochemical markers that are demonstrated in the normal striatum (Bjorklund et al., 1980b). In a comprehensive study, Isacson and colleagues (1985) made a cell suspension out of cells removed from the striatal region of the foetal rat brain at 14-15 days gestation. This cell suspension was injected into the lesioned striatum of an adult rat 5-7 days after lesioning and the transplant was left to grow for 1, 4 or 20 weeks. The animals were sacrificed and their brains were prepared for immunohistochemistry and the brain sections were subsequently analysed with light microscopy. The results of this study demonstrated that the depletion
of neurons in the striatum caused by excitotoxic lesion appeared to be reversed by the transplantation of the foetal striatal neuron suspension. The intrastriatal foetal striatal transplant survived, grew and established new striatal neurons within the host striatum (Isacson et al., 1985). The phenotype of striatal neuron transplants was analysed by Graybiel and co-workers (1989) using immunohistochemical techniques to assess the neurochemical organisation of the striatal transplants. These studies often showed that the foetal striatal cell transplants displayed the same cell phenotypes as the normal striatum. In particular, calbindin, which is a marker of medium spiny efferent projection neurons was present within the transplants and was very similar to the calbindin staining demonstrated in the host striatum (Bjorklund et al., 1980a; Das and Altman, 1971; Dunnett and Svendsen, 1993; Faull et al., 1995; Graybiel et al., 1989). Foetal striatal transplants also contained the normal striatal interneuron populations; Morris et al. (1989) used in situ hybridisation techniques to demonstrate the presence of interneurons that contained neuropeptide Y in foetal striatal cell transplants. The presence of choline-acetyl transferase and glutamic acid decarboxylase positive neurons has also been demonstrated in striatal transplants containing foetal striatal tissue (Isacson et al., 1985; Morris, 1989; Morris et al., 1989). The striatal neuron transplants display all of the immunohistochemical and histochemical characteristics of the interneurons in the normal striatum.

If the transplantation of foetal cells is to be used as a therapeutic treatment, one of the essential characteristics of foetal striatal cell transplants is that they must have the capacity to form new connections with the host striatum and they must be able to project axons to the striatal target projection areas, i.e. the globus pallidus and substantia nigra. In a comprehensive tract tracing study Wictorin and colleagues (1990) demonstrated that after a foetal striatal transplant into the excitotoxically lesioned rat striatum the neurons in the transplant had projected axons into the globus pallidus (Wictorin et al., 1990). The connections between the transplants and the host globus pallidus were assessed using an iontophoretically injected anterograde tracer that was detected immunohistochemically in the globus pallidus, demonstrating that projections had connected the striatum and globus pallidus. Once the striatal transplants have projected axons to the striatal projection areas they must form functional synapses on the cell bodies, axons and dendrites in the host.
projection areas. One of the difficult aspects regarding foetal cell transplantation has been to prove that the projections form functional connections in the globus pallidus. In studies designed to specifically test the hypothesis that transplanted cells can form fully functioning synapses and that neuronal activity can be restored Nakao and colleagues (1999) performed electrophysiological tests on the transplanted neurons that had formed synapses in the globus pallidus. The results of their research showed that in quinolinic acid lesioned rats the level of spontaneously firing pallidal neurons was high; this was a result of the loss of inhibitory GABAergic striatopallidal fibres to the globus pallidus. Five months after placement of a striatal neuron transplant results showed that there was a significant decrease in the rate of spontaneously firing pallidal neurons compared with the lesioned normals (Nakao et al., 1998; Nakao et al., 1999). These results demonstrate that transplanted neurons exhibit a normal functional inhibitory effect similar to that demonstrated in the normal brain.

In the human, clinical transplantation trials have begun (Kopyov et al., 1998; Lindvall, 1995). However, the results so far have been varied and the number of patients trialed has been small. Human foetal striatal tissue from elective abortions has been used as the source of transplant cells and about 6-8 foetuses are required for each transplant. The use of human foetal tissue as a source of transplant material has alone raised significant ethical controversy. The main ways of assessing the success of the transplants has involved assessment of the changes in patient symptomatology and also using imaging modalities such as computerised tomography (CT) or magnetic resonance imaging (MRI) (Kopyov et al., 1998). To date the success of intrastriatal transplants has failed to show robust alleviation of the symptoms or the ongoing neuropathology of HD. The lack of success may be caused by the variability in surgical placement of the transplants, poor survival of the transplants or poor integration of the transplanted cells. Whichever is the cause of the poor outcomes, it is apparent that unless transplant technology improves it may never be a viable method for the routine treatment of HD.
2.6.2 Progenitor cell replacement

Since transplantation has not proved to be effective for the routine treatment of HD other methods of cell replacement have been investigated. Making full use of the body’s own ability to produce new neurons in response to degenerative diseases is the most recent of neural replacement therapy approaches. The notion that the brain can produce new neurons that migrate into the area of neuronal damage in response to the damage is a hypothesis recently tested in experiments performed on rats (Arvidsson et al., 2002; Parent, 2003; Parent et al., 2002a). In one such experiment, the middle cerebral artery was occluded which led to a non-fatal medium sized stroke in the striatal brain region adjacent to the SEL in adult rats. The number of progenitor cells in the SEL and around the stroke affected area was measured using BrdU; migrating cells were counted using doublecortin and neurons were counted after being stained for NeuN. The results demonstrated not only that neuroblasts from the SEL proliferated in response to the stroke but also that they migrated toward the site of the stroke and became mature striatal neurons that demonstrated immunoreactivity for the striatal neuron markers Meis2 and DARP-32. In order to determine that the new neurons at the site of the stroke had originated from the SEL, and not from local progenitors within the striatum and lesion affected area, the antimitotic drug cytosine-β-D-arabinofuranoside (Ara-C) was selectively administered to SEL cells via a lateral ventricle infusion. The results demonstrated that in Ara-C treated brains, there was very little cell proliferation in the SEL and very few cells that had migrated from the SEL to the site of the stroke; thus no new neurons were demonstrated near the stroke site. This demonstrates that the progenitor cells and neuroblasts were being produced in the SEL. This study demonstrated the ability of the rodent brain to produce new replacement neurons in response to degeneration of neurons within the adult rodent brain (Arvidsson et al., 2002).

The research in this thesis addresses the question of the potential of progenitor cells in the SEL overlying the caudate nucleus in the normal and HD human brain. In this thesis I will investigate 4 main aspects of the SEL: (1) the general organisation of cell types and
cytoarchitecture of the normal and HD adult human SEL with an attempt to identify progenitor cell populations; (2) the **proliferative capacity** of progenitor cells in the normal and HD adult human SEL to determine whether proliferating cells are involved in neurogenesis or gliogenesis; (3) the **distribution** of proliferating progenitor cells in the normal and HD SEL; and, (4) the range of **mature cell types** (neuronal and glial subtypes) that are present in the normal and HD adult human SEL.
Chapter 3

ANATOMY OF THE SUBEPENDYMAL LAYER IN THE NORMAL AND HUNTINGTON’S DISEASE HUMAN BRAIN

3.1 Introduction

In the developing brain the subependymal layer (SEL) is the birthplace of neurons and the time course of the development of young neurons from stem/progenitor cells in the SEL has been well documented (Kandel et al., 2000). During development the SEL contains cells that are mitotically active and morphologically heterogeneous (Privat and Leblond, 1972; Smart, 1972; Smart, 1973; Smart, 1976). In the adult rat and mammalian brain, cells in the SEL are still mitotically active and retain a neurogenic potential; however, in the adult brain the thickness of the SEL is reduced as the brain matures postnatally and the SEL becomes considerably less active as the brain becomes developmentally mature (see Garcia-Verdugo 2002 for review) (Garcia-Verdugo et al., 2002). There have been very few studies on the anatomy and heterogeneity of the cells in the SEL in the adult mammalian brain.

The SEL in the adult human brain has not been previously described in terms of its histological characteristics. Doetsch et al (1997) described the histological anatomy of the SEL in the normal adult mouse brain. In their study in the mouse SEL, 5 main cell types were described; cell types A-E. Type A cells were described as migrating neuroblasts, believed to migrate within the rostral migratory stream to the olfactory bulb where they formed replacement interneurons. Type B cells were described as being glial cells that support the migration and differentiation of the type A cells in the SEL; it is suggested that type B cells provide trophic support for other cells in the SEL (Doetsch et al., 1997). Type C cells were identified as putative precursor cells believed to be capable of
differentiation down a number of different cell lineages. Type D cells were described as very rare tanyocytes, which were randomly distributed within the SEL. Type E cells were ciliated ependymal cells that maintained a barrier between the cerebrospinal fluid of the lateral ventricle and the SEL and caudate nucleus parenchyma (Doetsch et al., 1997). Doetsch et al (1997) identified these various cell types in the mouse SEL according to their distribution, morphology and staining characteristics. In this study of the human brain, the same criteria have been adopted for classifying the various cell types in the SEL of the normal and HD human brain.

In HD, it is the caudate nucleus and putamen that are the principal regions of degeneration and they lie immediately adjacent to the SEL, but the normal anatomy and the changes that occur in the SEL in HD have never been previously studied in the human brain. The plasticity present in the SEL during development suggests there may be significant alterations in the SEL in response to HD. This chapter will therefore be directed towards investigating: (1) the normal anatomy of the human SEL; (2) a comparison of the thickness of the adult human SEL in normal and HD brains; (3) the quantification of cells in the SEL in normal and HD brains; and (4) comparison of the numbers of different cell types present in the adult human SEL in normal and HD brains.

3.2 Materials and methods

3.2.1 Human tissue collection

For this study, the basal ganglia from post-mortem human brains were obtained from the Neurological Foundation of New Zealand Human Brain Bank at the Department of Anatomy with Radiology, the University of Auckland. The full consent of all families was obtained and the University of Auckland Human Subjects Ethics Committee approved the protocols used in these studies. Normal brains (detailed in table 3.1) were received from cases with no history of neurological disease and on pathological examination showed no neurological abnormalities. The ages of normal cases ranged from 46-72 years of age (average = 59.6 years of age), and the post-mortem delays
ranged from 5-16 hours (average = 10.75 hours). HD brains (detailed in table 3.1) were received from patients with a family and clinical history of HD. The diagnosis of HD was confirmed by genetic analysis of the CAG repeat length in both copies of the IT15 gene, the gene affected in HD. The ages of the HD cases ranged from 35-80 years of age (average = 54.8 years of age), and the post-mortem delays ranged from 6-24 hours (average = 15.22 hours).

### Table 3.1

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Table detailing the case number, age, sex, post-mortem delay, CAG repeat length in the IT15 gene, HD neuropathological grade. H= normal cases; HC = Huntington's disease cases.

For the histochemical studies the brains were fixed by perfusion through the basilar and internal carotid arteries. Initially the brains were perfused with phosphate buffered saline (PBS) with 1% sodium nitrite to clear the brain's circulation, followed by 15% formalin in 0.1 M phosphate buffer pH 7.4. Following the perfusion, the brains were dissected into regions and the basal ganglia blocked and post-fixed in the same fixative for 24 hours.
Subsequently these blocks were cryo-protected in 20% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for 4-5 days, and then in 30% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for a further 4-5 days.

### 3.2.2 Tissue processing

Human tissue blocks containing the caudate nucleus that had been fixed in the standard way were dehydrated and processed for wax embedding using standard histological protocols for the processing of human brain tissue. The wax impregnated tissue blocks were then cut on a microtome at a thickness of 7 μm and were floated from warm water onto glass slides in preparation for histochemical staining.

### 3.2.3 Heamotoxylin & eosin and luxol fast blue staining

The sections, mounted on a glass slide, were dewaxed in xylene and were hydrated to 95% alcohol before being incubated for 2 hours at 60°C in a Luxol Fast Blue solution containing:

- Luxol Fast Blue: 1 gm
- Methanol (absolute): 1000 mls
- 10% Acetic acid: 5 mls

Reagents were mixed and filtered. The sections were washed in 70% alcohol before being washed in tap water. Sections were differentiated in saturated lithium carbonate solution until the grey/white matter were distinguished and they were then washed again in tap water.

The sections were stained in Meyer’s haematoxylin (2-5 minutes), washed in running tap water, placed in Scot’s tap water substitute for a few seconds until ‘blueing’ occurred before washing again thoroughly in running tap water. Sections were then stained in Moores buffered eosin (6-12 dips), washed quickly in tap water, dehydrated through ascending grades of alcohol (70%, 80%, 90% for five minutes each and 2 x 100% for ten minutes each), then cleared in 3 changes of xylene (ten minutes each) and coverslipped with Hystomount (Hughes and Hughes) mounting medium.
3.2.4 Image digitisation

For the quantitative and morphological studies the mounted and coverslipped brain sections were photographed using a digital camera (Kontron ProgRes 3008) mounted on a conventional transmitted light microscope (Leica DMR). A calibration slide was also photographed at the same magnification for accurately determining cell size. For very low power macrographs, a Nikon SLR digital camera was used.

3.2.5 Image analysis

In order to make measurements, count cells and to determine which types of cells were present in the SEL, the sections were digitised using a digital camera (Kontron ProgRes 3008) mounted on a conventional transmitted light microscope (Leica DMR). A calibration slide was also photographed at the same magnification as the brain slices so that calibration and accurate measurements could be made.

The number of each cell type was counted in the normal brain and in HD grades 1-3 brains. The thickness measurements of the SEL were made from macro photographs of the caudate nucleus sections with the SEL included. The distances from the deep part of the EPL to the superficial part of the myelin layer was measured at five equidistant points along the SEL and averaged. The averaged thicknesses of the SEL have been displayed in terms of μm.

The number of cells present in the normal and HD SEL were determined by counting every haemotoxylin & eosin cell present along the dorsal to ventral length of the SEL overlying the caudate nucleus between the deep surface of the EPL to the superficial surface of the myelin layer. Because the length of the sections varied from case to case, the number of cells counted was divided by the length of the SEL in mm, thus the results have been displayed as cells/mm.
In order to count the number of each cell type present on haemotoxylin & eosin stained sections, each brain examined had digital photomicrographs (63 x magnification) taken of the SEL in three regions so the numbers of each cell type within the SEL could be counted. The number of each cell type was averaged across the three counts and thus the cell numbers have been displayed as the number of cells in a 230 μm (the width of a single frame at 63 x magnification) length of SEL.

Once the sections were digitised, all distance measurements were made using SCION image, which enabled calibrated measurements to be made. For the statistical comparison of the data in different groups general ANOVAs with subsequent T-tests, corrected for multiple comparisons using Bonferroni’s method, were used.

3.3 Results

3.3.1 The anatomy of the SEL in the normal and Huntington’s disease brain (see figure 3.1)

In the normal adult human brain the ependymal region is comprised of two layers, the ependymal layer (EPL) and the subependymal layer (SEL). The EPL is a single cell layer that is made up of epithelial cells that form a barrier between the cerebrospinal fluid of the lateral ventricle and the underlying brain tissue (see figure 3.1). The ependymal cells were tightly packed together and surrounded the full extent of the lateral ventricle. Ependymal cells stain darkly with haemotoxylin & eosin stain (see figure 3.1). Immediately beneath the EPL lies the SEL, which is a heterogeneous layer bound superficially by the EPL and by a deep myelin layer adjacent to the caudate nucleus (see figure 3.1). Within the SEL there was a superficial region beneath the EPL in which there
Figure 3.1

Anatomy of the ependymal and subependymal region in the normal brain

This figure demonstrates a haemotoxylin & eosin and luxol fast blue stained section from the lateral wall of the ventricle overlying the caudate nucleus in the normal human brain showing the anatomy of the ependymal layer (EPL) and subependymal layer (SEL). The superficial layer is the EPL, which consists of a single layer of cells that line the wall of the lateral ventricle. Immediately beneath the EPL is the SEL; this is a heterogeneous layer comprised of a superficial area that is devoid of cell body staining and a deeper region that contains cells of various morphology that can be identified on the basis of size, shape and staining intensity (see figure 3.2). The SEL is bounded by a layer of myelin that is stained (blue) with luxol fast blue. The myelin layer contains myelin that is tangentially oriented to the SEL and cell bodies are interspersed between the myelin fibres. Beneath the myelin layer is the caudate nucleus, which contains a heterogeneous cell population and a sparse amount of myelin in the superficial region. The scale bar = 40 μm.
Anatomy of the ependymal and subependymal region in the normal brain
were very few cell bodies labelled with haemotoxylin & eosin and luxol fast blue histochemical stains. Beneath this the SEL was comprised of a broad band of cellular staining that contained morphologically heterogeneous cells (see figure 3.1). Beneath the SEL was a dense band of myelin staining (blue in figure 3.1) which separated the SEL from the caudate nucleus of the striatum. The band of myelin contained tangentially oriented fibres, and cell bodies were distributed throughout the myelin layer. The thickness of the myelin layer varied throughout the dorsal to ventral extent of the SEL region. Beneath the myelin layer was the parenchyma of the caudate nucleus in which the myelin fibres were very dispersed. Throughout the caudate nucleus, haemotoxylin & eosin positive cells were evenly distributed. The cells in the caudate nucleus were more sparsely distributed compared with that of the SEL.

**3.3.1.1 Histological classification of the cells in the adult human subependymal layer (see figure 3.2)**

In order to determine what types of cells are present in the subependymal region, the cells of the SEL have been classified according to their size, shape and histochemical staining characteristics as demonstrated with haemotoxylin & eosin staining. The classification of these cells follows the criteria for the classification of the SEL cells in the adult mouse brain by Doetsch et al (1997) (Doetsch et al., 1997).

As in the mouse brain; in the human lateral ventricle wall, 4 major types of cells were identified within the SEL region; types A, B, C and E (see figure 3.2). The appearance of each of these cell types is described below.

**Type A** cells had an elongated, irregularly shaped nucleus that often had a tear drop appearance. The type A cells had darkly haemotoxylin & eosin labelled nuclear membranes and often had a small amount of lightly stained nucleoplasm (compare with type B cells below) (a typical type A cell is surrounded by a yellow box in figure 3.2A). Thus the nucleoplasm was uniformly labelled in type A cells (see figure 3.2A). **Type B** cells were subdivided into type B1 and type B2 cells. Type B1 cells had large, round,
Figure 3.2

Cell types in the subependymal layer of the normal adult human brain

A. This figure demonstrates the range of cell types present in the adult normal brain SEL. Haemotoxylin & eosin and luxol fast blue histochemical staining demonstrated the 4 major cell types in the SEL. Type A cells had densely stained, medium sized nuclei that were predominantly located in the upper region of the SEL (a typical type A cell is surrounded by a yellow box). Type B cells had large nuclei that had lightly labelled nucleoplasms with dark punctate chromatin staining within. Following the classification of Doetsch et al (1997), type B cells that were round were classified as type B1 cells (a typical type B1 cell is surrounded by a yellow box), and type B cells that had elongated nuclei were classified as type B2 cells (a typical type B2 cell is surrounded by a yellow box). Type B1 cells were the most common cell type in the SEL. Type C cells had small, round and very densely stained nuclei and were predominantly located in the lower part of the SEL (a typical type C cell is surrounded by a yellow box in figure 3.2A). Type E cells were ependymal cells and these cells lined the lateral ventricle by forming a single cell layer of epithelial-like cells.

Protoplasmic astrocytes were identified in the myelin layer and caudate nucleus (see red circles in figure A). Also, perithelial cells were demonstrated surrounding blood vessels in the SEL (see blue circle in figure A).

B. The graph in figure 3.2B demonstrates the average number of type A, B and C cells in the normal brain SEL. These cell counts were made from 6 normal brains and are displayed as the number of cells present per 230 μm. Type A and C cells were present in approximately even numbers; however, there were about 3 times as many type B cells as there were type A or C cells.
Cell types in the subependymal layer of the normal adult human brain

Comparison of the different cell types present in the normal human subependymal layer

Cell numbers

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Type A</th>
<th>Type B</th>
<th>Type C</th>
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<tr>
<td></td>
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Cell types
vesicular nuclei whose nucleoplasm was lightly stained with haematoxylin & eosin. Within the nucleoplasm there were multiple dark, punctate stained regions of densely labelled chromatin in the nucleus. The nuclear membrane of Type B cells was a little more densely labelled than the nucleoplasm. Surrounding some type B cell nuclei a small amount of cytoplasm could be seen that was lightly stained with haematoxylin. Also, close to the nuclear membrane, some type B cells had small nucleoli that were strongly haematoxylinophilic. Type B₁ cells had the largest nuclei of any cell in the SEL (see figure 3.2A where a typical type B₁ cell is surrounded by a yellow box). Type B₂ cells had large, elongated nuclei that had a densely haematoxylin & eosin labelled nuclear membrane but had nucleoplasm staining much like that of type B₁ cells (a typical type B₂ cell is surrounded by a yellow box in figure 3.2A).

**Type C** cells had small, mostly round nuclei that had very densely stained chromatin and had very little nucleoplasm. The nuclear membrane of type C cells was indistinguishable from the nucleoplasm because of the dense labelling in both regions. The labelling in the nucleoplasm demonstrated no punctate staining; instead, there was just very dense labelling throughout (a typical type C cell is surrounded by a yellow box in figure 3.2A).

**Type E** cells were ependymal cells that had irregularly shaped nuclei and labelled with haematoxylin & eosin with a mixed density. The type E cells formed a single cell thick layer that separated the lateral ventricle from the subependymal cells. Type E cell nuclei had a heterogeneous array of shapes but were predominantly cuboidal to columnar in shape. However, almost all of the cells in the EPL were densely stained with haematoxylin & eosin and had no punctate intranuclear or cytoplasmic staining (see figure 3.2A).

Within the SEL there were a few cells that did not fit into the classification outlined by Doetsch et al (1997). One such cell type was identified as perithelial cells (see blue circle in figure 3.2A) that were located around blood vessels in the SEL. The perithelial cells were flat, elongated cells that formed a circular shape with the lumen of the blood vessel. These cells were only ever found around blood vessels.
Another type of cell present in the SEL was **protoplasmic astrocytes** (see red circles in figure 3.2A). These cells were occasionally seen in the myelin layer beneath the SEL and are only found in neuron rich grey matter. They are cells with round nuclei that are smaller than type C cell nuclei and stain very darkly with haemotoxylin & eosin.

### 3.3.1.2 Localisation of the different cell types in the subependymal layer
(see figure 3.2)

Within the SEL the various cell types identified were present in different parts of the SEL. The type A cells were predominantly present in the more superficial part of the SEL closer to the EPL (see figure 3.2A). The type B cells were evenly distributed throughout the SEL and were not confined to any particular part of the SEL (see figure 3.2A). The type C cells were almost exclusively found in the deep region of the SEL near the myelin layer (see figure 3.2A). Protoplasmic astrocytes were only demonstrated in the myelin layer and caudate nucleus and were never demonstrated in the SEL itself. The perithelial cells were only present in blood vessel walls, where they appeared to surround the vessel (see blue circle around the cells in figure 3.2A).

The number of cells of each cell type, A, B and C, in the normal human brain were counted in 6 normal brains (see table 3.1). The proportion of each cell type could be expressed as a ratio of A:B:C - 1:3:1 (see figure 3.2B). The cell numbers demonstrated in figure 3.2B represent the number of each cell type present per 230 μm (per frame photographed at 63 x magnification). Thus the most common type of cell present in the normal brain SEL were type B cells; on average there were 3 times more type B cells than type A and C cells (see figure 3.2B).

### 3.3.2 Comparison of the thickness of the subependymal layer in normal and Huntington’s disease brains (see figures 3.3 – 3.6)

To examine the difference between the normal adult brain SEL and the HD SEL, comparison was made of the thickness (μm) of the SEL from the EPL to the superficial
surface of the myelin layer. This comparison was made on 7 μm thick caudate nucleus sections; three measurements were taken; dorsal, middle and ventral. Measurements were taken from 6 normal and 9 HD brains whose pathological grades ranged from 1 to 3 (see table 3.1) (Vonsattel and DiFiglia, 1998; Vonsattel et al., 1985). The mean thickness of the SEL in normal brains was 50.90 ± 28.07 μm (mean ± standard deviation), whilst the mean thickness of the SEL in HD brains was 141.06 ± 65.92 μm (see figure 3.3). Thus there was a statistically significant difference between the SEL thickness of normal and HD brains (p<0.01) (see figure 3.3 and 3.4). On average the SEL in the HD brain was 2.8 times thicker than that of the normal brain SEL.

There were also differences seen across the three grades of HD examined (HD grade 1-3) (see figure 3.5 and 3.6). Grade 1 HD brains had an average thickness of 90.59 ± 42.80 μm (median = 84.40 μm). Grade 2 HD brains SEL had an average SEL thickness of 142.57 ± 65.67 μm (median = 116.56 μm). Grade 3 brains SEL had an average thickness of 190.03 ± 100.75 μm (median = 216.15 μm). Thus, as the grade of HD increased so did the thickness of the SEL (see figure 3.5 and 3.6). There was no significant difference between the normal brain SEL thickness and the HD grade 1 brains (p<0.06); however, there was a significant difference between the thickness of the normal and grades 2 (p<0.01) and 3 (p<0.01) HD brains. There was a significant difference between the SEL thickness of grade 1 and grade 3 brains (p<0.01). There was no significant difference between grade 1 and 2 or between grade 2 and 3, however the overall trend (R^2 = 0.9974) was that as the grade of HD increased the thickness of the SEL also increased. Comparison of the means showed that in comparison with the normal brains, there was a 77% increase in the thickness of the SEL in HD grade 1 brains, a 180% increase in the thickness of the SEL in HD grade 2 brains, and, a 273% increase in the thickness of the SEL in HD grade 3 brains.
Figure 3.3

Comparison of the thickness of the subependymal layer in normal and Huntington’s disease brains

This figure demonstrates that in the normal human brain the SEL is relatively thin compared with the much thicker SEL in the HD brain (see arrows). These sections are 7 μm thick and have been stained with haemotoxylin & eosin and luxol fast blue. Scale bar = 40 μm.
Comparison of the thickness of the subependymal layer in normal and Huntington’s disease brains

A

EPL

SEL

CN

40 μm

Normal

B

EPL

SEL

CN

40 μm

Huntington’s disease
Figure 3.4

Thickness of the subependymal layer in normal and Huntington’s disease brains

This graph demonstrates the thickness of the SEL in normal brains and in HD brains. In normal brains the average thickness of the SEL was 50.90 ± 28.07 µm (mean ± standard deviation) compared with the average thickness of the SEL in HD brains of 141.06 ± 65.92 µm. Thus there was a 2.8 fold increase in the thickness of the SEL in the HD brain compared with normals; the increase in the thickness of the SEL in the HD brains was statistically significant (p<0.01).
Thickness of the subependymal layer in normal and Huntington's disease brains

![Bar chart showing thickness comparison]

- Normal
- Huntington's disease

$p < 0.01$
Figure 3.5

Thickness of the subependymal layer in normal and Huntington’s disease (grade 1-3) brains

This graph demonstrates the relationship of increasing HD pathological grade on the thickness of the SEL. The normal brains had an average thickness of $50.90 \pm 28.07 \, \mu m$ compared with $90.59 \pm 42.80 \, \mu m$ in HD grade 1, $142.57 \pm 65.67 \, \mu m$ in HD grade 2 and $190.03 \pm 100.75 \, \mu m$ in grade 3. The SEL in each grade of HD was thicker than that of the normal brains SEL, and with increasing neuropathological grades there is an increase in the thickness of the SEL ($R^2 = 0.9974$). There was a significant difference between the normal brain and the grade 2 brain (p<0.01), grade 3 brain (p<0.01) and also between grade 1 and grade 3 (p<0.01).
Thickness of the subependymal layer in normal and Huntington's disease (grade 1-3) brains.
Figure 3.6

The subependymal layer in normal and Huntington’s disease (grades 1 and 3) brains

This figure demonstrates that as the grade of HD increases so does the thickness of the SEL. In the normal case (A) the SEL is very thin; however in HD grade 1 (B) the SEL is thicker, and in HD grade 3 (C) the SEL is considerably thicker than the SEL in the normal case or grade 1 HD case. Scale bar = 40 μm.
The subependymal layer in normal and Huntington's disease (grades 1 and 3) brains

A

\[ 40 \mu m \]

Normal

B

\[ 40 \mu m \]

Grade 1

C

\[ 40 \mu m \]

Grade 3
3.3.3 Quantification of cells in the subependymal layer in normal and Huntington’s disease brains (see figure 3.7, 3.8)

Having demonstrated a significant difference between the thickness of the SEL in normal and HD brains (see figure 3.4 and 3.5), the same six normal and nine HD brains (see table 3.1) were used to count and compare the number of cells in the SEL. The number of cells that were present in 7 μm haemotoxylin & eosin and luxol fast blue histochemically stained sections was examined by counting the number of cells that were present within the SEL, extending from the deep surface of the EPL to the superficial surface of the myelin layer, and from the dorsal to ventral extent of the SEL (see figure 3.1). The cells that were in this region were counted regardless of their cellular morphology or histochemical staining intensity. The number of cells counted was divided by the length of the SEL in the section (in mm); thus the cell counts are expressed as cells/mm. The average number of cells/mm in the normal brains was 103.23 ± 23.41 cells/mm (median = 100.10 cells/mm); in the HD brains the average number was 291.62 ± 132.15 cells/mm (median = 244.20 cells/mm). There was a statistically significant difference between the number of cells/mm present in the normal and HD brains (p<0.01; see figure 3.7). Thus, comparing the means, the HD brain had a 2.8 fold increase in the number of cells in the SEL compared to that of the normal brain SEL.

In order to determine whether particular grades of HD contained more cells/mm than other HD grades, the number of cells/mm were compared across the three grades of HD examined (HD grades 1, 2 and 3). As shown in figure 3.8, HD grade 1 brains had an average of 228.10 ± 24.99 cells/mm (median = 240.80 cells/mm). Grade 2 brains had an average of 328.06 ± 157.59 cells/mm (median = 212.10 cells/mm). Grade 3 brains had an average of 318.71 ± 187.74 cells/mm (median = 289.12 cells/mm). Statistical analysis was performed on the three different HD grades and the normal cases examined which demonstrated a strong trend (R² = 0.8515) that as the HD grade increased so did the number of cells/mm in the SEL (see figure 3.8). Despite the very evident trend there was no statistically significant difference between the three grades of HD, however there
Figure 3.7

Number of cells/mm in the subependymal layer of normal and Huntington’s disease brains

This graph demonstrates the effect HD has on the number of cells in the SEL that were counted on 7 µm thick haemotoxylin & eosin and luxol fast blue stained sections. The number of cells in the length of the SEL was divided by the length of the SEL (mm). Normal brain SEL (6 cases) contained 103.23 ± 23.41 cells/mm, compared with 291.62 ± 132.15 cells/mm in the HD brain SEL (9 cases; grades 1-3). In HD cases there was a statistically significant increase in the number of cells/mm in the SEL (p<0.01).
Number of cells/mm in the subependymal layer of normal and Huntington's disease brains

- Normal: [Bars for cell counts in normal brains]
- Huntington's disease: [Bars for cell counts in Huntington's disease brains]

$p < 0.01$
Figure 3.8

Number of cells/mm in the subependymal layer of normal and Huntington’s disease (grades 1-3) brains

This graph demonstrates the effect of increasing HD pathological grade on the number of cells/mm in the SEL. In the SEL, the normal brains had an average number of 103.23 ± 23.41 cells/mm whereas HD grade 1 cases had an average of 228.10 ± 24.99 cells/mm. HD grade 2 SEL had an average of 328.06 ± 157.59 cells/mm, and HD grade 3 cases had an average of 318.71 ± 187.74 cells/mm. Compared to the normal brain SEL the number of cells present in the SEL in each grade of HD was significantly higher. Normal compared with grade 1 (p<0.01), grade 2 (p<0.01) and grade 3 (p<0.02).
Number of cells/mm in the subependymal layer of normal and Huntington's disease (grades 1-3) brains

- Normal: 100 cells/mm
- Grade 1: 200 cells/mm
- Grade 2: 300 cells/mm
- Grade 3: 300 cells/mm

Statistical differences:
- p < 0.01
- p < 0.02
was a significant difference between the normal brains and each of the HD grades examined. Comparison of the mean number of cells in the SEL of each HD grade with the normal brains demonstrated that there was: a 2.2 fold increase in the number of cells in the HD grade 1 brains (p<0.01); a 3.2 fold increase in the number of cells in HD grade 2 brains (p<0.01); and, a 3.1 fold increase in the number of cells in HD grade 3 brains (p<0.02).

3.3.4 Comparison of the different cell types present in the adult subependymal layer in normal and Huntington's disease brains (see figure 3.9, 3.10)

In the HD brain SEL the same cell types that were present in the normal brain SEL could be identified (see figure 3.9). The distribution of the various cell types was the same as that demonstrated in the normal SEL. The type A cells were predominantly located in the more superficial part of the SEL closer to the EPL, type B cells were homogenously distributed throughout the SEL beneath the cell devoid area beneath the EPL, and the type C cells were located closer to the myelin layer in the deep part of the SEL (see figure 3.9).

In order to determine which type of SEL cells were increased in numbers in the HD brains, the number of each cell type was counted in the normal brain and in HD grades 1-3 brains. The cell numbers have been displayed as the number of cells in a 230 µm (the width of a frame at 63 x magnification) length of SEL. In the normal SEL the average number of: type A cells was 4.00 ± 1.7; type B cells was 11.16 ± 3.7; and, type C cells was 4.00 ± 1.4 (see figure 3.9A and 3.10). Thus the ratio of type A:B:C cells was approximately 1:3:1.

In HD grade 1 brains the average number of type A cells was 7.30 ± 1.5, type B cells was 24.66 ± 4.0 and type C cells was 6.00 ± 3.4 (see figure 3.9B and 3.10). Thus the approximate ratio of A:B:C was 1:4:1, which indicates that the type B cells had the most
Figure 3.9

Cell types in the subependymal layer and ependymal layer of the normal and Huntington’s disease brain

This figure demonstrates the range of cell types present in the adult normal and HD brain (grade 3) SEL. The cell types were classified on the basis of their morphological appearance in sections stained with haemotoxylin & eosin and luxol fast blue using the nomenclature of Doetsch et al (1997) (Doetsch et al., 1997). Four major cell types were identified. **Type A** cells (densely stained, medium sized nuclei) were predominantly located in the superficial region of the SEL. **Type B** cells (large, lightly labelled nucleoplasm and dark puctate stained chromatin in the nucleoplasm). Type B cells were sub-classified by shape; type B₁ cells had round and type B₂ cells had elongated nuclei. Type B₁ cells were the most common cell type in the SEL. **Type C** cells (small, round and very densely stained nuclei) were predominantly located in the lower part of the SEL. **Type E** cells were ependymal cells, which lined the lateral ventricle forming a single cell layer of cuboidal-columnar epithelial cells within the EPL.

Protoplasmic astrocytes were identified in these sections, located in the myelin layer and caudate nucleus (see red circles). Also, perithelial cells were demonstrated surrounding blood vessels in the SEL (see blue circle). Scale bar = 40 μm.

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Cell types in the subependymal layer and ependymal layer of the normal and Huntington's disease brain

A

SEL

B

SEL

40 μm

Normal

40 μm

Huntington's disease
Figure 3.10

Comparison of the numbers of the different cell types present in the normal and Huntington's disease (grades 1-3) subependymal layer

The normal brain SEL had an average of 4.00 ± 1.7 type A cells, 11.16 ± 3.7 type B cells and 4.00 ± 1.4 type C cells. HD grade 1 brain SEL had an average of 7.30 ± 1.5 type A cells, 24.66 ± 4.0 type B cells and 6.0 ± 3.4 type C cells. HD grade 2 brain SEL had an average of 8.66 ± 5.5 type A cells, 40.33 ± 10.6 type B cells and 7.33 ± 3.0 type C cells. The HD grade 3 brain SEL had an average of 5.66 ± 3.2 type A cells, 41.66 ± 24.4 type B cells and 6.66 ± 3.5 type C cells. Thus with increasing grades of HD there were proportionally more type B cells present in the SEL. Statistical comparison of the number of type B cells in the normal and HD brain SEL demonstrated that grade 1 (p<0.01), grade 2 (p<0.01) and grade 3 (p<0.01) HD brains had significantly more type B cells than the normal brain SEL. Also, on average there was an increase (approximately 50%) in the number of type A and C cells in the HD brain SEL compared with the normal brain SEL.

This figure demonstrates that the normal brain had less type A, B and C cells compared with each grade of HD (grades 1-3). The type B cells demonstrated the greatest increase in cell numbers in the SEL.
Comparison of the numbers of the different cell types present in the normal and Huntington's disease (grades 1-3) subependymal layer.
substantial increase in cell numbers (220%) compared with normals (see figure 3.10). However, there was also a 54% increase in the number of type A cells and a 50% increase in the number of type C cells in HD grade 1 brains.

In HD grade 2 brains, the average number of type A cells was 8.66 ± 5.5, type B cells was 40.33 ± 10.6, and type C cells was 7.33 ± 3.0 (see figure 3.10). Thus the approximate ratio of type A:B:C cells was 1:5.5:1. There were increases in the number of type A and C cells compared with normals, however the major increase in the number of cells present in the SEL was due to an increase in the number of type B cells.

In HD grade 3 brains, the average number of type A cells was 5.66 ± 3.2, type B cells was 41.66 ± 24.4 and the type C cells was 6.66 ± 3.5 (see figure 3.10). Thus the ratio of type A:B:C was approximately 1:7.5:1. Again the major increase in the number of cells in the SEL in HD grade was caused by an increase in the number of type B cells. There was also an increase in the number of type A and C cells in each of the HD grades compared with the normal brain SEL. Statistical comparison of the number of type B cells in the normal brain SEL compared with the number present in the HD brain SEL demonstrated that grade 1 (p<0.01), grade 2 (p<0.01) and grade 3 (p<0.01) each had significantly more type B cells than the normal brain SEL.

In summary, as the grade of HD increases so does the ratio of type B cells to type A and C cells. In the normal brains there was a ratio of type A:B:C cells of 1:3:1, where as in HD grade 3 there is a ratio of 1:7.5:1. Despite such a large increase in the number of type B cells present in the HD SEL compared with the normal SEL, there is also an increase in the number of type A and type C cells in the HD SEL that is relatively unchanged with HD grade.

3.4 Discussion

3.4.1 The general anatomy of the subependymal layer

The human SEL is comprised of a heterogeneous cell population that can be categorised on the basis of morphology, location and histochemical staining characteristics. Using the
same classification of cell types in the SEL that was used in the mouse SEL by Doetsch et al (1997) (Doetsch et al., 1997). I have identified 4 major types of cells in the human SEL. My study here is the first demonstration of the various cell types in the human SEL that have been previously identified in the rodent SEL. The type A cells, described by Doetsch et al (1997) as migrating neuroblasts (Doetsch et al., 1997), are present in the human SEL and are predominantly located in the upper region of the SEL immediately beneath the cell-sparse gap beneath the ependymal layer. Within the SEL many type B cells were also present, these cells had large, round and lightly stained nuclei (type B1) or had large, elongated and lightly stained nuclei (type B2) and were evenly distributed throughout the SEL beneath the cell-sparse region. The type C cells were also demonstrated in the human SEL near the myelin layer. Type C cells have been described by Doetsch et al (1997) as being putative precursors in the mouse brain. Type E (ependymal) cells lined the lateral ventricle and separated the cerebrospinal fluid from the SEL. This is the first demonstration of a stratified, compartmentalised arrangement of SEL cell types in the human brain and represents a significant advancement of the knowledge of the human SEL anatomy. Although my findings and descriptions of the various cell types present in the human SEL are in general agreement with the study of Doetsch et al (1997) the anatomy demonstrated in the mouse brain appears not to have a stratified arrangement of cell types within the SEL. The stratification is probably less evident in the mouse brain due to the smaller size of, and lower number of cells in, the SEL. Because of the larger size of the human SEL, if the type B cells are to provide the trophic support suggested by Doetsch et al (1997), then it would be vital that the type B cells have close contact with the type A and C cells (Doetsch et al., 1997).

3.4.2 The Huntington's disease subependymal layer is thicker than that of the normal brain subependymal layer.

In these experiments the thickness of the SEL in normal and HD human brains was measured. When the mean thickness of the SEL was compared between normal and the HD SEL, the HD SEL was on average 2.8 times thicker than that of normal (see figure 3.4). This increase in thickness of the SEL was not strongly correlated with the number of
cells in the SEL. But as the grade of HD increased, so did the thickness of the SEL (90.59 ± 42.80 μm in HD grade 1, 142.57 ± 65.67 μm in HD grade 2 and 190.03 ± 100.75 μm in grade 3; R² = 0.9974).

3.4.3 The Huntington’s disease brain subependymal layer contains more cells than that of the normal subependymal layer.

The results from the cell counts of the number of cells present on haemotoxylin & eosin and luxol fast blue stained sections demonstrated that the HD SEL had a 2.8 fold increase in the number of cells in the SEL compared with normals (see figure 3.7). Furthermore, as the grade of HD increased so did the number of cells in the SEL (see figure 3.8). The dramatic increase in the number of cells in the HD SEL (103.23 ± 23.41 cells/mm in normal brains compared with 291.62 ± 132.15 cells/mm in HD) gives one indication as to why the thickness of the SEL is increased in HD. As the pathology increased in the HD caudate nucleus, there was an increase in the number of cells in the SEL which must be brought about by either astrocytosis, oligodendrogliosis, neurogenesis or a combination of the three. Increased numbers of cells in the SEL were reported by Arvidsson and colleagues, in experiments aimed at demonstrating the effect that a stroke has on the SEL in the rodent brain (Arvidsson et al., 2002). In their experiments Arvidsson et al (2002) used an ipsilateral middle cerebral artery occlusion (MCAO) model of stroke with subsequent administration of the mitotic marker BrdU, which demonstrated a 1.6 fold increase in the number of new cells in the SEL on the ipsilateral, ischaemic side of the brain (Arvidsson et al., 2002). Using BrdU to label the dividing cells, they demonstrated the presence of increased numbers of labelled cells not only in the SEL but also in the ischaemic striatum on the ipsilateral side.

In another study investigating the proliferation of cells in the SEL, Parent et al (2002) induced status epilepticus by injecting pilocarpine into the peritoneum of rats. This animal model of epilepsy induces seizures within one hour of pilocarpine injection and can be stopped by the injection of diazepam (Parent et al., 2002a). 1 and 2 hours prior to sacrificing the animals, BrdU was administered (I.P.) to label any mitotically active cells.
in the SEL. In response to pilocarpine induced seizures, there was an increase in the numbers of BrdU positive cells in the SEL, this was evident in some animals by day 4 after seizure induction but was most evident by 7-14 days post-seizure (Parent et al., 2002a). However, at day 1 and after day 21 on post-seizure, there was no significant increase in the BrdU positive cell numbers in the SEL compared with the normal SEL (Parent et al., 2002a).

Taken together, these studies indicate the plasticity and capacity of the SEL to respond to neurodegenerative pathology by increasing the production of new cells in response to the nearby cellular injury. Thus it is most likely that the increased pathology that I have seen in the caudate nucleus in HD is driving more new cell production as demonstrated by increased cell numbers in the HD SEL.

3.4.4 There are more type A, B and C cells in the Huntington’s disease brain subependymal layer compared with the normal brain subependymal layer.

In the present study the number of cells of each cell type (using the Doetsch classification which is based on cell morphology) in the SEL was counted in normal and HD brain sections. In the HD brains there were more A, B and C type cells present compared with the normal brains. In particular, on average (average of HD grades 1-3) there was a 1.8 fold increase in type A cells, 3.2 fold increase in type B cells and a 1.7 fold increase in type C cells in the HD brains. However, as the grade of HD increased so did the proportion of type B cells. HD grade 1 brain SEL had an average of 7.30 ± 1.5 type A cells, 24.66 ± 4.0 type B cells and 6.0 ± 3.4 type C cells. Thus there was an increase in the ratio of type B cells to type A and C type cells. In the normal brain the ratio of A:B:C type cells was 1:3:1 where as here in HD grade 1 there is an A:B:C ratio of 1:4:1.

HD grade 2 brain SEL had an average of 8.66 ± 5.5 type A cells, 40.33 ± 10.6 type B cells and 7.33 ± 3.0 type C cells. The ratio of type A:B:C type cells is again increased relative to the normal brain. The ratio in grade 2 HD brains is 1:5.5:1 compared with 1:3:1 in normal brains.
The HD grade 3 brain SEL had an average of $5.66 \pm 3.2$ type A cells, $41.66 \pm 24.4$ type B cells and $6.66 \pm 3.5$ type C cells. Once again there is major increase in the proportion of type B cells to type A and C cells. In HD grade 3 brains there is a 1:7.5:1 ratio of type A:B:C cells. There was a significant increase in the number of type B cells in each grade of HD; grade 1 (p<0.01), grade 2 (p<0.01) and grade 3 (p<0.01) each had significantly more type B cells than the normal brain SEL.

Based on the morphological identification of the cell types there seems to be more migrating neuroblasts and more putative precursor cells in the HD SEL. These results, indicating increased cell numbers in the SEL in HD, indicate that migrating neuroblasts possibly migrate into the diseased striatum in HD. The putative precursors may become neurons, astrocytes or oligodendrocytes depending on their location and cues from the local environment; numerous studies have demonstrated that there are precursor cells in the rodent brain capable of becoming new neurons and glial cells. In particular the evidence has come from in vitro studies in which the SEL is dissected from the rodent brain and grown in culture to form spheres of immature cells. The cells within the spheres give rise to neurons, astrocytes and oligodendrocytes (Chiasson et al., 1999; Reynolds and Weiss, 1996; Tropepe et al., 1999; Vescovi et al., 1993; Weiss et al., 1996a). The major increase in the number of type B cells (identified by Doetsch et al (1997) as glial cells) suggests that some of the type B cells are a result of increased astrocytosis but also may also provide trophic support for the migrating and differentiating cell in the SEL (Doetsch et al., 1997; Flax et al., 1998). However, it is impossible without in vitro experiments to determine the potential of new cells. Irrespective of these considerations, the classification here is remarkably comparable to the mouse where in vitro experiments have shown the presence of progenitor cells that have the potential to form new neurons and glia (Chiasson et al., 1999; Craig et al., 1996; Gates et al., 1995; Morshead et al., 1994; Pardo and Honegger, 2000).
3.5 Summary

The results from this chapter demonstrate that the human SEL is a heterogeneous region mostly comprised of type A, B and C cells. This is in good keeping with the results from studies of the mouse SEL performed by Doetsch et al (1997). Also, my results demonstrate for the first time, that there is a compartmental organisation of the human SEL. Beneath the EPL there is a small area predominantly devoid of cell body staining. The type A cells are present in a layer beneath the cell-devoid area; based on morphology these cells appear to be migrating neuroblasts. Type B cells are evenly spread throughout the SEL, but their location is not confined to any particular part of the SEL. In the lower part of the SEL, above the myelin layer superficial to the caudate nucleus, the type C cells are located and beneath the type C cells a layer of myelin separates the SEL from the caudate nucleus.

In HD, the SEL was on average 2.8 times thicker than that of normal brains. Also, there were 2.8 times as many cells in the HD brain SEL compared with normal brains. The major increase in cell numbers was a result of a major increase in the number of type B cells (normal vs. HD brains p<0.01), however there was also an increase in the number of type A and C cells in the HD SEL. As the HD grade increased so did the thickness of the SEL and number of cells in the SEL; also, as the grade of HD increased so did the ratio of B type cells to A and C type cells.

3.6 Conclusion

This chapter has demonstrated a remarkable resemblance between the cell types present in the normal adult human brain SEL and that of the adult mouse brain (as demonstrated by Doetsch et al (1997) (Doetsch et al., 1997)). The present study has, however, demonstrated that there are proportionally fewer type A (migrating neuroblasts) in the human SEL compared with the mouse brain SEL. Instead of a large number of type A cells the human brain SEL contains a large number of type B cells. However, the most
important discovery here is the demonstration for the first time that the SEL is not only 2.8 times thicker in HD brains, but it also contains 2.8 times more cells than in the normal brain SEL. Furthermore, I have demonstrated that there are increased numbers of type A, B and C cells but in particular the bulk of the increase in cell numbers was made up of type B cells in the HD SEL. Also, as the grade of HD increases, so does the ratio of type B cells to type A and C cells. Taken together, these results raise the exciting possibility that the increase in type A and C cells in HD brains may serve to provide for the replacement of neurons and the increase in type B cells may represent astrocytosis. The following chapter will examine the possibility that the cell proliferation in the SEL represents gliogenesis and/or neurogenesis.
Chapter 4

PROGENITOR CELL PROLIFERATION AND NEUROGENESIS IN THE NORMAL AND HUNTINGTON’S DISEASE BRAIN

4.1 Introduction

A particularly exciting and novel development in the treatment of neurodegenerative diseases is the suggestion from both animal and human studies that the transplantation of embryonic neurons or stem/progenitor cells may offer a potential treatment strategy for neurodegenerative disorders such as Parkinson’s disease, Huntington’s disease and Alzheimer’s disease (Freed et al., 2001; Svendsen et al., 1997; Svendsen and Smith, 1999). Although in recent years the transplantation of embryonic cells into the diseased human brain has emerged from the realm of the theoretical to that of the practical, it is associated with ethical, technical and immunological problems (Freed et al., 2001). Thus, the demonstration of endogenous stem/progenitor cells in the hippocampus and the SEL overlying the caudate nucleus in the adult mammalian brain has raised the exciting possibility that these undifferentiated cells may be able to generate new neurons for cell replacement in neurodegenerative diseases such as Huntington’s disease (HD). Indeed, neural stem cells in the rodent brain SEL adjacent to the caudate nucleus have recently been shown to proliferate and differentiate into striatal neurons suggesting they may provide a source of replacement neurons (Arvidsson et al., 2002; Morshhead et al., 1994; Parent et al., 2002a). In this regard it is especially interesting that recent studies on the normal adult human brain have shown evidence of neurogenesis in the hippocampus (Eriksson et al., 1998), but no previous study has yet shown neurogenesis in the SEL of the normal or diseased human brain.
In chapter 3, more cells were demonstrated in the SEL in the HD brain compared with the normal brain. Therefore, in this chapter, western blot analysis, immunohistochemistry, light and laser scanning confocal microscopic techniques have been used to investigate whether progenitor cell proliferation and neurogenesis occur in the SEL adjacent to the caudate nucleus in response to cell death in the caudate nucleus of the adult human HD brain. In particular, the following questions have been addressed:

1. Does progenitor cell proliferation occur in the SEL of the adult normal and HD brains?
2. Does neurogenesis occur in the SEL of the adult normal and HD brains?
3. Does gliogenesis occur in the SEL of the adult normal and HD brain?
4. Are young neurons and glial cells evenly distributed throughout the SEL?

4.2 Materials and methods

4.2.1 Human tissue collection

For this study the basal ganglia from post-mortem human brains were obtained from the Neurological Foundation of New Zealand Human Brain Bank at the Department of Anatomy with Radiology, University of Auckland. The full consent of all families was obtained at the time of autopsy and the University of Auckland Human Subjects Ethics Committee approved the protocols used in these studies. Normal brains were received from cases with no history of neurological disease and on pathological examination showed no neurological abnormalities. The 6 normal post-mortem cases ranged from 42-73 years of age (average = 55.3 years of age) and had post-mortem delays ranging from 10-18 hours (average = 14 hours). 9 HD brains were received from cases with a family and clinical history of HD. The diagnosis of HD was confirmed by genetic analysis of the CAG repeat length in both copies of the IT15 gene, the gene affected in HD. The HD post-mortem cases ranged from 41-74 years of age (average = 62.8 years of age) and had post-mortem delays ranging from 5-19 hours (average = 10.2 hours) (see figure 4.1A).

For immunohistochemical, histochemical and immunofluorescent studies, the brains were fixed by perfusion through the basilar and internal carotid arteries. Initially the brains were perfused with phosphate buffered saline (PBS) with 1% sodium nitrite to clear the
brain’s circulation, followed by 15% formalin in 0.1 M phosphate buffer pH 7.4. Following the perfusion, the brains were dissected into regions and the basal ganglia were removed en-bloc and post-fixed in the same fixative for 24 hours. Subsequently these blocks were cryo-protected in 20% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for 4-5 days, and then in 30% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for a further 4-5 days.

For western blotting fresh brain tissue was removed from the region of interest and frozen at -80° C for further processing (see Western blotting section).

4.2.2 Immunohistochemistry

4.2.2.1 Section preparation

The basal ganglia blocks were sectioned at a thickness of 50 μm on a freezing sledge microtome (Zeiss HM440), from the rostral to caudal axis of the lateral ventricle and were stored in serial order in individual wells of a 90 well plate in a solution of PBS and 1% sodium-azide. The sections were removed from PBS and 1% sodium-azide and were washed 3 times in PBS containing 0.2% triton-x (wash buffer) before being further processed free floating in culture wells for immunostaining.

Sections that were to be stained for PCNA were first processed for antigen retrieval to optimise the PCNA staining. The sections were incubated overnight in a citric acid buffer solution containing citric acid (Na₃ salt) and Na₂HPO₄; pH 4.5 HCL at 4° C. The next day the citric acid from each of the wells was replaced with fresh citric acid solution (room temperature) and the sections were microwaved for 30 seconds on high in a 600 W microwave oven. The sections were allowed to cool for approximately one hour before the citric acid solution was removed.

All sections were thoroughly washed (3 x 10 minute washes) in PBS containing 0.2% triton-x (wash buffer) before being incubated for 20 minutes in a 50% methanol solution containing distilled H₂O, methanol and 1% H₂O₂ to block endogenous peroxidase staining in the tissue. The sections were again thoroughly washed (3 x 10 minute washes) in wash buffer. Subsequently, primary, secondary and tertiary antibodies were applied as detailed below.
4.2.2 2 Primary antibodies
The following primary antibodies were applied to sections from the basal ganglia blocks. For the PCNA staining, 3 different primary antibodies were trialed and compared:
1) Mouse anti-Proliferating Cell Nuclear Antigen (PCNA PC10 Santa Cruz) at a dilution of 1:500
   Rabbit anti-PCNA (FL-261 Santa Cruz) at a dilution of 1:1000
   Mouse anti-PCNA (Chemicon) at a dilution of 1:500
All three antibodies demonstrated identical patterns of labelling in human tissue.
2) Rabbit anti-Glial Fibrillary Acidic Protein (GFAP Dako) at a dilution of 1:5000
3) Mouse anti-βIII-tubulin (SDL.3D10 Sigma) at a dilution of 1:500
4) Mouse anti-neuronal nuclei (NeuN Chemicon) at a dilution of 1:1000
5) Mouse anti-vimentin (Chemicon) at a dilution of 1:500
Antibodies were diluted in a solution of PBS, 0.2% triton-x, 1% normal goat serum and 0.04% merthiolate. 1 ml of primary antibody was put in each well with the floating sections and was incubated on a shaker for 48 hours at 4°C. The sections were then washed (3 x 10 minute washes) with wash buffer and secondary antibodies were applied.

4.2.2.3 Secondary antibodies
1) Anti-rabbit Ig biotinylated (from donkey, Amersham Life Sciences).
2) Anti-mouse Ig biotinylated (from sheep, Amersham Life Sciences).
The secondary antibodies were diluted in PBS, triton-x, normal goat serum and merthiolate at a dilution of 1:500. The sections were incubated in secondary antibody, 1 ml per well, over night at room temperature before being washed (3 x 10 minutes washes).

4.2.2.4 Tertiary antibodies
Streptavidin biotinylated horseradish peroxidase (Amersham Life Sciences), was applied at a dilution of 1:1000 diluted with normal goat serum, PBS triton-x and merthiolate for 3 hours. The sections were thoroughly washed again and 200 μl 3,3 diamino-benzidine (DAB), 0.4 M phosphate buffer and 1% H2O2 was applied for 20 minutes. Sections were thoroughly washed and mounted onto chrome alum dipped slides (3 g gelatine, 0.3 g chromium potassium sulphate, 600 ml dH2O), slides were dipped in this solution and dried.
in an oven). The mounted sections were air dried for 24 hours before being coverslipped by immersing them in graded 70%, 80% and 90% alcohol (5 minutes each), then 2 x 100% alcohol and 3 x xylene (10 minutes each). Each slide was removed from xylene and a thin smear of Hystomount (Hughes & Hughes) was applied to the slide followed by a coverslip.

4.2.3 Immunofluorescence

4.2.3.1 Section preparation
The 50 μm thick striatal sections included the full ventral to dorsal aspect of the lateral ventricle. The sections underwent the standard PCNA antigen retrieval protocol, methanol/peroxide and washing steps as detailed in 4.2.2.1.

4.2.3.2 Primary antibodies
Sections were serially incubated in:

1. Rabbit anti-PCNA (FL-261 Santa Cruz) at a dilution of 1:100

or

Mouse anti-PCNA (PC-10 Santa Cruz) at a dilution of 1:100

Following this incubation the sections were washed (3 x 10 minute washes). The second primary antibodies were then applied; these were either:

2. Mouse anti-βIII-tubulin (SDL.3D10 Sigma) at a dilution of 1:250

or

Rabbit anti-GFAP (Dako) at a dilution of 1:500

The rabbit anti-PCNA antibody was used in conjunction with the mouse anti-βIII-tubulin antibody. The mouse anti-PCNA antibody was used in conjunction with the rabbit anti-GFAP antibody. Using two primary antibodies from different species sequentially on the same section ensured that the secondary antibody or fluorochrome did not bind in a non-specific manner.

Antibodies were diluted in a solution of PBS, 0.2% triton-x, 1% normal goat serum and 0.04% merthiolate.
1 ml of primary antibody was added to each floating section and the sections were incubated on a shaker for 48 hours at 4°C. The sections were subsequently washed with wash buffer (3 x 10 minute washes); fluorescent chromagens were then applied.

4.2.3.3 Fluorochromes
After the sections had been serially incubated in the primary antibodies they were incubated in pooled solutions of mouse and rabbit specific fluorochromes:

1. Anti-mouse Alexa 594 (Molecular Probes Inc.) at a dilution of 1:200 (emission fluoresces red)

and

2. Anti-rabbit FITC (Sigma) at a dilution of 1:100 (emission fluoresces green).

Antibodies and fluorochromes were diluted in a solution of PBS, 0.2% triton-x, 1% normal goat serum and 0.04% merthiolate. The sections were incubated on a shaker for 12 hours at room temperature. The sections were subsequently washed in wash buffer, 3 times for 10 minutes per wash. Each section was then incubated in a Hoechst stain (33258 Sigma) (fluoresces blue when excited in the UV spectrum), diluted 1:500 in PBS, for 30 minutes. Hoechst labels DNA in the cell nucleus. Sections were again washed and mounted from water onto glass slides and were immediately coverslipped using Citifluor (Agar Scientific). The fluorescently labelled sections were imaged using a laser scanning confocal microscope (Leica TCS SP2) equipped with UV, argon, argon/krypton and helium/neon lasers. Each fluorescent label was imaged serially to eliminate detection of bleed through and other artificial fluorescence. The confocal images were captured in a Z-series with an interslice gap of 1 μm.

4.2.3.4 Image analysis and cell counting
Cell counts of double-labelled fluorescent cells in normal and HD brain SEL was achieved by imaging the entire length of the SEL at 20 x magnification (field of view), taking slices 1 μm apart through the Z-direction of the specimen. After the images had
been acquired, each stack was separated into 10 slice stacks with a 5 µm interstack gap to ensure that PCNA positive cells were not counted twice. Each 10-slice stack was made into a projection image for the purposes of counting PCNA positive cells; but the stacks could also be played as a cine loop for the purposes of counting the double-labelled cells. The cell counts from each 10-slice stack were combined to give the number of cells per field of view for the entire Z-direction. The total numbers of cells across all fields of view were divided by the number of fields of view for the entire length of the SEL. T-tests were used to compare the mean number of cells counted in the normal and HD brain SEL.

4.2.4 Western blotting

4.2.4.1 Preparation of gels
All the western blot experiments were performed using the Bio-Rad Mini-PROTEAN II electrophoresis system. Discontinuous polyacrylamide gels were cast using a 15% acrylamide gel solution for the running gel and a 4% acrylamide gel solution for the stacking gel. Acrylamide gel solutions contained the following:

<table>
<thead>
<tr>
<th>15% Acrylamide running gel</th>
<th>4% Acrylamide stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide</td>
<td>30% acrylamide</td>
</tr>
<tr>
<td>19.8 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCL pH 8.8</td>
<td>0.5 M Tris-HCL</td>
</tr>
<tr>
<td>10 ml</td>
<td>2.52 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10% SDS</td>
</tr>
<tr>
<td>400 µl</td>
<td>100 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>dH₂O</td>
</tr>
<tr>
<td>9.6 ml</td>
<td>6 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>TEMED</td>
</tr>
<tr>
<td>20 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>10% APS</td>
</tr>
<tr>
<td>200 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

The glass for the casting chamber was cleaned with alcohol and the casting chamber was assembled using sandwich clamps and was inserted into the casting stand ready for the gels to be poured. The first four components of the stacker gel were added to, and gently mixed in a falcon tube to produce a monomer solution. Ammonium persulfate (APS) and TEMED were subsequently added to the monomer one at a time (thus creating a polymer) and the falcon tube was very gently inverted to allow mixing to occur. In a timely manner the polymer was poured between the sandwiched glass plates using a
pipette. A small amount of room was left at the top of the sandwich glass to allow for the stacking gel that would overlay the running gel. Water-saturated butanol was added to the top of the gel so that the gel would not dry out. The gel was left to set for 1 hour before the butanol was poured off and the top of the gel was washed with dH2O. The stacking gel was made in the same way as the running gel and was poured on top of the running gel. A 10-space comb was inserted into the stacking gel solution and the stacking gel was left to polymerise for 1 hour. Once the stacking gel set, the casting assembly was transferred to the electrophoresis chamber. The electrophoresis chamber was filled with running buffer that contained:

**Running buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.42 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 ml</td>
</tr>
<tr>
<td>Made up to 1 litre of dH2O</td>
<td></td>
</tr>
</tbody>
</table>

The comb was removed from the gel and the protein samples were loaded into the spaces left by the comb.

**4.2.4.2 Preparation of samples**

For this study the basal ganglia from post-mortem human brains (see table 4.1) were obtained from the Neurological Foundation of New Zealand Human Brain Bank (Department of Anatomy with Radiology, University of Auckland). Representative western blots have been displayed in the figures.

The striata, including the SEL, was removed en-bloc from the HD and normal brains and was frozen onto cryostat chucks in the cryostat and was left for 30 minutes while the tissue temperature equilibrated. 30 μm sections were cut onto uncoated glass microscope slides and were immediately frozen on dry ice so as to minimize the activity of endogenous proteases in the tissue. The sectioned brain tissue was stored at -80°C prior to isolation of the SEL. With the assistance of a dissecting microscope the SEL was selectively separated from the caudate nucleus and the SEL from each brain were kept in separate eppendorf tubes on dry ice. Approximately 150 mg of tissue was collected from each HD and normal brain. 250 μl of homogenisation buffer was added to each SEL.
### Table 4.1

**Normal and Huntington’s disease cases used for Western blotting analysis**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Post-mortem delay (hrs)</th>
<th>CAG repeats</th>
<th>HD grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>H114</td>
<td>42</td>
<td>Male</td>
<td>11</td>
<td>unavailable</td>
<td>Normal</td>
</tr>
<tr>
<td>H121</td>
<td>64</td>
<td>Female</td>
<td>5</td>
<td>18/23</td>
<td>Normal</td>
</tr>
<tr>
<td>H128</td>
<td>34</td>
<td>Female</td>
<td>18.5</td>
<td>17/19</td>
<td>Normal</td>
</tr>
<tr>
<td>H123</td>
<td>78</td>
<td>Male</td>
<td>7.5</td>
<td>17/20</td>
<td>Normal</td>
</tr>
<tr>
<td>H131</td>
<td>73</td>
<td>Male</td>
<td>13</td>
<td>17/17</td>
<td>Normal</td>
</tr>
<tr>
<td>H132</td>
<td>63</td>
<td>Female</td>
<td>12</td>
<td>15/19</td>
<td>Normal</td>
</tr>
<tr>
<td>HC74</td>
<td>42</td>
<td>Female</td>
<td>12</td>
<td>17/42</td>
<td>1</td>
</tr>
<tr>
<td>HC72</td>
<td>63</td>
<td>Female</td>
<td>24</td>
<td>17/42</td>
<td>2</td>
</tr>
<tr>
<td>HC73</td>
<td>47</td>
<td>Male</td>
<td>4</td>
<td>19/49</td>
<td>2</td>
</tr>
<tr>
<td>HC76</td>
<td>71</td>
<td>Male</td>
<td>16</td>
<td>17/42</td>
<td>2</td>
</tr>
<tr>
<td>HC80</td>
<td>45</td>
<td>Female</td>
<td>15</td>
<td>23/43</td>
<td>2</td>
</tr>
<tr>
<td>HC82</td>
<td>74</td>
<td>Male</td>
<td>16</td>
<td>15/42</td>
<td>2</td>
</tr>
<tr>
<td>HC75</td>
<td>41</td>
<td>Male</td>
<td>5.5</td>
<td>18/52</td>
<td>3</td>
</tr>
<tr>
<td>HC85</td>
<td>61</td>
<td>Female</td>
<td>19</td>
<td>24/44</td>
<td>3</td>
</tr>
<tr>
<td>HC91</td>
<td>63</td>
<td>Female</td>
<td>5.5</td>
<td>23/44</td>
<td>3</td>
</tr>
<tr>
<td>HC93</td>
<td>56</td>
<td>Female</td>
<td>17</td>
<td>20/43</td>
<td>3</td>
</tr>
</tbody>
</table>

Sample and the tissue was homogenised using a hand-held tissue homogeniser for 30 seconds per sample. The homogenisation buffer contained sucrose 150 mM, Hepes pH 7.9 15 mM, KCL 60 mM, EDTA 5 mM, EGTA 1 mM, and 1 Complete™ Mini tablet protease inhibitor cocktail (Roche) per 10 ml. After homogenisation, 12.5 ml of 20% triton was added to the homogenised tissue samples (final dilution of 1% triton) and the samples were placed on ice for 1 hour. Subsequently, the samples were spun in a cooled centrifuge for 10 minutes at 14,000 rpm. The supernatant (soluble fraction) was removed and stored in aliquots of 50 μl in the −80°C freezer.

As the samples were required they were thawed on ice and the protein concentrations of each sample determined using a Bio-Rad protein assay as described by the manufacturer. Approximately 50 μg of sample protein in 25 μl of homogenisation buffer was used and this was heated at 95°C for 10 minutes in 2 x Laemli sample loading buffer. The protein samples were cooled and loaded onto the acrylamide gel and were resolved.
electrophoretically at 200 V at variable amperage for 45 minutes. One lane of the gel contained a protein ladder (MagicMark, Invitrogen Life Technologies), a weight marker for identification of the different weight proteins.

The acrylamide gel was removed from the running apparatus and was equilibrated for 15 minutes in transfer buffer that contained the following:

**Transfer buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3.00 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.42 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>112.50 ml</td>
</tr>
</tbody>
</table>

Made up to 1 litre with dH₂O

Filter paper, transfer pads and polyvinylidene di-fluoride (PVDF) (Hybond P, AmershamPharmacia Biotec RPN303f) transfer membrane was also equilibrated in transfer buffer.

The pads and the filter paper were used to ensure a tight fit between the gel and the PVDF membrane, and were placed in the transfer chamber. The transfer chamber was filled with transfer buffer and surrounded with ice. Proteins were electrophoretically transferred to the PVDF membrane at 100 V at variable amperage for 1 hour. Post transfer the gel was discarded and the PVDF membrane was transferred to a container for immunoblotting.

### 4.2.4.3 Immunoblotting

The PVDF membrane containing the transferred proteins was initially incubated for about 1 minute in Ponceau-S to allow visualisation of the protein on the blot. The membrane was thoroughly washed in dH₂O before being incubated in tris-buffered saline-tween 20 (TBST, 0.1% tween) containing 5% non-fat milk powder (Anchor) for 1 hour on a shaker at room temperature. The membrane was incubated overnight at 4°C in a sealed bag that contained the primary antisera diluted in TBST with 5% bovine serum albumin (BSA). The membrane was washed extensively (3 x quickly followed by 3 x 5 minute washes) in TBST followed by species-specific incubation in biotinylated secondary antiserum (Amersham Life Sciences) (1:2,000) diluted in TBST and 5% milk for 1 hour at room temperature. The membrane was again washed extensively and the antibody complex was visualised using the ECL+ chemiluminescence system (Amersham) in accordance with the manufacturer’s instructions.
The membrane was placed in a flat cassette and the film (Hyperfilm ECL, AmershamPharmacia Biotech) was exposed to the chemiluminescent membrane. The film was subsequently developed in Kodak D-19 developer, fixed, washed and dried.

To probe the blots a second time, bound antibody was stripped from the membranes using a buffer that contained 625 mM Tris pH 6.8, 2% SDS and 0.7% β mercaptoethanol (60°C for 30 minutes). The stripped membranes were then washed in TBST and non-specific binding sites were again blocked in 5% non-fat milk powder (Anchor) for 1 hour on a shaker at room temperature before the antibody steps were repeated.

4.3 Results

4.3.1 Progenitor cell proliferation in the human normal and Huntington’s disease subependymal layer (Figure 4.1A)

In order to investigate whether the SEL contained a population of progenitor cells in normal and HD brains, 6 normal and 9 HD brains were immunostained for PCNA. PCNA labels a proliferating cell protein that is specifically expressed in the late phases of G1 and in the first 35% of the S-phase of the cell cycle (Kurki et al., 1986; Mathews et al., 1984; Takahashi and Caviness, 1993). Thus only those cells dividing at the time of death express the protein PCNA. All the HD brains tested positive for an expanded CAG triplet repeat on exon 1 of the IT15 gene located on chromosome 4 (see figure 4.1A), and the extent of the neuropathology was independently examined by a neuropathologist and was graded according to the Vonsattel grading scale (see figure 4.1A) (Vonsattel and DiFiglia, 1998; Vonsattel et al., 1985). Thus 9 HD brains were examined which included 3 HD brains of each grade 1, 2, and 3 brains and 6 normal brains were examined for the presence of proliferating progenitor cells in the SEL. The normal and HD SEL had PCNA positive cells that were distributed throughout the SEL beneath the cell sparse layer which was immediately beneath the EPL. The nuclei of these cells stained positively for PCNA demonstrating small round or oval nuclei that often had a granular appearance. The caudate nucleus contained very few PCNA positive cells. A difference
was evident in the number of PCNA positive cells in the SEL between the normal and HD brains.

4.3.2 Comparison of the number of proliferating cells in the normal and Huntington’s disease subependymal layer (Figure 4.1B)

Whereas a small number of PCNA positive cells were detected in the SEL of the normal human brains the number of proliferating cells was considerably increased in HD brains (see figure 4.1B). All the comparisons were made from sections taken at the same level of the caudate nucleus. The PCNA positive cells in the normal SEL were present in a thin band beneath the EPL just beneath a small cell sparse region. PCNA predominantly labelled the nucleus of cells that were round or oval in shape and had a slight granular appearance throughout. In the HD brain SEL, the PCNA positive cells were located beneath the EPL in the SEL. Within the SEL there was a small cell sparse area immediately beneath the EPL that was predominantly devoid of PCNA positive cells and beneath this region the PCNA cells were present. In the HD brain SEL the cells were evenly spread throughout the SEL regardless of the HD grade. The thickness of the SEL and the number of cells was examined in the normal and HD brain SEL. Using a qualitative PCNA grading scale (0 = small, + = few, ++ = moderate, +++ = many, and ++++ = large numbers of PCNA positive cells), the thickness of the SEL and the number of PCNA positive cells in the normal brain and HD brains were rated by an observer blind to the case numbers. The resulting ratings were tested using a Mann-Whitney U test and showed a statistically significant difference in the thickness of the SEL and in the number of PCNA positive cells in the SEL between the normal and HD brains (Mann-Whitney U test, normal median = 0.5; HD median = 2.0; U = 52.5; p<0.01; (see figure 4.1A and B).
Figure 4.1

The number of proliferating cells in the Huntington’s disease subependymal layer increase as the grade and CAG repeat length increase

PCNA immunoreactivity is increased in the SEL in HD cases. (A) Table summarising the age, post-mortem delay, pathological grade, CAG trinucleotide repeat length in the IT15 gene, and PCNA grade for each HD case examined. A qualitative five-point grading scale (PCNA grade) was used to assess the level of PCNA immunoreactivity in the SEL in normal and HD SEL. (B) Compared with the normal brain, the thickness of the SEL and the number of PCNA positive cells in the SEL increased as the pathological grade of HD increased (HD grade 1 to grade 3). In each case, an arrow indicates the boundary of the SEL with the caudate nucleus (CN). (EP = ependymal layer; LV = lateral ventricle) scale bar, 80 μm. (C) Graph showing a significant correlation between PCNA grade and pathological grade in the HD cases ($R^2 = 0.8972$, $p<0.003$). (D) Graph showing a significant correlation between CAG trinucleotide repeat length in the IT15 gene and PCNA grade in the HD cases ($R^2 = 0.8168$, $p<0.02$).
### A

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>PM Delay (Hours)</th>
<th>Pathological grade</th>
<th>CAG repeats (IT15 gene)</th>
<th>PCNA grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC68</td>
<td>55</td>
<td>11</td>
<td>HD-1</td>
<td>17/42</td>
<td>++</td>
</tr>
<tr>
<td>HC92</td>
<td>72</td>
<td>5</td>
<td>HD-1</td>
<td>17/41</td>
<td>+</td>
</tr>
<tr>
<td>HC74</td>
<td>74</td>
<td>12</td>
<td>HD-1</td>
<td>17/42</td>
<td>++</td>
</tr>
<tr>
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<td>6</td>
<td>HD-2</td>
<td>17/47</td>
<td>+++</td>
</tr>
<tr>
<td>HC89</td>
<td>71</td>
<td>12</td>
<td>HD-2</td>
<td>17/44</td>
<td>++</td>
</tr>
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<td>15/42</td>
<td>++</td>
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<td>18/52</td>
<td>+++</td>
</tr>
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<td>61</td>
<td>19</td>
<td>HD-3</td>
<td>24/44</td>
<td>+++</td>
</tr>
<tr>
<td>HC91</td>
<td>63</td>
<td>5.5</td>
<td>HD-3</td>
<td>23/44</td>
<td>+++</td>
</tr>
</tbody>
</table>

### B

- LV
- EP
- SEL
- CN

- Normal
- Grade 1
- Grade 2
- Grade 3

### C

**Correlation between pathological grade and PCNA grade**

Correlation coefficient: $r = 0.8972$, $p < 0.003$

### D

**Correlation between CAG repeat number and PCNA grade**

Correlation coefficient: $r = 0.8168$, $p < 0.02$
4.3.3 Comparison of the pathological grade of Huntington’s disease with the number of proliferating cells in the subependymal layer (Figure 4.1B and C)

The grade of PCNA staining was compared with the HD neuropathological grade to determine whether the increase in PCNA positive cells was the same in each grade or was HD grade dependent. When HD (grade 1-3) brains SEL were examined it was apparent that in HD grade 1 brains there were only a few or moderate numbers of PCNA positive cells (qualitative grade average = 1.6, see figure 4.1A) whereas in HD grade 2 cases there were many PCNA positive cells (qualitative grade average = 2.3, see figure 4.1A) and in the HD grade 3 cases there were large numbers of PCNA positive cells (qualitative grade average = 4, see figure 4.1A). Statistical analysis revealed that the grade of PCNA staining in HD cases significantly correlated with the HD neuropathological grade (p<0.003; R^2 = 0.8992; see figure 4.1C). Thus the more severe the neuropathology the more PCNA positive cells were present in the SEL.

4.3.4 Comparison of the number of CAG repeats in the IT15 gene in Huntington’s disease with the number of proliferating cells in the subependymal layer (Figure 4.1D)

The PCNA grade was also compared to the length of the CAG repeat on exon 1 of the IT15 gene on chromosome 4. Statistical analysis also demonstrated a significant correlation between the grade of PCNA staining in the SEL of HD cases and the number of CAG repeat expansions in the expanded allele of the HD IT15 gene (p<0.02; R^2 = 0.8168; see figure 4.1D). The case with the lowest number of CAG repeats (41 repeats) on the IT15 gene had the lowest PCNA grade (1), whereas the case that had the highest number of CAG repeats (52 repeats) had a PCNA grade of 4. Thus, the more severe the genetic defect on the IT15 gene in terms of CAG repeat expansions the more PCNA positive cells were present in the HD SEL.

In order to demonstrate that the increase in PCNA positive cell labelling was indeed due to increased cellular proliferation in the HD SEL and not simply due to shrinkage of the
caudate nucleus, the dorsal to ventral length of the caudate nuclei were measured at rostral, central and caudal levels (all brains were processed identically, thus any section shrinkage was the same for each section). Statistical analysis of the measurement demonstrated no significant reduction in the SEL length in the HD brains compared with normal brains (15.78 ± 3.00 mm HD; 16.63 ± 3.36 mm normal). Furthermore, no correlation was found between the PCNA grade and the age, sex or post-mortem delay period of the cases examined.

4.3.5 Analysis of the specificity of the PCNA antibody for detecting cell proliferation

It has been suggested that the PCNA antibody not only detects dividing cells but also cells that are undergoing DNA repair or apoptosis (Tomasevic et al., 1998). To determine whether there were cells undergoing DNA repair or apoptosis, TUNEL (Transferase mediated 14-deoxyUridine triphosphate Nick End Labelling) histochemical staining was performed which labels short nucleosomal-sized fragments; these fragments can be labelled by terminal deoxynucleotidyl transferase and visualised immunohistochemically (Gates et al., 1995). TUNEL was performed on the caudate nucleus sections including the SEL from normal and HD brains on the same cases that had been stained for PCNA. Very few TUNEL positive cells were detected in the SEL in all the normal and HD cases examined; also, the number of TUNEL positive cells in the SEL was similar between normal and HD brains. However, within the caudate nucleus of the HD cases there were a large number of TUNEL positive cells but only the occasional PCNA positive cell was identified in the caudate nucleus. This work correlated with previously published TUNEL experiments (Butterworth et al., 1998).

4.3.6 Western blot analysis of the subependymal layer (Figure 4.2)

Western blotting was used to examine the specificity of the PCNA, βIII-tubulin and GFAP antibodies by determining the molecular weight of the proteins of interest in the SEL. In these experiments the EPL and SEL were selectively removed from the caudate nuclei of fresh frozen normal and HD brain sections. The brain tissues were homogenised
and subsequently run on a polyacrylamide gel and transferred to a PVDF membrane where the proteins were immunoblotted with the above antibodies. In both the normal and HD cases (see table 4.1), the PCNA antibody demonstrated a single clear band that was evident at 36 kDa, which is the calculated weight of the PCNA protein (see figure 4.2A). The difference in density between the normal and HD homogenates was measured using SCION image; although visually there appears to be a difference between the density of staining between the normal and HD cases SCION image analysis showed no significant difference between the density of the PCNA protein between normal and HD tissue.

The PVDF bound protein was also subject to immunoblotting with the βIII-tubulin antibody. One dark consistent band was demonstrated which ran at approximately 60 kDa for each sample examined (see figure 4.2B). The density of the bands was approximately the same regardless of whether the proteins were from normal or HD brains and no significant difference was detected between the different grades of HD examined.

The blot was stripped and immunoblotted with the GFAP antibody. A strong band was detected at 50 kDa and a number of less dense bands were demonstrated between 40 kDa and 50 kDa. The calculated weight of GFAP is 50 kDa. The band density was approximately the same between normal and HD cases and no significant difference was detected in the density of the bands between the HD grades (see figure 4.2C). Each of the three antibodies examined by immunoblotting demonstrated protein weights that were consistent with the weights calculated from their protein sequences.

4.3.7 The localisation of proliferating, neuronal and glial cell markers in the subependymal layer (Figure 4.3)

To investigate the morphology and pattern of proliferative cell labelling, young neuronal and glial cell labelling, serial 50 μm sections from the caudate nucleus that included the SEL were immunostained with various cell specific markers. βIII-tubulin was used to specifically label and identify neurons early in development (Lee et al., 1990; Menezes and Luskin, 1994), and neuronal nuclei (NeuN) that labels mature neurons were used to
Figure 4.2

**Western blot analysis of normal and Huntington’s disease subependymal layer homogenates**

Western immunoblotting with normal human (three cases) and Huntington’s disease (three HD grade 2 and 3 cases) SEL homogenates (representative Western blots have been shown; see table 4.1). (A) Immunoblotting with the PCNA antibody demonstrates a single strong band at 36 kDa, which demonstrates antibody specificity as this is the calculated molecular weight of the PCNA protein. (B) Immunoblotting with the βIII-tubulin antibody demonstrates a single strong band at 49 kDa, which is the calculated molecular weight of the β-tubulin isotype III protein. (C) Immunoblotting with the GFAP antibody demonstrates a number of bands; however, the strongest band was demonstrated at 50 kDa, which is the calculated molecular weight of the GFAP protein. The other lighter proteins that are also detected by the GFAP antibody are likely to be cleavage products of the GFAP protein.
A Proliferating cell nuclear antigen

kDa
40 -
30 -

Normal Huntington's disease

B βIII-tubulin

Normal Huntington's disease

C Glial fibrillary acidic protein

Normal Huntington's disease
label the neuronal populations in the SEL. For the detection of mature glial cells, antibodies to GFAP were used, and antibodies to Vimentin were used to label young glial cells. The use of these cell markers enabled comparison of different cell type locations within the SEL. Representative photomicrographs displaying one label for proliferative, neuronal and glial markers have been demonstrated in the figures. PCNA positive cells were located within the SEL and were homogenously distributed throughout the SEL (see figure 4.3A). The PCNA labelling was predominantly nuclear and had a granular appearance when viewed at high magnification. The nuclei that were PCNA positive were round or oval in shape and had a diameter that ranged from 3–10 μm. The EPL was very densely immunoreactive for PCNA with labelling that was evident throughout the dorsal to ventral extent of the EPL (see figure 4.3A). PCNA positive cells were occasionally seen just at the medial edge of the caudate nucleus but were very infrequently seen in the lateral parts of the caudate nucleus. βIII-tubulin mostly stained fibres that arose from cell bodies that were located in the deeper aspect of the SEL close to the caudate nucleus (see figure 4.3B). The fibres spread up toward the region of the SEL that was devoid of cell body staining and occasionally as far as the EPL. These fibres were very thin but were densely immunoreactive for βIII-tubulin. The cell bodies from which the fibres arose were small and labelled darkly with βIII-tubulin. The EPL was devoid of βIII-tubulin immunoreactivity (see figure 4.3B). The caudate nucleus had βIII-tubulin positive fibres that stained throughout, although there were fewer cells and fibres that were positively labelled in the caudate nucleus compared with the SEL. NeuN labelling was also demonstrated to be in the region of the SEL close to the caudate nucleus. NeuN immunoreactivity consisted of darkly labelled neuron-like cells that were present in the SEL and caudate nucleus but never in the EPL. GFAP and Vimentin positive cells were scattered throughout the SEL and caudate nucleus but a slightly denser band of labelling was detected immediately next to the EPL (see figure 4.3C). The GFAP and Vimentin positive staining was cytoplasmic and the cells had processes that were characteristic of glial cells.
Localisation of proliferating, neuronal and glial cells in the Huntington’s disease subependymal layer

PCNA positive, βIII-tubulin positive and GFAP positive cells are located within the SEL as demonstrated with bright-field immunohistochemical serial sections through the EPL (EP), SEL and caudate nucleus (CN) of a grade 2 HD brain (HC89). (A) PCNA positive cells are evenly distributed within the SEL and the EP is also densely stained. Arrows demonstrate examples of PCNA positive cell bodies. (B) βIII-tubulin positive fibres and cell bodies are present in the lower part of the SEL. Arrows indicate cell bodies with immunoreactive fibres. (C) GFAP positive cells are distributed homogeneously throughout the SEL and in the CN. Arrows indicate examples of GFAP cell bodies.
Thus, in summary, there was a stratified array of proliferating, neuronal and glial cell staining within the SEL such that the PCNA (proliferating) cells were located homogenously throughout the SEL but were not present in the caudate nucleus. The neuronal cells were predominantly located in the SEL close to the caudate nucleus and the glial cells were located evenly across the SEL and caudate nucleus with a slightly denser band of immunoreactivity close to the EPL.

4.3.8 Neurogenesis and gliogenesis in the human subependymal layer (Figure 4.4)

To detect the presence of neurogenesis and gliogenesis in the SEL, multilabel fluorescence laser scanning confocal microscopy was used. To detect neurogenesis, the SEL in HD brain sections was triple-labelled with antibodies to PCNA, βIII-tubulin (which labels young neurons) and Hoechst (which labels DNA). To detect gliogenesis, PCNA was combined with GFAP and Hoechst. The use of Hoechst was to confirm that the PCNA positive cell labelling was in the same location as the Hoechst labelled nuclear DNA.

In all the normal and HD cases examined, PCNA positive cells were co-localised with βIII-tubulin immunoreactivity and a Hoechst stain was present in the same cell. The demonstration that βIII-tubulin-stained processes co-localised with PCNA positive cell nuclei in the HD SEL (see figure 4.4A-E) indicates that some of these PCNA positive cells exhibit a neuronal phenotype. The PCNA labelling was evident in cells that had either round or oval shaped nuclei and the size of the labelled nuclei ranged from 4-10 μm. The βIII-tubulin labelling was predominantly present on fibres, which were in continuity with the PCNA labelled cells. The PCNA/βIII-tubulin positive cells were located mainly in the deeper regions of the SEL adjacent to the caudate nucleus (see figure 4.3 and 4.4A). These findings show that neurogenesis occurs in the HD SEL (see figure 4.4A-E).
Neurogenesis and gliogenesis occur in the subependymal layer in Huntington’s disease

Newly generated cells in the SEL of the HD brain exhibit either a neuronal or glial phenotype. (A) Triple-immunofluorescence laser scanning confocal microscopy demonstrates that the SEL of the HD brain contains newly generated cells that co-express PCNA (red) and βIII-tubulin (green) and stain with a Hoechst stain (blue). The new neurons are located in the lower part of the SEL, (scale bar, 6 μm). (B-D) Higher magnification of the neuron that is outlined with a box in A (scale bar, 3 μm). (B) Hoechst (blue) stains the nucleus of cells in the SEL. (C) PCNA (red) labels the nucleus of a new cell and has a granular appearance. (D) βIII-tubulin (green) labels the processes and cell body of neurons early in their development. (E) The merged image demonstrates co-expression of PCNA and βIII-tubulin in the same cell that displays a Hoechst positive stain, indicating that neurogenesis occurs in the SEL of the HD brain. (F) Triple-immunofluorescence laser scanning confocal microscopy demonstrates that the SEL of the HD brain also contains newly generated cells that co-express PCNA (red) and GFAP (green) and stain with a Hoechst stain (blue). These new glial cells are located in the upper part of the SEL in the HD brain, (scale bar, 18 μm). (G-I) Higher magnification of the glial cell that is outlined with a box in F, (scale bar, 9 μm). (G) Hoechst (blue) stains the nucleus of cells in the SEL. (H) PCNA (red) labels the nucleus of cells with punctate staining. (I) The astrocytic marker GFAP (green) labels the cytoplasm of the cell. (J) The merged image demonstrates co-expression of PCNA and GFAP in the same cell that displays a Hoechst stain, indicating the occurrence of gliogenesis. Images A-E and F-J were each obtained using different excitation wavelengths, and the signal was detected at different emission wavelengths.
Fluorescent laser scanning confocal microscopy using antibodies against PCNA and GFAP combined with a Hoechst stain demonstrated large numbers of cells in the SEL that had intense GFAP cytoplasmic staining (see figure 4.4I), with the nuclear region clearly delineated by PCNA labelling and Hoechst staining (see figure 4.4G-I). The GFAP labelling had a characteristic star-like appearance surrounding a GFAP immunonegative nuclear region; this appearance was in keeping with the GFAP staining that was demonstrated using 3,3 diamino-benzidine as a chromogen. The demonstration that the GFAP stained cytoplasm co-localised with PCNA positive nuclei in the HD SEL (see figure 4.4J) indicates that these PCNA positive cells exhibit a glial phenotype. The PCNA/GFAP positive cells were identified mainly in the more superficial region of the SEL adjacent to the EPL (see figure 4.3C and 4.4F) and comprised approximately 50% of the PCNA positive cells in the SEL. These findings show that gliogenesis occurs in the HD SEL (see figure 4.4F-J).

To ensure that the fluorescence detected was not simply accounted for by autofluorescence or artifact, sections were also imaged from normal and HD brains omitting the βIII-tubulin or GFAP primary antibodies. No βIII-tubulin or GFAP-like immunofluorescence was detected in these tissues, but the PCNA immunofluorescence was the same as that seen in the sections that had PCNA and either βIII-tubulin or GFAP antibody treatment. The same test was performed omitting the PCNA primary antibody and PCNA-like immunofluorescence was undetectable, but normal βIII-tubulin and GFAP signal was detected, indicating that there was no cross reactivity between the primary antibodies and no bleed through between excitation wavelength channels. To test for autofluorescence, sections that had not been exposed to antibody treatment were imaged, with and without Hoechst staining; also, sections where the primary antibodies were omitted from the incubation were imaged. The autofluorescence that was detected from these sections was present in all wavelength channels, was homogeneously spread across the section, and was not cell specific.
4.3.9 The number of PCNA/βIII-tubulin double-labelled cells in the normal and Huntington’s disease subependymal layer (Figures 4.5, 4.6 and 4.7)

In this study I have immunolabelled the normal (n = 4) and HD (n = 3) SEL with antibodies to PCNA and βIII-tubulin. These labels were detected using fluorochromes and were analysed using laser scanning confocal microscopy. The entire length of the SEL was imaged at 20 x magnification taking slices 1 μm apart through the Z-direction of the specimen. After the images had been acquired, each stack was separated into 10 slice thick stacks with a 5 μm interstack gap to ensure that PCNA positive cells were not counted twice. The 10 slice stacks were made into a projection image for the purposes of counting PCNA positive cells, but the stacks could also be played as a cine loop for the purposes of counting the double-labelled cells. The cell counts from each 10 slice stack were combined to give the number of cells per field of view for the entire Z-direction and the numbers of cells in each field of view were averaged across each field of view for the entire length of the SEL.

In this study I observed a significant increase (p<0.01) in the number of PCNA positive cells that were fluorescently labelled in the HD SEL (143.89 ± 38.33 cells) compared with the normal brain SEL (57.53 ± 7.84 cells) (see figure 4.5A and figure 4.6). Of the cells that were fluorescently stained for PCNA a proportion co-localised with the early neuronal marker βIII-tubulin.

The normal brains had an average of 1.87 ± 1.10 PCNA/βIII-tubulin double-labelled cells per frame. Thus 3.25% of the PCNA positive cells in the normal SEL were also βIII-tubulin positive indicating that these cells had recently undergone cell division and also had a neuronal phenotype. In the HD SEL there was 4.88 ± 1.87 double-labelled PCNA/βIII-tubulin cells per frame (see figure 4.5B and figure 4.7). Thus 3.39% of the PCNA positive cells in the HD SEL were also βIII-tubulin positive. The normal and HD SEL each contain a similar proportion of PCNA positive cells that colocalise with βIII-
A. The number of fluorescently labelled PCNA positive cells in the normal and Huntington’s disease subependymal layer

In this study I counted the number of fluorescently labelled PCNA positive cells in the SEL of 4 normal brains and 3 HD brains (grade 2, 3). The normal brain had $57.53 \pm 7.84$ cells/frame that were fluorescently labelled for PCNA compared with the HD brain SEL ($143.89 \pm 38.33$ cells/frame) that had significantly more PCNA positive cells ($p<0.01$). The number of fluorescently labelled cells in the normal and HD SEL was counted so that the proportion of double-labelled cells could be determined (see figure 4.5B).

B. The number of PCNA/βIII-tubulin positive cells in the normal and Huntington’s disease subependymal layer

The number of PCNA/βIII-tubulin positive fluorescently double-labelled cells in the normal brain was $1.87 \pm 1.10$ cells/frame compared with the HD brain SEL ($4.88 \pm 1.87$) that had significantly more double-labelled cells ($p<0.04$). This demonstrates that in response to HD the SEL responds by increasing the amount of neurogenesis that occurs in the human brain. As demonstrated in figures 4.1B and 4.5A, there were more PCNA positive cells in the HD SEL and the increase in the number of new neurons in the HD brain SEL was almost exactly proportional to the increase in the number of PCNA positive cells: 3.25% of the PCNA positive cells in the normal brain SEL were βIII-tubulin positive, whereas 3.39% of the PCNA positive cells in the HD brain SEL were also βIII-tubulin positive. Thus the proportion of PCNA positive cells that are also positive for βIII-tubulin in the normal and HD SEL is approximately the same. However, because there are significantly more PCNA positive cells in the HD SEL there is a 2.6 fold increase in the production of new neurons in the HD SEL.
A

The number of fluorescently labelled PCNA positive cells in the normal and Huntington's disease subependymal layer

![Bar graph showing the number of PCNA-positive cells in normal vs. Huntington's disease brains.](chart_a)

B

The number of PCNA/βIII-tubulin positive cells in the normal and Huntington's disease brains

![Bar graph showing the number of PCNA/βIII-tubulin-positive cells in normal vs. Huntington's disease brains.](chart_b)
Figure 4.6

Neurogenesis in the normal subependymal layer

This figure demonstrates that the normal human SEL contains PCNA (A) positive cells and βIII-tubulin (B) positive cells. A small proportion (3.25%) of PCNA positive cells also co-label with βIII-tubulin (C) (see boxes surrounding cells). This demonstrates that neurogenesis occurs in the normal brain SEL; however, there are relatively few PCNA positive cells in the normal brain SEL and a correspondingly low number of PCNA/βIII-tubulin double-labelled cells.
Neurogenesis in the normal subependymal layer

A

PCNA

SEL

B

βIII-tubulin

SEL

C

PCNA/

βIII-tubulin

SEL
Figure 4.7

Neurogenesis in the Huntington’s disease subependymal layer

This figure demonstrates that the HD subependymal layer (SEL) contains PCNA (A) positive cells and βIII-tubulin (B) positive cells. A small proportion (3.39%) of PCNA positive cells also co-label with βIII-tubulin (C) (see boxes surrounding cells). The HD SEL contains more PCNA positive cells than the normal SEL. Also, there was approximately 2.6 fold more double-labelled cells in the HD SEL which indicates that in response to degeneration of the caudate nucleus more neurogenesis occurs in the HD SEL.
Neurogenesis in the Huntington's disease subependymal layer

A

PCNA

SEL

B

βIII-tubulin

SEL

C

PCNA/βIII-tubulin

SEL
tubulin, however because there were so many more PCNA positive cells in the HD SEL there were significantly more PCNA/βIII-tubulin positive cells. The increase in the number of double-labelled PCNA/βIII-tubulin positive cells in the HD SEL compared with the normal SEL is statistically significant (p<0.04) and represents a 2.6 fold increase in the number of cells that undergo neurogenesis in the HD SEL (see figure 4.5B).

4.4 Discussion

This chapter has examined progenitor cell proliferation in the normal and HD SEL and has used triple-immunofluorescence laser scanning confocal microscopy to determine the presence and extent of neurogenesis and gliogenesis in the SEL in the normal and HD brain.

4.4.1 Increased numbers of progenitor cells in the HD subependymal layer

Progenitor cell proliferation is a well-established phenomenon in the adult brain of non-human primates and other mammals (Craig et al., 1996; Luskin, 1993; Luskin et al., 1997; Martens et al., 2002; Rakic, 2002; Stewart et al., 2002). In addition, progenitor cell proliferation has been recently demonstrated in the hippocampus of the normal adult human brain (Eriksson et al., 1998). But until the present study, the presence of increased cell proliferation in the SEL in response to degeneration of the adjacent caudate nucleus in HD had not been demonstrated. My results are the first to show an increase in the thickness of the SEL and in the number of PCNA positive cells in the SEL in the HD brain compared with the normal brain SEL (p<0.01; see figure 4.1A). Furthermore, I have demonstrated that as the neuropathological grade of HD increased so did the number of PCNA positive progenitor cells in the HD SEL (p<0.003; R² 0.8972; see figure 4.1B, C); that is to say increased numbers of PCNA positive cells correlate with increased pathological grade. My results have also shown a significant correlation between the number of PCNA positive cells in the HD SEL and the CAG trinucleotide repeat length in the IT15 gene (p<0.02; R² 0.8168; see figure 4.1D). Thus as the CAG
repeat length increases so does the number of PCNA positive cells in the HD SEL. Various models of brain injury have demonstrated that neuronal death can trigger increased cell production in the SEL of the rodent brain (Arvidsson et al., 2002; Parent, 2003; Parent et al., 2002a). In particular, my results are in keeping with studies in rodent models of neurodegenerative disorders in which cell proliferation in the SEL has been demonstrated in response to an experimentally induced pathology. In one study, Arvidsson et al (2002) demonstrated increased numbers of proliferating progenitor cells in the SEL overlying the caudate nucleus in a middle cerebral artery occlusion (MCAO) induced stroke model whereby a 2.2 fold increase in proliferating cells was demonstrated on the stroke affected side compared with the contralateral side. Also, Parent et al (2002) demonstrated, in a pilocarpine induced seizure model of epilepsy in rats, approximately a 4 fold increase in the numbers of proliferating cells compared with controls 14 days after seizure induction. Further supporting these observations, in experiments carried out by Luthi-Carter et al (2000) looking at changes in mRNA levels in wild type compared with R6/2 transgenic HD mice littermate controls, there was an approximate 2 fold increase in PCNA mRNA in the striatum of 12 week old animals (Luthi-Carter et al., 2000). However, the present study is the first to show increased numbers of progenitor cells in the SEL of the adult human brain in response to neurodegeneration of the caudate nucleus.

4.4.2 Neurogenesis and gliogenesis in the Huntington's disease subependymal layer

This study has not only demonstrated increased numbers of progenitor cells in the SEL of the HD human brain but also, that these progenitor cells exhibit either a neuronal or glial phenotype as evidenced by the βIII-tubulin positive or GFAP positive staining in the SEL (see figure 4.4A-E and F-J). Neurogenesis has never previously been demonstrated in the human HD brain nor has it been demonstrated in the adult human normal brain in the SEL. However, neurogenesis has been demonstrated in the normal hippocampus. In these studies Eriksson et al (1998) used post-mortem brain tissue from cancer patients who, as a part of their oncological treatment, had been administered BrdU which labels dividing cells and radiosensitises cells to radiation therapy treatment. BrdU was detected in the
progeny of the dividing cells and subsequent analysis of the hippocampal dentate gyrus cell population demonstrated that they stained for neuronal or glial markers (Eriksson et al., 1998). In particular, they demonstrated co-localisation of BrdU with the neuronal markers neuronal nuclei (NeuN), neuron specific enolase (NSE) and calbindin. Fewer calbindin positive cells were co-localised with BrdU than with NSE or NeuN, probably because calbindin is only present in a subset of neurons in the hippocampus. They also demonstrated that GFAP co-localised on a proportion of BrdU positive cells indicating the presence of gliogenesis. In their study, Eriksson et al (1998) also examined the SEL adjacent to the caudate nucleus for the presence of BrdU positive cells but identified very few BrdU positive cells in the SEL and none of them appeared to co-localise with neuronal or glial markers. My results are in keeping with these results in so far as the human brain has the capacity to produce new neurons and glial cells. However, my results differ in that I have demonstrated increased progenitor cell proliferation, neurogenesis and gliogenesis in the SEL overlying the caudate nucleus. The most likely explanation for the lack of BrdU cells in the SEL in the study by Eriksson et al (1998) is the length of the delay between BrdU administration and the death of the patient (which ranged from 16-781 days) (Eriksson et al., 1998). Because the SEL cells have a fast cell cycle time it is likely that many of the SEL progenitor cells had diluted the BrdU such that it was undetectable, or alternatively that the cells had migrated out of the SEL by the time the SEL was immunostained to detect the presence of BrdU in the specimens (Eriksson et al., 1998; Morshead and van der Kooy, 1992).

In a rodent model of neurodegeneration, Arvidsson et al (2002) demonstrated that in response to a middle cerebral artery occlusion stroke, not only was there increased numbers of progenitor cells in the SEL but also there were cells that migrated toward the site of the injury that had the characteristic morphology and immunostaining of neurons. In particular, they demonstrated that BrdU positive cells also contained immunoreactivity for the specific striatal neuron markers Meis-2 and DARP-32 thus indicating that the progenitor cells from the SEL not only proliferate and migrate but that they can also differentiate to form the same type of cell that degenerated due to stroke induced injury (Arvidsson et al., 2002). Parent et al (2002) also demonstrated injury site-specific
migration of BrdU positive cells from the SEL to the injured striatum after an ischaemic episode to the striatum. The BrdU positive cells also had a striatal phenotype (Parent et al., 2002b). Taken together these results demonstrate the capacity of the SEL to produce new replacement neurons in response to cell loss in a particular part of the brain. It is impossible to monitor the migration of progenitor cells from the SEL in brains that have not first been treated with a cell cycle marker that is incorporated into the cell i.e. BrdU, and therefore under normal circumstances cannot be given to humans. Thus it is impossible to determine whether the progenitor cells in this study underwent cell migration from the SEL.

In order to ensure that the PCNA, βIII-tubulin and GFAP antibodies were binding specifically to their respective antigens at the calculated weight, I performed Western blot analysis on tissue dissected from the normal and HD brain SEL. A single band demonstrated that the approximate weight of PCNA was 36 kDa (see figure 4.2A). The βIII-tubulin antibody was demonstrated as a single band on the western blot at approximately 49 kDa (see figure 4.2B). The GFAP antibody however, demonstrated a number of bands very close together at approximately 50 kDa that most likely represents post-translational modifications of the GFAP protein (see figure 4.2C). Each antibody detected bands on the PVDF membrane that were approximately at the calculated weight for each protein. In controls, where the primary antibody was omitted, no bands were detected. The bands demonstrated on the Western blots were always demonstrated at the calculated weight for the protein of interest. Thus it can be concluded that the primary antibody was specific for the protein of interest.

Interestingly, this study has also demonstrated that there is a stratified arrangement of neurogenesis and gliogenesis in the SEL. In particular the PCNA/βIII-tubulin positive cells were predominantly located in the deeper parts of the SEL very close to the caudate nucleus, which suggests that neurogenesis occurs mostly very close to the caudate nucleus. Whereas the PCNA/GFAP positive cells were predominantly located in the more superficial region of the SEL (see figure 4.3), this suggests that gliogenesis occurs mainly in the region of the SEL that is closest to the EPL.
4.4.3 Increased neurogenesis in the Huntington's disease subependymal layer

The present study has demonstrated not only that neurogenesis occurs in the HD SEL but also that there was on average a 2.6 fold increase in the number of PCNA/βIII-tubulin double-labelled cells in the HD SEL (4.88 ± 1.87 cells/frame) (see figure 4.5B and figure 4.7) compared with the normal SEL (1.87 ± 1.10 cells/frame) (see figure 4.5B and figure 4.6), which is significantly more than in the normal brain SEL (p<0.04; see figure 4.5B). In the normal brain, approximately 3.25% of PCNA positive cells were double-labelled with the neuronal marker βIII-tubulin; this is comparable to the HD brain SEL where 3.39% of PCNA positive cells were also double-labelled with βIII-tubulin. Thus, overall there is increased neurogenesis in the HD SEL that represents an increase that is proportionally comparable to the increase in the number of PCNA positive cells in the HD SEL. My study is in keeping with the study performed by Arvidsson et al (2002) where they demonstrated increased neurogenesis on the ipsilateral side in the SEL after a unilateral MCAO induced stroke (Arvidsson et al., 2002). In particular, they demonstrated that up to 5 weeks after the striatal insult there were still BrdU labelled cells that were also NeuN positive near the lesion site (Arvidsson et al., 2002). Many of the BrdU positive cells that were in the striatum were also doublecortin positive which indicated that the cell was a migrating neural cell. To ensure that these migrating neural cells were not arising from some place other than the SEL, they infused the mitotic inhibitor cytosine-β-D arabinofuranoside (via a mini osmotic pump) that dramatically reduced the number of dividing cells in the SEL. The massive reduction in the number of BrdU positive cells in the SEL and around the lesion site demonstrated that the migrating and differentiating cells had originated in the SEL. This study demonstrates the importance of the SEL for the production of new neurons and glial cells for the replacement of cells in the adjacent brain regions undergoing degeneration.

Our findings indicate a high level of neural plasticity in the adult human brain in response to the degenerating environment that is present in the caudate nucleus in HD. I also note: (1) the close relationship that the SEL has with the caudate nucleus, the part of the brain
that is most severely and preferentially affected in HD; and (2) the location of the PCNA/βIII-tubulin positive double-labelled cells very close to the caudate nucleus. Since in early neural development cells migrate from the SEL to form the caudate nucleus it would not be unreasonable to suggest that these immature neurons are migrating toward the caudate nucleus in response to the neuronal cell loss occurring in the HD brain. Furthermore, the observation of increased progenitor cells in the HD SEL with advancing neuropathological grades of HD would indicate that the proliferation is a response to greater numbers of degenerating neurons in the caudate nucleus. It is not clear what the factors are that signal the observed cellular proliferation, migration and differentiation. Neural progenitor cells have been shown to respond to a variety of mitogenic growth/trophic factors and cytokines in vivo such as epidermal growth factor (EGF), fibroblast growth factor (FGF-2), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), transforming growth factor α (TGFα), erythropoietin and others by facilitating the proliferation, migration and differentiation of progenitor cells in the adult central nervous system (Arsenijevic and Weiss, 1998; Arsenijevic et al., 2001; Ciccolini, 2001; Craig et al., 1996; Fallon et al., 2000; Fiore et al., 2003; Kuhn et al., 1997; Pencea et al., 2001; Reynolds et al., 1992; Reynolds and Weiss, 1996; Tirassa et al., 2003; Yoshimura et al., 2001; Zigova et al., 1998b). In one such in vivo study the growth factor EGF was infused into the lateral ventricle of adult mice; the results demonstrated a dramatic increase in the number of proliferating cells in the subependymal layer of the lateral ventricle (Craig et al., 1996). Immunohistochemical analysis revealed that more than 95% of the cells present in the subependymal layer were immunopositive for the EGF receptor and were also nestin positive, a well-established marker of proliferating neural progenitor cells (Craig et al., 1996). Also, in these experiments EGF induced the migration of progenitor cells into the parenchyma away from the wall of the lateral ventricle. More recent studies have demonstrated the effect of intraventricular infusion of the growth factors EGF and FGF-2. In one such study Kuhn and colleagues (1997) demonstrated a strong mitotic effect of FGF-2 on progenitor cells in the subventricular zone throughout the period in which FGF-2 was infused; however, during this period migration of progenitor cells was diminished (Kuhn et al., 1997). Shortly after FGF-2 administration ceased the progenitor
cells in the subventricular zone appeared to undergo rapid migration. Still other studies have demonstrated that the neurotrophic factor BDNF plays an important role in differentiating progenitor cells into mature neurons in both neurogenic and non-neurogenic regions of the adult brain and has neuroprotective properties (Benraiss et al., 2001; Chmielnicki et al., 2004; Pencea et al., 2001; Takahashi et al., 1998; Zigova et al., 1998b). TGFβ, administered to the rat striatum after a lesion induced Parkinson’s disease, also increased the level of progenitor cell proliferation and migration from the SEL to the striatum (Fallon et al., 2000). Cytokines, such as erythropoietin, also enhance the proliferation and differentiation of endogenous neural progenitors (Shingo et al., 2001; Yu et al., 2002). Erythropoietin, a member of the heamatopoietic cytokine superfamily, not only serves to enhance proliferation and differentiation of neural progenitor cells but also acts as an anti-apoptotic factor in the embryonic brain (Shingo et al., 2001; Yu et al., 2002).

Apart from endogenous stimulants of progenitor cells, pharmacological compounds such as lithium and fluoxetine (both commonly prescribed antidepressant drugs for recurrent mood disorders) have been shown to increase the number of proliferating cells in the brain (Duman et al., 2001; Malberg et al., 2000; Manev et al., 2001). A study performed by Chen and colleagues (2000), in which mice were chronically administered lithium and the cell cycle marker BrdU demonstrated a 25% increase in the number of proliferating cells in the dentate gyrus of the hippocampus compared to normal (Chen et al., 2000; Senatorov et al., 2004). Lithium has been shown to increase the levels of B-cell lymphoma protein-2 (bcl-2) that exerts a major anti-apoptotic neuroprotective effect in vitro and in vivo and may also exert a trophic effect as well (Chen et al., 2000; Manji et al., 1999; Manji et al., 2000a; Manji et al., 2000b; Nonaka et al., 1998).

It is clear that the increase in progenitor cells demonstrated in this study is insufficient to compensate for the progressive cell loss observed in the HD brain. If the potential for endogenous neural replacement could be augmented pharmacologically with the use of exogenous growth factors or pharmaceuticals that increased the rate of neural progenitor formation, neural migration, and neural maturation, then the rate of cell loss may be
slowed and clinical improvements may be observed. The results of the present study indicate that the adult human brain has the potential to repair itself after injury or disease. Combined with the knowledge that various mitogenic growth factors and pharmacological agents can enhance the proliferation and differentiation of endogenous progenitor cells; this suggests that novel therapeutic strategies could be developed to enhance the brain's natural repair mechanism in response to brain injury and disease.

4.5 Summary

In this chapter I have studied the proliferative capacity of the human SEL with the use of the proliferative cell marker PCNA. In particular this study has demonstrated that there is a significant increase in the number of proliferating progenitor cells in the HD SEL compared with the normal brain SEL (p<0.01; see figure 4.1A, B). Furthermore, this study has shown that as the pathological grade of HD increases so does the number of PCNA positive cells in the HD SEL (p<0.003; R² = 0.8972; see figure 4.1C); that is, there is a very strong correlation between the pathological grade in HD and progenitor cell proliferation in the HD SEL. Also, as the number of CAG trinucleotides increases in the IT15 gene, there is also an increase in the number of PCNA positive cells in the HD SEL (p<0.02; R² = 0.8168; see figure 4.1A, D).

In order to determine the fate of progenitor cells in the SEL, triple-immunofluorescence laser scanning confocal microscopy was undertaken on sections of the SEL that were fluorescently labelled for PCNA and either the neuronal marker βIII-tubulin or the glial marker GFAP to determine whether SEL progenitor cells become neurons or glial cells respectively. Our results demonstrated that βIII-tubulin co-localised with PCNA positive cells indicating that neurogenesis had occurred in the HD SEL (see figure 4.4A-E), and GFAP also co-localised with some PCNA positive cells demonstrating that gliogenesis was also present in the HD SEL (see figure 4.4F-J). This finding demonstrates for the first time that neurogenesis occurs in the human SEL in HD brains. This chapter has demonstrated that the newly formed neurons were generally located in the deeper part of
the SEL immediately adjacent to the degenerating caudate nucleus, whereas the newly produced glial cells were almost exclusively located in the superficial part of the SEL close to the EPL. These results suggest that the developing neurons may be migrating towards the damaged caudate nucleus.

In this study, the number of PCNA/βIII-tubulin double-labelled cells were counted in the normal SEL and compared with the HD SEL using fluorescently stained sections imaged with the laser scanning confocal microscope. The results demonstrated that there was on average a 2.6 fold increase in the number of newly formed neurons in the HD SEL compared with the normal brain SEL. This represents a significant increase in the number of new neurons in the HD SEL (p<0.04; see figure 4.5).

The findings in this chapter demonstrate a high level of plasticity in the HD SEL to respond to degeneration of the caudate nucleus in HD by producing increased numbers of progenitor cells that become neurons and glial cells. It is clear that the extent of neurogenesis in the SEL in HD is insufficient to replace all the degenerating cells in the caudate nucleus and to alleviate the symptoms of HD. However, it is well established that growth factors and pharmacological agents can increase the rate of neurogenesis in vivo. Therefore, if the endogenous increase in neurogenesis and the replacement of neurons in the HD affected areas could be augmented by the use of pharmacological agents, then the findings in this study may be of major importance in the development of novel and imaginative therapeutic approaches for the treatment of neurodegenerative diseases.

4.6 Conclusion

The results in this chapter have demonstrated that the number of progenitor cells is increased in the SEL in response to degeneration of the caudate nucleus in HD. As the pathological grade of HD increased so did the number of progenitor cells in the HD SEL. Furthermore, as the CAG trinucleotide repeat length increased so did the number of progenitor cells in the HD SEL. I have also demonstrated for the first time, that the
normal and HD human brain SEL produces new neurons and glial cells in the HD SEL. Thus the brain has a high level of plasticity such that it can respond to degeneration in adjacent brain regions by producing more new cells. Finally, my results also showed that neurogenesis is significantly increased in the HD SEL compared with the normal SEL. Thus for the first time I have demonstrated that the human brain has the potential to increase neurogenesis in an attempt to replace the degenerating cells in the adjacent caudate nucleus in HD. If the extent of endogenous neurogenesis could be enhanced using pharmacological therapies, as various animal studies suggest, then manipulation of neurogenesis in the adult human brain may offer hope for the development of novel cell replacement therapies for neurodegenerative diseases.

In order for the progenitor cells to be useful for cell replacement, the distribution of these cells would ideally extend across the entire ventricular surface of the caudate nucleus. The following chapter will examine the distribution of progenitor cells throughout the SEL overlying the caudate nucleus.
Chapter 5

DISTRIBUTION OF PROGENITOR CELLS IN THE SUBEPENDYMAL LAYER OF THE LATERAL VENTRICLE

5.1 Introduction

Cytoarchitectural studies of the mammalian subependymal layer (SEL) of the lateral ventricle have demonstrated that the SEL is a heterogeneous layer comprised of neuroblasts, glial cells, precursor cells and a number of other cell types. Two major studies on the rodent and bovine SEL have been recently published investigating the cellular morphology and the heterogeneity of cells in the SEL.

In particular, Doetsch and colleagues (1997) demonstrated that in the normal mouse brain there was a 3:2:1 ratio of migrating neuroblasts (type A cells): glial cells (type B cells): and precursor cells (type C cells). However, they did not study the localisation of these cells throughout the wall of the lateral ventricle. In the bovine brain, Rodriguez-Perez and colleagues (2003) undertook a very detailed regional analysis of the SEL by dividing the lateral ventricle into a rostral horn, central body, temporal (inferior) horn and occipital horn. The wall of the lateral ventricle was characterised by dividing the lateral ventricle into different wall types based on the presence, and orientation of certain cell types. **Type 1** ventricle wall consisted mostly of astrocytes and a few subependymal neuroblasts; very few cells were PCNA positive. **Type 1** ventricle wall was located on the upper and medial surfaces of the lateral ventricle in the rostral half of the lateral ventricle wall and the entire caudal half of the lateral ventricle was made up of the type 1 ventricle wall architecture (Rodríguez-Pérez, 2003). **Type 2** ventricle wall comprised of subependymal neuroblasts and PCNA positive cells in the region close to the EPL.
Further away from the EPL there were many astrocytes that were interspaced with subependymal neuroblasts and PCNA positive cells. **Type 2** ventricle wall was located on the **rostral half of the ventricle**, but only on the **lateral aspect** of the ventricle wall overlying the caudate nucleus (Rodríguez-Pérez, 2003). **Type 3** ventricle wall was comprised of radially oriented astrocytes and subependymal neuroblasts; close to the EPL were PCNA positive cells and subependymal neuroblasts. The location of **type 3** ventricle wall was restricted to a small segment of the **rostro-ventral SEL** in an area equivalent to the beginning of the rostral migratory stream described in rodents (Doetsch et al., 1997; Lois et al., 1996).

The area of particular interest in this chapter is the **type 2** region which overlies the caudate nucleus which is the brain region most affected in HD. The human SEL and ventricle wall has not been characterised as well as that of the bovine or rodent brain. However, a recent study has demonstrated some characteristics of the human SEL. Bernier and colleagues (2000) reported the presence of the neuroepithelial marker ‘nestin’ throughout the SEL and showed it to stain 4 major cell types identified in the rodent SEL by Doetsch and colleagues (1997) (Doetsch et al., 1997). Nestin was used as a general marker of the SEL and with this marker variations in the thickness of the human SEL were noted from ventral to dorsal. The thickest part of the SEL was at the level of the anterior commissure and the ventral striatum (Bernier et al., 2000). Some of the nestin staining was also present in the temporal and occipital horns of the lateral ventricle. Bernier and colleagues (2000) also demonstrated immunoreactivity for βIII-tubulin, GFAP, PSA-NCAM and PCNA; however the localisation of these cell types throughout the lateral ventricles was not detailed. Thus, the detailed distribution of proliferating cells throughout the lateral ventricle has not been reported. Although, the presence of cells that proliferate in the SEL has been previously demonstrated in the human brain (Bernier et al., 2000; Eriksson et al., 1998), their distribution in the normal brain and their presence and distribution in the HD brain has not been previously studied.

In this chapter the results from studies aimed at mapping the location of PCNA positive cells in the lateral ventricle, that overlies caudate nucleus, will be detailed. In these
studies approximately 600 serial sections of the SEL were cut through the entire body of the caudate nucleus of 3 normal and 3 HD brains and sections from specific levels of the caudate nucleus were examined in detail. This is the first study looking at the localisation and number of proliferating cells in the normal and HD brain SEL. The first part of these results will describe the ventral-dorsal distribution of PCNA positive cells followed by the rostral-caudal localisation; the results from the two parts will then be combined to give a 3-dimensional demonstration of the distribution of PCNA positive cells in the SEL of the lateral ventricle overlying the caudate nucleus.

5.2 Materials and methods

5.2.1 Human tissue collection

For this study the basal ganglia from six post-mortem human brains were obtained from the Neurological Foundation of New Zealand Human Brain Bank in the Department of Anatomy with Radiology, University of Auckland (see table 5.1). The full consent of all families was obtained at the time of autopsy and the University of Auckland Human Subjects Ethics Committee approved the protocols used in these studies. Three normal brains were received from cases with no history of neurological disease and on pathological examination showed no neurological abnormalities. Three HD brains were received from cases with a family and clinical history of HD. The diagnosis of HD was confirmed by genetic analysis of the CAG repeat length in both copies of the IT15 gene. The HD brains used were grades 2 and 3 because they best represent the average presentation of HD cases at post-mortem.

For the immunohistochemistry studies, the brains were fixed by perfusion through the basilar and internal carotid arteries. Initially the brains were perfused with phosphate buffered saline (PBS) with 1% sodium nitrite to clear the brain’s circulation, followed by 15% formalin in 0.1 M phosphate buffer pH 7.4. Following the perfusion, the brains were dissected into regions and the basal ganglia were removed intact and post-fixed in the
Table 5.1
Table of normal and Huntington’s disease cases examined for the distribution of PCNA positive cells in the subependymal layer

<table>
<thead>
<tr>
<th>Case number</th>
<th>Age</th>
<th>Sex</th>
<th>Post mortem delay (hrs)</th>
<th>CAG repeats</th>
<th>HD grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>69</td>
<td>Female</td>
<td>11.5</td>
<td>unavailable</td>
<td>Normal</td>
</tr>
<tr>
<td>N2</td>
<td>70</td>
<td>Male</td>
<td>16</td>
<td>unavailable</td>
<td>Normal</td>
</tr>
<tr>
<td>N3</td>
<td>87</td>
<td>Female</td>
<td>11</td>
<td>unavailable</td>
<td>Normal</td>
</tr>
<tr>
<td>HC1</td>
<td>66</td>
<td>Female</td>
<td>12</td>
<td>21/39</td>
<td>2</td>
</tr>
<tr>
<td>HC2</td>
<td>47</td>
<td>Male</td>
<td>24</td>
<td>17/50</td>
<td>3</td>
</tr>
<tr>
<td>HC3</td>
<td>65</td>
<td>Male</td>
<td>10</td>
<td>17/42</td>
<td>3</td>
</tr>
</tbody>
</table>

same fixative for 24 hours. Subsequently these blocks were cryo-protected in 20% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for 4-5 days, and then in 30% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for a further 4-5 days. The basal ganglia blocks were then sectioned on a freezing microtome (Zeiss HM 440) at a thickness of 50 μm and the sections were collected in PBS and 0.1% sodium-azide and stored in individual wells at 4°C for further immunohistochemical, histochemical or immunofluorescent processing.

5.2.2 Immunohistochemistry

5.2.2.1 Section preparation
The basal ganglia blocks were sectioned from the rostral pole of the caudate nucleus to the caudal part of the body of the caudate nucleus; approximately 600 sections were cut at a thickness of 50 μm on a freezing sledge microtome (Zeiss HM440) from each basal ganglia specimen. In order to preserve the exact serial order of the sections, each section was stored in an individual well in a solution of PBS and 1% sodium-azide. A 1 in 10 series of sections was processed for each immunostain. The sections were washed 3 times
in PBS containing 0.2% triton-x (wash buffer) before being further processed free floating in culture wells for immunostaining.

In order to maximise the immunostaining, antigen retrieval methods were utilised. To achieve this the sections were incubated overnight in a citric acid buffer solution containing citric acid (Na₃ salt) and Na₂HPO₄ (pH 4.5 HCL at 4°C). The next day, the citric acid from each of the wells was replaced with fresh citric acid solution (room temperature) and the sections were microwaved for 30 seconds on high in a 600 W microwave oven. The sections were allowed to cool for approximately one hour before the citric acid solution was removed.

In order to block the endogenous peroxidase staining in the tissue, all sections were thoroughly washed (3 x 10 minute washes) in wash buffer before being incubated for 20 minutes in a 50% methanol solution containing distilled H₂O, methanol and 1% H₂O₂. The sections were again thoroughly washed (3 x 10 minute washes) in wash buffer. Subsequently, primary antibodies were applied.

5.2.2 Primary antibodies
For the PCNA immunostaining, the following three primary antibodies were applied to sections from the basal ganglia blocks; the main antibody used for the PCNA distribution studies was:
(1) Mouse anti-Proliferating Cell Nuclear Antigen (PCNA PC10 Santa Cruz) at a dilution of 1:500.

The staining appearances were confirmed by using
(2) Mouse anti-Proliferating Cell Nuclear Antigen (PCNA Chemicon) at a dilution of 1:500 and,
(3) Rabbit anti-Proliferating Cell Nuclear Antigen (PCNA Santa Cruz) at a dilution of 1:100.

Antibodies were diluted in a solution of PBS, 0.2% triton-x, 1% normal goat serum and 0.04% merthiolate. 1 ml of primary antibody was put in each well with the floating
sections and was incubated on a shaker for 48 hours at 4°C. The sections were then washed (3 x 10 minute washes) with wash buffer and secondary antibodies were applied.

5.2.2.3 Secondary antibodies
The secondary antibodies used for these experiments were:
(1) Anti-mouse Ig biotinylated (from sheep, Amersham Life Sciences)
(2) Anti-rabbit Ig biotinylated (from sheep, Amersham Life Sciences)

The secondary antibodies were diluted in PBS, triton-x, normal goat serum and merthiolate at a dilution of 1:500. The sections were incubated in secondary antibody, 1 ml per well, over night at room temperature before being washed (3 x 10 minute washes) with wash buffer.

5.2.2.4 Tertiary antibodies
Third antibodies, streptavidin biotinylated horseradish peroxidase (Amersham Life Sciences), were applied at a dilution of 1:1000 diluted with normal goat serum, PBS triton-x and merthiolate for 3 hours. The sections were thoroughly washed again (3 x 10 minute washes) and 200 µl 3, 3 diamino-benzidine (DAB), 0.4 M phosphate buffer and 1% H₂O₂ was applied for 20 minutes. Sections were thoroughly washed and mounted onto chrome alum dipped slides (3 g gelatine, 0.3 g chromium potassium sulphate, 600 ml dH₂O, slides were dipped in this solution and dried in an oven). The mounted sections were air dried for 24 hours before being coverslipped by immersion in graded 70%, 80% and 90% alcohol for 5 minutes each, then 2 x 100% alcohol and 3 x xylene for 10 minutes each. Each slide was removed from xylene and a thin smear of Hystomount (Hughes & Hughes) was applied to the slide followed by a coverslip.

5.2.3 Image digitisation

In order to produce the figures, immunostained, mounted and coverslipped brain sections were photographed using a digital camera (Kontron ProgRes 3008) mounted on a conventional transmitted light microscope (Leica DMR). A calibration slide was also
photographed at the same magnification as the brain slices so that calibration and accurate measurements could be made.

5.2.4 Cell counting

On each section the SEL overlying the caudate nucleus was divided into equal dorso-ventral thirds, and detailed cell counts were made at the midpoint of each third i.e. from the ventral, middle and dorsal areas of the SEL (see figure 5.1). The counts were made from sections taken at 3 equally spaced regions of the SEL overlying the caudate nucleus i.e. rostral, central and caudal regions (see figure 5.2). The three regions examined were chosen based on easily identifiable anatomical landmarks. The first section from the caudal region was taken at the level of the first appearance of the horizontal limb of the anterior commissure. The first section from the central region was taken from the rostral pole of the globus pallidus externus. The first rostral region section was taken at an equidistant rostral level. For each normal and HD brain there were 5 or 6 sections counted per region. When a ‘region’ (i.e. rostral) was combined with an ‘area’ (i.e. ventral) the result was referred to as a ‘location’ (i.e. rostro-ventral location, see figure 5.2).

Two observers (Ellen B Penney and I) blinded to the disease status, area, region and brain from which the image was taken counted the number of PCNA positive cells. Cells were counted from digitised micrographs using SCION image software. Three rectangular background measurements were taken from areas of the section that did not contain PCNA positive cells and the background staining from each of these rectangles was averaged. Each cell in the micrograph had a dot placed on it so that no cell was counted twice. Also a density measurement was taken, from the point the dot was located, and recorded for each cell counted. The density measurements, which ranged from 0-255 (where 0 = white and 255 = black) were made of each cell; in order for the cell to be counted in the data it had to have a density measurement value of 25 density points higher than the average background measurements. This technique ensured that
Figure 5.1

Diagram of a coronal section of the human brain demonstrating ‘Areas’ within the subependymal layer

Diagram of the human brain in coronal section. The striatum and SEL are enlarged in the box below. The dorso-ventral extent of the SEL was divided into six equal parts; PCNA positive cell counts were made over 400 μm of the SEL at 1/6th (dorsal), 3/6th (middle) and 5/6th (ventral) areas. Only cells in the SEL were counted; no ependymal cells were included in the cell counts.
Figure 5.2

Diagram of a lateral section of the human brain demonstrating ‘Areas’, ‘Regions’ and ‘Locations’ within the subependymal layer

Schematic diagram showing a lateral projection of the caudate nucleus in the brain. The grid overlying the caudate nucleus (enlarged beneath brain diagram) demonstrates the levels from which PCNA positive cell counts were taken. Sections from each normal and HD brain (each section is represented by a blue line) were taken from 3 regions (rostral, central, caudal). At each region counts were made from 3 different areas (dorsal, middle, ventral; see figure 5.1). An area within a region makes up a ‘Location’. In total, cells were counted in 9 ‘Locations’ within the SEL overlying the caudate nucleus in each brain.
cells were not included or excluded from the cell counts based on the background density or intensity respectively. Each area sample area was 400 μm in length and cells were counted with the observer blinded to the location and disease status from which the sample was taken.

5.2.5 Statistical analysis

The data resulting from the cell counts were analysed with the statistical package ‘R’. Because the frame length of each image varied slightly throughout the cell counting phase of the experiment (due to factors outside of my control), a mathematical adjustment was made to the cell counts so that cell counts represented data taken from a uniform length of SEL. The magnitude and significance of the covariates of interest was assessed with a log linear mixed effect model that included fixed effects for HD, area, region and interactions between area and region (location) and between HD and region. p-values for these effects are shown in table 5.2. The differences between brains with the same disease status (normal or HD) were included in the error as a random effect. A log transformation was applied to the cell count data so that statistical outliers did not significantly sway the results. Transformation ensured that significance levels and averages reflected honestly, significant differences and central values; hence the quoted (displayed) values are the medians for each group. Welch T-tests, corrected for multiple comparisons by Tukey’s method, were used to obtain p-values. These values are more conservative than the mean and are influenced less by some of the very high cell counts observed in some groups of sections. These methods allowed us to examine whether the area effect depended on the region; and also allowed us to test whether the effect of HD was also dependent on the area and region.
Table 5.2

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</tr>
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</table>

5.3 Results

In chapters 3 and 4 the results demonstrated that there were more cells present in the HD SEL compared with the normal SEL and that neurogenesis occurs in the normal and HD SEL. But no previous study has investigated the distribution of dividing cells throughout the extent of the SEL that overlies the caudate nucleus. Thus the principal aims of this study were to: (1) investigate the number of proliferating progenitor cells in HD brain SEL compared with the normal human brain SEL; (2) demonstrate the localisation of proliferating cells in the various areas and regions throughout the lateral ventricle in the normal and HD brain SEL; and (3) demonstrate the area by region differences in the PCNA cell numbers in normal and HD brains. Proliferating progenitor cells in the human brain were detected using a range of antibody clones raised against PCNA. The antibodies were applied to 1 in 10 serial sections taken from the rostral to caudal extent of the SEL overlying the caudate nucleus; the dorsal to ventral axis of the SEL was thus available to be counted on each section taken. More than 150 samples were counted from both the normal and HD brains at different locations within the SEL of the lateral ventricle. Each antibody I used returned a remarkably similar result, but for consistency sake the data represented in this chapter were from cell counts performed using the mouse anti PCNA (PC10) Santa Cruz antibody.
5.3.1 Comparison of PCNA positive cell numbers in the normal and Huntington’s disease subependymal layer (see figure 5.3)

When the data from the SEL cell counts was divided into normal and HD groups there were significantly more PCNA positive cells in the HD SEL (56.75 ± 4.87; median ± standard error) compared with the normal SEL (20.89 ± 1.36; see figure 5.3). Thus there is a 2.7 fold increase in the number of proliferating cells in the SEL of the HD brain compared with normals (see figure 5.3).

5.3.2 The number of PCNA positive cells in the subependymal layer in dorsal, middle and ventral areas of normal and Huntington’s disease brains (see figure 5.4)

The number of PCNA positive cells was counted in each of 3 areas throughout the dorso-ventral axis (dorsal, middle and ventral areas; see figure 5.1). The HD brain SEL contained significantly more PCNA positive cells in each of these areas than did the normal brain SEL (see figure 5.4). The ventral area contained the greatest number of PCNA positive cells in both the HD and normal brains. The area that contained the lowest numbers of PCNA positive cells was the dorsal area for the SEL in both the HD and normal brain (see figure 5.4). The normal brains demonstrated significantly more cells ventrally (32.45 ± 3.61) than in the middle (16.75 ± 1.86; p<0.02) area and there were more cells in the ventral area compared with the dorsal (16.45 ± 1.88; p<0.03) area. However, there was no significant difference between the dorsal and middle areas of the normal brain (p<0.85). In contrast the HD brains demonstrated an increase in cell numbers from the dorsal (43.13 ± 6.95) to the ventral (77.27 ± 11.36) areas. The middle area contained 54.98 ± 8.03 PCNA positive cells. A significant difference was shown between the number of PCNA positive cells in the ventral area compared to the middle (p<0.02) and dorsal (p<0.01) areas in the HD brains. In the HD brains there was no significant difference between the dorsal and middle areas (p<0.85).
Figure 5.3

Comparison of the number of PCNA positive cells in the normal and Huntington’s disease subependymal layer

This bar graph demonstrates that overall the HD brains contained 2.7 fold more PCNA positive cells than the normal brains. The median number of cells in the normal brain was 20.89 ± 1.36 (median ± standard error) compared with the HD brains that contained 56.75 ± 4.87. This represents a significant difference between the numbers of cells in the HD brain compared to normals (p<0.01). The HD cases from which cell counts were made were HD grade 2 and 3. Beneath the columns of the graph are photomicrographs illustrating the substantial increase in the number of cells in the HD brain SEL compared with the number in the normal brain SEL.
Comparison of the number of PCNA positive cells in the normal and Huntington's disease subependymal layer.

Cell number

0 10 20 30 40 50 60 70

Normal Huntingdon's disease

p < 0.01
Figure 5.4

The number of PCNA positive cells in each area (dorsal, middle, ventral) of the subependymal layer in normal and Huntington’s disease brains

This graph shows that in all 3 areas counted (dorsal, middle, ventral) there were significantly more PCNA positive cells present in the HD SEL than in the normal SEL (p<0.01). In the normal brain SEL the dorsal area had 16.45 ± 1.88 cells, the middle area had 16.75 ± 1.86 cells and the ventral area had 32.45 ± 3.61 cells. Thus, the ventral area has twice as many cells as the dorsal and middle areas. In the HD brains, the ventral area (77.27 ± 11.36 cells) contained more PCNA positive cells than did the dorsal (43.13 ± 6.95 cells) and middle areas (54.98 ± 8.03). The dorsal and middle areas contained comparable numbers of PCNA positive cells. However, there was a major increase in the number of PCNA positive cells in each of the areas examined in the HD brain SEL compared with the normal brain SEL.
The number of PCNA positive cells in each area (dorsal, middle, ventral) of the subependymal layer in normal and Huntington's disease brains.
5.3.3 The number of PCNA positive cells in the subependymal layer in rostral, central and caudal regions of the normal and Huntington’s disease brains (see figure 5.5)

The number of PCNA positive cells in the SEL was counted in 3 different regions throughout the rostro-caudal axis (rostral, central and caudal regions; see figure 5.2). In the SEL of HD brains there were significantly more PCNA positive cells in the central (p<0.01) and caudal (p<0.01) regions compared with the normal SEL. However, in the rostral area there was no significant difference between the number of PCNA positive cells in the SEL of HD and normal brains (p<0.06; see figure 5.5).

In the normal brain SEL there was no significant difference between the numbers of PCNA positive cells present in the rostral (22.64 ± 2.87), central (23.87 ± 2.63) and caudal (16.90 ± 1.84) regions (ranging from p<0.39 – 0.95; see figure 5.5). However, in the HD brains more PCNA positive cells were located in the central (83.56 ± 9.49) region compared to the rostral (35.00 ± 5.67) and caudal (61.20 ± 10.18) regions but there was no significant difference between the number of PCNA positive cells in the central and caudal regions (p<0.88; see figure 5.5).

5.3.4 The number of PCNA positive cells in the subependymal layer in different locations in the normal and Huntington’s disease brains (see figure 5.6)

Within the SEL there was a variation in the number of PCNA positive cells at different locations (area within region) of the SEL overlying the caudate nucleus. In the normal brain the rostral-dorsal location had 25.37 ± 6.30 cells in the SEL compared with 38.22 ± 12.56 cells in the HD SEL. The rostral-middle location had 21.33 ± 5.02 cells in the normal SEL compared with 41.83 ± 12.87 in the HD brain SEL, and the rostral-ventral location had 21.19 ± 4.40 in the normal brain compared with 26.16 ± 6.49 cells in the HD brain. There was no significant difference (ranging from p<0.16 – p<0.22) between the number of cells present in normal and HD brains in the rostral region, but there was a trend of increased cell numbers in HD (see figure 5.6). In the central-dorsal location in
Figure 5.5

The number of PCNA positive cells in each region (rostral, central, caudal) of the subependymal layer in normal and Huntington's disease brains

This graph shows that there were comparable numbers of PCNA positive cells in the normal brain SEL in the rostral (22.64 ± 2.87 cells), central (23.87 ± 2.63 cells) and caudal (16.90 ± 1.84 cells) regions (ranging from p<0.39 - p<0.95). In contrast, in the HD brain SEL there were significantly more PCNA positive cells in the central (83.56 ± 9.49 cells; p<0.01) and caudal (61.20 ± 10.18 cells; p<0.01) regions than in the normal brain SEL. However, in the rostral region the HD brain SEL had 35.00 ± 5.67 cells, which was not significantly (p<0.06) more than the normal brain SEL in this region but there was a trend of more PCNA positive cells in the HD brain SEL. The caudal region in the normal brain contained the lowest number of PCNA positive cells of any region counted, and the central region in the HD brain SEL contained the most PCNA positive cells.
The number of PCNA positive cells in each region (rostral, central, caudal) of the subependymal layer in normal and Huntington’s disease brains
Figure 5.6

The number of PCNA positive cells in different locations of the subependymal layer in normal and Huntington’s disease brains

This graph demonstrates the difference in the number of PCNA positive cells at various ‘locations’ (area within region) within the SEL. The ventral-caudal location in HD brains contained the most cells (126.15 ± 30.85) compared with the ventral-middle location in normal brains that contained the lowest number of cells (11.99 ± 2.24). In the caudal and central regions there was a trend that demonstrated increasing numbers of cells from dorsal to ventral. Also, in these locations there was a significant difference between the cell numbers in the SEL of normal and HD brains (p<0.01). In the rostral region there was no significant trend on the area axis for normal or HD brains. Each rostral location in HD brains contained similar numbers of PCNA positive cells; at the rostral location there was no significant difference between the numbers of PCNA positive cells in the HD brain compared with normal brains (ranging from p<0.16 – p<0.22). However, there was a trend because in each area there were more PCNA positive cells in the SEL in HD brains compared with normal brains. Above each of the normal and HD brain graphs is the overall percentage increase in the number of cells in the HD brain compared with the normal brain SEL.
The number of PCNA positive cells in different locations of the subependymal layer in normal and Huntington's disease brains
normal brains there were 14.11 ± 2.73 cells in the SEL compared with the HD brains that had 59.24 ± 13.42 cells in the SEL. This represents an approximate 4 fold increase in the number of PCNA positive cells in HD brains in the central-dorsal location. The central-middle location had 18.62 ± 3.29 cells in the normal SEL compared with 78.18 ± 14.38 cells in the HD SEL, which is more than a 4 fold increase in the number of PCNA positive cells in the HD brain in the central middle location. The central-ventral location had 48.79 ± 8.05 cells in the normal SEL and 125.37 ± 22.39 in the HD brains. Thus there is a 2.5 fold increase in the number of PCNA positive cells in the HD brain compared with normal brains in the central-ventral location (see figure 5.6). In each of these locations there is a statistically significant increase in the number of PCNA positive cells in the HD brain SEL compared with the normal brain SEL.

In the caudal-dorsal location there was 12.10 ± 1.85 cells in the SEL compared with 35.24 ± 11.40 cells in the HD SEL. This represents more than a 2.5 fold increase in the number of PCNA positive cells in the HD brain SEL compared with normal brain SEL in the caudal-dorsal location. The caudal-middle location in the normal brain had 11.99 ± 2.24 cells in the SEL compared with the HD SEL, which had 50.57 ± 14.58 cells.

There was greater than a 4 fold increase in the number of PCNA positive cells in the HD brain SEL compared with the normal brain SEL in the caudal-middle location. In the caudal-ventral location the normal brain had 31.80 ± 6.54 cells in the SEL compared with 126.15 ± 30.85 cells in the HD SEL. Likewise, in the caudal-ventral location there was an approximate 4 fold increase in the number of PCNA positive cells in the HD brain SEL compared with the normal brain SEL. There was a significant difference between the numbers of cells in the normal brain SEL compared with the HD brain SEL for caudal-ventral, caudal-middle and caudal-ventral locations examined (p<0.01, p<0.01, p<0.01 respectively; see figure 5.6).

Thus, in all locations examined, there were more cells in the HD SEL compared with the normal brain SEL. The greatest numbers of cells in the HD SEL were seen in ventral area of the HD brain SEL where there was more than a 2.5 fold increase in the number of
PCNA positive cells compared with the normal brains. Furthermore, there was a significant difference between the numbers of cells in normal and HD brains in all locations except in those sections that were most rostral however, there was a trend in the rostral region toward an increase in the SEL cell numbers in HD brains compared with normal brains.

5.4 Discussion

In this chapter I have investigated the regional distribution of proliferating cells in both the normal and HD human SEL overlying the caudate nucleus. The results presented in this chapter demonstrate that in the HD brain there is an increase in the number of dividing cells in the SEL that in response to the degenerating caudate nucleus may provide cells for replacement or repair in the diseased brain.

5.4.1 There is a variation in the regional distribution of PCNA positive cells in the normal brain subependymal layer

In the normal brain SEL stained for PCNA, low numbers of PCNA positive cells were demonstrated in the dorsal and middle areas (see figure 5.4). However, in comparison the ventral area had approximately twice as many cells present, which demonstrated that in normal brains the ventral area had the greatest number of proliferating cells (see figure 5.4). In the rostral, central and caudal regions of the normal brain SEL overlying the caudate nucleus, the number of PCNA positive cells was approximately the same with the cell numbers ranging from $16.90 \pm 1.84$ to $23.87 \pm 2.63$ cells (see figure 5.5). These results indicate that the normal brain, with the exception of the ventral area, has an approximately even distribution of PCNA positive cells throughout the SEL that overlies the caudate nucleus.

5.4.2 There is a variation in the regional distribution of PCNA positive cells in the Huntington’s disease brain subependymal layer
Within the HD brain there were more PCNA positive cells in each area examined compared with the normal brain, with a gradient of PCNA positive cell numbers ranging from the lowest dorsally to the highest ventrally. In the dorsal area there were approximately 2.6 times the number of PCNA positive cells present in the HD SEL compared with the normal brain SEL (see figure 5.4). In the middle area there was approximately a 3.3 fold increase in the number of cells present in the HD SEL compared with the normal brain SEL. In the ventral area there was approximately a 2.4 fold increase in the number of PCNA positive cells in the HD SEL compared with the normal brain SEL (see figure 5.4). However, despite the proportional increase in PCNA positive cell numbers being lower relative to normal brains in the ventral area, there were more cells overall in the ventral area in HD brains compared with the other two areas examined.

In the rostral region the HD SEL contained fewer PCNA positive cells than any other region or area of the HD brain. Also, there was only a very small increase in the number of cells in the HD SEL compared with the normal SEL. The central region in the HD SEL contained the greatest number of PCNA positive cells with a 3.6 fold increase in cell numbers compared with the control SEL (see figure 5.5). The caudal region of the SEL in HD brains had fewer cells present than the central region and more than the rostral region. There was an approximate 3.8 fold increase in the number of PCNA positive cells in the HD SEL compared with the normal SEL in the caudal region (see figure 5.5).

5.4.3 There is a variation in the distribution of PCNA positive cells in different location of the subependymal layer

In this study I not only looked at the distribution of PCNA positive cells in the 3 areas and 3 regions of the normal and HD brain SEL, I also examined the areas within each region thus giving 9 locations that were examined.

In the normal brain SEL, the rostro-dorsal, rostro-middle and rostro-ventral locations each had similar numbers of PCNA positive cells (see figure 5.6). The HD brain SEL had
slightly more PCNA positive cells but the increase in the cell numbers compared with the normal brains was not significant. In the central locations in the normal brain SEL, the central-dorsal and central-middle locations had similar numbers of PCNA positive cells, in contrast with the central-ventral location that had more than a 2.6 fold increase in the number of PCNA positive cells.

In the HD brain SEL there was a gradient of PCNA positive cell numbers from central-dorsal which contained the lowest number of cells in the central region (59.24 ± 13.42) to the central-ventral location that had the greatest number of cells in the central region (125.37 ± 22.39 cells; see figure5.6). In the normal brains, the caudal-dorsal and caudal-middle locations contained similar numbers of cells and the lowest numbers of cells of any location (11.99 ± 2.24); whereas there were approximately 3 times more cells in the caudal-dorsal and caudal-middle locations in the normal brains. In the HD brains in the caudal locations, there was an approximate 3.6 fold increase in cell numbers from the caudal-dorsal location (35.24 ± 11.40 cells) to the caudal-ventral location (126.15 ± 30.85 cells). The caudal-ventral location in the HD brains contained more cells than any other location examined.

This study is the first to demonstrate the variation in the numbers of proliferating cells in the human SEL overlying the caudate nucleus in normal and HD brains. The demonstration that proliferating cells are present in normal brains in the SEL overlying the caudate nucleus in uniformly low levels and that this uniform pattern of distribution changes markedly in the HD brain demonstrates that this region of the SEL in the mature human brain has a high level of plasticity which adapts to the disease process. The dorsal aspect of the striatum is preferentially affected in HD but there is no evidence of more proliferation in the dorsal area of the SEL, which indicates that there is not a simple relationship between degeneration and the production of new cells. It is however, possible that early on in the disease process there are more cells present in the dorsal area of the SEL or that vascularity or some other factor limits the production of progenitor cell in particular areas. The major increase in the number of dividing cells in HD demonstrates the plasticity present in the adult SEL in that it can respond to degeneration.
in the adjacent caudate nucleus by increasing the number of proliferating cells in the SEL. These results are in keeping with studies in rodent models of neurodegenerative disorders in which cell proliferation in the SEL has been demonstrated in response to the experimentally induced pathology. In these studies Arvidsson et al (2002) and Parent et al (2002) have demonstrated increased numbers of proliferating cells in the SEL overlying the caudate nucleus in a middle cerebral artery occlusion induced stroke model (2.2 fold increase in proliferating cells on the stroke affected compared with the contralateral side) and in a pilocarpine induced seizure model of epilepsy in rats (approximately a 4 fold increase in the number of proliferating cells compared with controls at 14 days), respectively.

5.5 Summary

The experiments performed in this chapter represent the first demonstration of the distribution of proliferating cells throughout the SEL overlying the caudate nucleus. In this study I performed a very detailed regional analysis of the number of PCNA positive cells in the different parts of the SEL and have found that there are a similar number of PCNA positive cells in the normal brain throughout the SEL overlying the caudate nucleus except in the ventral area where there was approximately twice as many proliferating cells present compared with the middle and dorsal areas. In the HD brains there was, on average, 2.8 times more PCNA positive cells in the SEL compared with the normal brain SEL. In particular, in the HD brain SEL there was a gradient of PCNA positive cell numbers from the lowest in the dorsal area (35.24 ± 11.40) to the highest in the ventral area (126.15 ± 30.85 cells). The ventral area had the greatest number of PCNA positive cells of any area examined.

In the rostral region, there was no significant difference between the number of PCNA positive cells in the normal and HD brain SEL, whereas in the central and caudal regions there was as much as a 3.6 fold increase in the number of PCNA positive cells in the HD brains compared with normal brains. The areas within regions (locations) demonstrated significant increases in PCNA positive cell numbers in the HD SEL compared with the
normal SEL except in the rostral region. However, even in the rostral region there was a
trend of more PCNA positive cells present in the HD SEL.
These results demonstrate the plasticity of the SEL in that it can respond to the extensive
degeneration of the caudate nucleus by producing new cells throughout the SEL that
overlies the caudate nucleus. Because the proliferation of PCNA positive cells occurs in
all locations of the SEL, these results highlight the potential use of these progenitor cells
in the development of a cell replacement and cell repair therapy that targets the various
areas and regions of the degenerating caudate nucleus in HD.

5.6 Conclusion

The results in this chapter have demonstrated the localisation and number of proliferating
cells in the normal adult human brain SEL. Furthermore, the present study has
demonstrated a massive increase in the number of proliferating cells in the HD brain in
the SEL that overlies the degenerating caudate nucleus. A significant increase in cell
proliferation in the HD brain has been demonstrated in all areas and regions of the SEL
overlying the caudate nucleus except in the rostral region, however even rostrally there is
a trend of more proliferating cells in HD brains compared with normals. The
demonstration of increased numbers of PCNA positive cells in the HD SEL is in keeping
with previous studies in rodent models of neurodegeneration where experimentally
induced neuropathology resulted in an increase in the number of dividing cells in the SEL
(Arvidsson et al., 2002; Parent et al., 2002a). My results not only demonstrate the
plasticity in the SEL but also further indicate the potential that progenitor cells in the SEL
have to divide and possibly migrate into the degenerating caudate nucleus to provide for
cell replacement and repair in neurodegenerative diseases such as HD.

In the next chapter I will investigate the range of mature cell types that are present in the
SEL in the normal and HD brain and will also look at the most common GABA<sub>A</sub> receptor
subunits present in the SEL.
Chapter 6

CHEMICAL ANATOMY OF THE
SUBEPENDYMAL LAYER

6.1 Introduction

The striatum develops from progenitor cells in the SEL and I have demonstrated that the SEL in the human brain contains progenitor cells that are active throughout life (see chapters 4 and 5). However, the SEL may also contain mature cell types similar to that of the striatum (see figure 2.6). In this chapter the results of experiments looking at mature neuronal and glial cell types and GABA_A receptor subunits in the SEL, EPL and striatum will be presented. This chapter will focus on the results of immunohistochemical studies investigating whether neurochemical markers present in the mature striatum are also present in the SEL.

The SEL in the adult brain is comprised of uncommitted progenitor cells, neurons and glial cells (Reynolds and Weiss, 1992); however, most of the neuron-like cells in the rodent SEL do not display immunoreactivity for mature neuron markers. Lois (1996) and Doetsch (1997) termed the neurons in the SEL ‘migrating neuroblasts’ destined for the olfactory bulb but these did not display mature neuron markers in the SEL (Doetsch et al., 1997; Lois et al., 1996). In contrast the neighbouring striatum displays a variety of mature cell types that are present in the normal brain (Cicchetti et al., 2000; Davies and Roberts, 1988; Ferrante et al., 1987b; Graybiel and Chesselet, 1984; Graybiel and Ragsdale, 1978; Holt et al., 1997).

Most of the neurons in the striatum contain the neurotransmitter γ-amino butyric acid (GABA), which is the major inhibitory neurotransmitter in the human brain. GABA is found in all striatal projection neurons (i.e. medium spiny projection neurons) which are
the major group of neurons in the striatum; these projection neurons project from the striatum to the globus pallidus and substantia nigra (DiFiglia et al., 1989; Holt et al., 1997; Hontanilla et al., 1998; Waldvogel et al., 1991). The medium spiny projection neurons contain not only GABA, but also enkephalin (ENK) or substance P (SP) depending on which nucleus they project to. ENK co-localises with GABA on striatal projection neurons that project to the globus pallidus externus (Graybiel and Chesselet, 1984; Haber and Elde, 1981; Holt et al., 1997). SP co-localises with GABA on striatal projection neurons that project to the globus pallidus internus or substantia nigra (Haber and Elde, 1981; Holt et al., 1997). Regardless of where GABAergic striatal projection neurons project to, they all express the calcium binding protein calbindin. Other cell types in the striatum are interneurons; there are 4 different types that can be identified by immunolabelling for specific markers (Kawaguchi et al., 1995). Parvalbumin is a calcium binding protein that specifically labels a subset of GABAergic interneurons. Cholinergic interneurons in the striatum can be detected using an antibody raised against choline-acetyl transferase (ChAT); this is an enzyme involved in the synthesis of acetylcholine (Kawaguchi et al., 1995). ChAT positive cells are large-sized interneurons in the striatum. A subset of small aspiny interneurons in the striatum can be identified by the presence of neuropeptide Y (NPY) immunoreactivity. All GABAergic neurons could be labelled with antibodies to glutamic acid decarboxylase, which is one of the GABA synthesising enzymes.

Within the striatum there are also non-neuronal (glial) cell types such as astrocytes and microglia; most astrocytes label with GFAP and immature astrocytes label with vimentin. Microglial cells can be labelled with ferritin.

Apart from the localisation of neurotransmitters and calcium binding proteins, the various categories of striatal neurons can also be identified by their particular configuration of GABA<sub>A</sub> receptor subunits. There are 19 GABA<sub>A</sub> receptor subunits (α1-6, β1-3, γ1-3, δ, ε, π, θ, ρ1-3). The GABA<sub>A</sub> receptor is a ligand-gated chloride ion channel comprised of 5 trans-membrane spanning subunits (Olsen and Tobin, 1990; Seeburg et al., 1990; Veenman et al., 1994; Waldvogel et al., 1990; Wisden and Seeburg, 1992). The
most common subunits in the brain are \( \alpha_1, \beta_2, 3 \) and \( \gamma_2 \). In the basal ganglia the parvalbumin and glutamic acid decarboxylase positive neurons have the \( \alpha_1, \beta_2 \) and \( \gamma_2 \) combination of \( \text{GABA}_A \) receptor subunits. In the striatum, calbindin positive medium spiny neurons contain \( \beta_2,3 \) and \( \gamma_2 \) \( \text{GABA}_A \) receptor subunits. However, the distribution of these receptor subunits has not been previously demonstrated in the SEL of the adult human brain.

In HD there is considerable cell loss in the striatum, which follows a specific pattern of cellular degeneration. The first group of projection neurons to be lost in HD are the \( \text{GABA}/\text{ENK} \) striatal neurons that project from the striatum to the external segment (externus) of the globus pallidus (striatopallidal fibres); followed secondly by loss of \( \text{GABA}/\text{SP} \) striatal neurons that project from the striatum to the substantia nigra (striatonigral fibres); and thirdly the loss of \( \text{GABA}/\text{SP} \) striatal neurons that project from the striatum to the internal segment (internus) of the globus pallidus (Glass et al., 2000; Reiner et al., 1988). Thus the striatal projection neuron markers calbindin, ENK and SP are present in considerably reduced numbers in the HD striatum. In HD, some striatal interneurons are relatively spared, such as NPY interneurons that only degenerate in very advanced cases of HD (Beal et al., 1991; Beal et al., 1986; Ferrante et al., 1987b).

Therefore, in this study investigating whether or not there are ‘mature’ neuron types in the SEL, the distribution and staining pattern of calbindin, ENK, SP, parvalbumin, calretinin, ChAT, NPY, GFAP, vimentin, ferritin and \( \text{GABA}_A \) receptor subunits \( \alpha_1, \beta_2, 3, \gamma_2 \) have been investigated.

### 6.2 Materials and methods

#### 6.2.1 Human tissue collection

For this study the basal ganglia from 15 post-mortem human brains were obtained from the Neurological Foundation of New Zealand Human Brain Bank at the Department of Anatomy with Radiology, the University of Auckland. The full consent of all families was obtained at the time of autopsy and the University of Auckland Human Subjects
Ethics Committee approved the protocols used in these studies. Normal brains were received from cases with no history of neurological disease and on pathological examination showed no neurological abnormalities. HD brains were received from patients with a family and clinical history of HD. The diagnosis of HD was confirmed by genetic analysis of the CAG repeat length in both copies of the IT15 gene (see table 6.1), the gene affected in HD. All of the cases examined in this chapter were from HD grade 1, 2 or 3 cases because these are the most common grades of HD encountered at post-mortem (see table 6.1). Representative photomicrographs are presented in the figures.

For immunohistochemical and histochemical studies, the brains were fixed by perfusion through the basilar and internal carotid arteries. Initially the brains were perfused with phosphate buffered saline (PBS) with 1% sodium nitrite, followed by 15% formalin in 0.1 M phosphate buffer pH 7.4. Following perfusion, the brains were sliced into 1 cm thick tissue blocks and post-fixed in the same fixative for 24 hours. Subsequently, these blocks were cryo-protected in 20% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for 4-5 days, and then in 30% sucrose in 0.1 M phosphate buffer with 0.1% sodium-azide for a further 4-5 days. The basal ganglia blocks were then sectioned on a freezing microtome (Zeiss HM 440) at a thickness of 50 μm and the sections were collected in PBS and 0.1% sodium-azide and stored in individual wells at 4°C for further immunohistochemical, histochemical or immunofluorescent processing.

### 6.2.2 Immunohistochemistry

#### 6.2.2.1 Section preparation

The basal ganglia blocks were sectioned, at a thickness of 50 μm on a freezing sledge microtome (Zeiss HM440), from the rostral to caudal direction and the sections were stored in individual wells in a solution of PBS and 1% sodium azide. The sections were removed from PBS and 1% sodium azide and were washed (3 x 10 minute washes) in wash buffer and then further processed free floating in culture wells for immunostaining.

For immunohistochemistry all sections were thoroughly washed (3 x 10 minute washes)
Table 6.1

Normal and Huntington’s disease cases examined for the presence of mature cell markers in the subependymal layer

<table>
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<tr>
<th>HD cases</th>
<th>Age</th>
<th>Sex</th>
<th>Post-mortem delay (hrs)</th>
<th>CAG repeats</th>
<th>HD grade</th>
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in wash buffer before being incubated for 20 minutes in a 50% methanol solution containing distilled water, methanol and 1% $\text{H}_2\text{O}_2$ to block the endogenous peroxidases in the tissue. The sections were again thoroughly washed (3 x 10 minute washes) in wash buffer. Subsequently, primary antibodies were applied.

6.2.2 2 Primary antibodies

The following primary antibodies were applied to sections of the basal ganglia:

<table>
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<tr>
<th>Neuronal markers</th>
<th>Anti-</th>
<th>Dilution</th>
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<td>Gift from Piers Emson</td>
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<td>Enkephalin</td>
<td>Mouse</td>
<td>1:10,000</td>
<td>Sera lab</td>
</tr>
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<td>Substance P</td>
<td>Rabbit</td>
<td>1:20,000</td>
<td>Watpa</td>
</tr>
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<td>Rabbit</td>
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<td>Gift from Piers Emson</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Rabbit</td>
<td>1:5,000</td>
<td>Swant</td>
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<td>Choline-acetyl transferase</td>
<td>Rabbit</td>
<td>1:1,500</td>
<td>Chemicon</td>
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<td>Neuropeptide Y</td>
<td>Rabbit</td>
<td>1:8,000</td>
<td>Sigma</td>
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<tr>
<td>Vimentin</td>
<td>Mouse</td>
<td>1:500</td>
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<table>
<thead>
<tr>
<th>GABA&lt;sub&gt;A&lt;/sub&gt; receptor subunits</th>
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<th>Source</th>
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<tr>
<td>$\beta_2$, 3 (bd17)</td>
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<td>Gift from Hanns Mohler</td>
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<tr>
<td>$\gamma_2$</td>
<td>Guinea pig</td>
<td>1:2,000</td>
<td>Gift from Hanns Mohler</td>
</tr>
</tbody>
</table>

Antibodies were diluted in a solution of PBS, 0.2% triton-x, 1% normal goat serum and 0.04% merthiolate. 1 ml of primary antibody was put in each well with the floating sections and was incubated on a shaker for 48 hours at 4° C. The sections were then washed (3 x 10 minute washes) in wash buffer and secondary antibodies were applied.
6.2.2.3 Secondary antibodies
1. Anti-rabbit Ig biotinylated (from donkey, Amersham Life Sciences).
2. Anti-mouse Ig biotinylated (from sheep, Amersham Life Sciences).
3. Anti-guinea pig Ig biotinylated (from goat, Sigma)

The secondary antibodies were diluted in PBS, triton-x, 1% normal goat serum and merthiolate at a dilution of 1:500. The sections were incubated in secondary antibody, 1 ml per well, overnight at room temperature before being washed (3 x 10 minute washes) in wash buffer.

6.2.2.4 Tertiary antibodies
Third antibodies:
Streptavidin biotinylated horseradish peroxidase (Amersham Life Sciences) or

Extravidin biotinylated horseradish peroxidase (Sigma),
were applied at a dilution of 1:1000 diluted with normal goat serum, PBS triton-x and merthiolate for 3 hours. The sections were thoroughly washed again and 200 μl 3,3 diaminobenzidine (DAB), 0.4 M phosphate buffer and 1% H2O2 was applied for 20 minutes. Sections were thoroughly washed and mounted onto chrome alum dipped slides (3 g gelatine, 0.3 g chromium potassium sulphate, 600 ml dH2O, slides were dipped in this solution and dried in an oven). The mounted sections were air dried for 24 hours before being coverslipped by immersing them in graded 70%, 80% and 90% alcohol for 5 minutes each, then 2 x 100% alcohol and 3 x xylene for 10 minutes each. Each slide was removed from xylene and a thin smear of Hystomount (Hughes & Hughes) was applied to the slide followed by a coverslip.

6.2.3 Image digitisation

In order to produce the figures, immunostained, mounted and coverslipped brain sections were photographed using a digital camera (Kontron ProgRes 3008) mounted on a conventional transmitted light microscope (Leica DMR). A calibration slide was also photographed at the same magnification as the brain slices so that accurate measurements could be made.
6.3 Results

6.3.1 Calbindin immunoreactivity in the subependymal layer

6.3.1.1 Normal (Figure 6.1A)
The SEL of the normal brain had a band of neuropil staining that was more intensely stained than that of the caudate nucleus or EPL. The cell bodies present in the SEL were scarce compared with the caudate nucleus. The boundary of the caudate nucleus with the SEL was thin and the two blended together making an indistinct boundary. There was a very small gap between the SEL and the EPL; the gap had only very light neuropil staining (see figure 6.1A). Throughout the entire length of the sections examined, only about 6-10 calbindin positive cells were demonstrated within the SEL. Within the caudate nucleus there were greater numbers of calbindin positive cells in the normal brains than in the HD brains. The calbindin positive cells were evenly distributed across the caudate nucleus and the neuropil staining was quite dense in the normal brain caudate nucleus. Calbindin positive cells were never seen in the EPL (see figure 6.1A).

6.3.1.2 Huntington’s disease (Figure 6.1B)
The calbindin stained HD SEL consisted of a barely distinguishable band of tissue that had a very small number of sparsely scattered calbindin positive cells (see figure 6.1B). The stained cells were elongated, medium sized cells that usually had a small number of thin processes. Calbindin positive cells were never seen to cluster within the SEL. The gap between the SEL and the EPL contained a small amount of punctate staining throughout the ventricle wall. The neuropil was very lightly stained in the SEL but the EPL was devoid of staining. Throughout the caudate nucleus there were a few medium sized, moderately stained and irregularly shaped cells that were homogenously distributed. These cells had multiple processes and were present in lower numbers in HD brains than in the normal brains.
Figure 6.1

Projection neuron markers in the subependymal layer of normal and Huntington's disease brains

These photomicrographs demonstrate the adult human ependymal layer (EPL), subependymal layer (SEL) and medial caudate nucleus (CN) that have been immunolabelled for various markers that characterise the striatal projection neurons. For each immunolabel used there is a normal and HD case demonstrated.

A and B show the immunoreactivity for the calcium binding protein calbindin, which is a marker of GABAergic medium spiny projection neurons.

C and D show the immunoreactivity for the neurotransmitter enkephalin, which is a marker of the GABAergic medium spiny neurons that project from the striatum to the globus pallidus (externus).

E and F show the immunoreactivity for the neurotransmitter substance P, which labels the GABAergic medium spiny neurons that project from the striatum to the globus pallidus internus and to the substantia nigra.
Projection neuron markers in the subependymal layer of normal and Huntington's disease brains

A Calbindin B

EPL SEL CN Normal Huntington's disease

C Enkephalin D

EPL SEL CN Normal Huntington's disease

E Substance P F

EPL SEL CN Control Huntington's disease
6.3.2 Enkephalin immunoreactivity in the subependymal layer

6.3.2.1 Normal (Figure 6.1C)
In normal brain sections immunostained for ENK, the SEL displayed no immunostaining of any form or in any region throughout the dorsal to ventral extent of the lateral ventricle. Likewise, the EPL contained no immunostaining throughout the ventricular wall (see figure 6.1C). The caudate nucleus in the normal brain contained many densely packed fibres and punctate staining. The fibres appeared to be made up of chains of punctate staining that were densely immunoreactive (see figure 6.1C). There was light neuropil immunoreactivity throughout the caudate nucleus; however there was variability in the density of ENK staining throughout the caudate nucleus.

6.3.2.2 Huntington’s disease (Figure 6.1D)
The HD SEL was predominantly devoid of immunostaining; however, very occasionally at high magnification, a small amount of terminal staining for ENK could be seen in the SEL. The background neuropil staining in the HD SEL stained more densely than in the normal SEL. The EPL was completely devoid of ENK immunoreactivity (see figure 6.1D). The caudate nucleus had a small amount of fibre and punctate staining throughout the caudate nucleus, but most notable was the large reduction in the number of ENK immunoreactive terminals in the HD brain caudate nucleus compared with the normal brain caudate nucleus. ENK positive cell bodies were never demonstrated in the HD SEL.

6.3.3 Substance P immunoreactivity in the subependymal layer

6.3.3.1 Normal (Figure 6.1E)
The normal brain SEL demonstrated light neuropil immunoreactivity for SP. The staining was predominantly background and no cell bodies were demonstrated. However, very occasionally small clusters of fine punctate staining were observed (see figure 6.1E). The EPL contained a small amount of background staining but cellular, neuropil or fibre staining was not evident (see figure 6.1E). The caudate nucleus contained very tightly packed punctate staining that was distributed evenly across the caudate nucleus. The
caudate nucleus contained a small number of SP immunoreactive cell bodies that were small to medium in size, were circular in shape and were moderately SP immunoreactive. The punctate staining that was observed appeared to be transected fibres or terminals of dentrites or axons.

6.3.3.2 Huntington’s disease (Figure 6.1F)
The HD SEL demonstrated a narrow band of fine punctate staining that was heterogenously distributed throughout the dorsal-ventral extent of the lateral ventricle; the SEL was indistinguishable from the caudate nucleus in some parts of the lateral ventricular wall (see figure 6.1F). The caudate nucleus contained a very lightly stained population of punctate fibre and terminal staining that was well dispersed throughout the caudate nucleus. The SP immunoreactivity was considerably weaker in the HD brains compared with normal brains. Within the caudate nucleus there were a small number of cell bodies that were small-medium in size and round in shape. These cells were lightly immunoreactive and were scattered throughout the caudate nucleus. The massive reduction of SP immunolabelling in HD brains compared with normal brains was the most obvious feature of the SP stained caudate nucleus sections (compare figure 6.1E and 6.1F). The HD EPL contained no cellular, neuropil, or fibre immunoreactivity for SP (see figure 6.1F).

6.3.4 Parvalbumin immunoreactivity in the subependymal layer
6.3.4.1 Normal (Figure 6.2A)
The normal brain SEL had no parvalbumin immunoreactivity from its dorsal to ventral extent. There was a very small amount of parvalbumin positive fibre immunoreactivity in the caudate nucleus very close to the SEL. The caudate nucleus had many parvalbumin positive fibres that were long, darkly stained and oriented in all directions (see arrows on figure 6.2A). In the caudate nucleus small numbers of medium sized, lightly parvalbumin positive cell bodies were seen evenly distributed throughout. The EPL demonstrated no immunoreactivity for parvalbumin.
Figure 6.2

Interneuron markers in the subependymal layer of normal and Huntington’s disease brains

These photomicrographs demonstrate the adult human ependymal layer (EPL), subependymal layer (SEL) and medial caudate nucleus (CN) that have been immunolabelled for the various markers of striatal interneurons. For each immunolabel used there is a normal and HD case demonstrated.

A and B show the immunoreactivity for the calcium binding protein parvalbumin, which labels a subpopulation of GABAergic interneurons in the striatum. The arrows indicate fibre staining in the caudate nucleus.

C and D show the immunoreactivity for the calcium binding protein calretinin, which is a marker of a subpopulation of GABAergic interneurons. The arrows indicate fibre staining in the caudate nucleus in C and fibre staining in the SEL in D.

E and F show the immunoreactivity for the marker of striatal cholinergic interneurons, choline-acetyl transferase. The arrows indicate cell bodies in the SEL in E and in the caudate nucleus in F.

G and H show the immunoreactivity for neuropeptide Y, a subset of striatal interneurons that are relatively spared in HD.
Interneuron markers in the subependymal layer of normal and Huntington’s disease brains

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<tr>
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</tr>
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<td>SEL</td>
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</tr>
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<td>CN</td>
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<tr>
<td>Normal</td>
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<td>CN</td>
</tr>
<tr>
<td>Normal</td>
<td>Huntington’s disease</td>
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6.3.4.2 Huntington's disease (Figure 6.2B)
The HD SEL contained no parvalbumin immunoreactivity on cells or fibres (see figure 6.2B). The caudate nucleus had the occasional fibre that was parvalbumin immunoreactive. The amount of fibre immunoreactivity was dramatically reduced in the HD caudate nucleus compared with that of normal brains. A small number of parvalbumin positive cells could be seen scattered throughout the caudate nucleus, but there were fewer parvalbumin positive cells in HD brains compared with normal brains. The EPL also demonstrated no immunoreactivity for parvalbumin.

6.3.5 Calretinin immunoreactivity in the subependymal layer
6.3.5.1 Normal (Figure 6.2C)
The normal brain SEL had only very light neuropil staining. There was a region of dense punctate immunoreactivity on the border of the SEL and the caudate nucleus. Within the border zone no cell bodies were demonstrated but some fibre staining was evident (see figure 6.2C). Calretinin positive fibres and puncta were seen in the SEL (see figure 6.2C), but cell bodies were never demonstrated in the SEL. The caudate nucleus had many moderately immunoreactive calretinin positive cell bodies that were medium sized, bipolar cells that had long processes (see arrows in figure 6.2C). Also, occasionally large, multipolar immunoreactive cells were seen with short processes. Calretinin positive cell bodies were homogenously distributed throughout the caudate nucleus and there was also considerable punctate staining throughout the caudate nucleus. The EPL was devoid of immunoreactivity.

6.3.5.2 Huntington's disease (Figure 6.2D)
The HD brain SEL was comprised of a band of light fibre and punctate calretinin positive staining (see arrows in figure 6.2D). The subependymal space was much wider in HD brains than in the normal brain and fibres from the SEL almost reached the EPL. There was very little background staining in the EPL and SEL (see figure 6.2D); however, in the caudate nucleus there was a gradient of staining from the dense staining to very light background fibre staining. Also, large sized, densely stained, multipolar neurons were
identified with a lower frequency than the medium sized, bipolar neurons; both cell types were randomly distributed throughout the caudate nucleus. There were fewer calretinin positive cells in the HD compared with the normal brain caudate nucleus.

6.3.6 Choline-acetyl transferase immunoreactivity in the subependymal layer

6.3.6.1 Normal (Figure 6.2E)
The normal brain SEL contained a small number of ChAT positive cells that were large in size, moderately stained with extended processes. These cells were randomly distributed throughout the SEL (see arrows in figure 6.2E). Within the SEL there was a lot of punctate staining. There was a region of the SEL immediately beneath the EPL that was devoid of cellular and fibre staining but did have some background staining (see figure 6.2E). The caudate nucleus contained many large and medium sized cells that stained with a moderate intensity (see figure 6.2E). The caudate nucleus had a lot of neuropil staining but did not contain as much punctate staining as the SEL. The EPL contained a large amount of fibre and punctate staining, but the distinct ChAT cell body staining seen in other brain regions was not evident in the EPL.

6.3.6.2 Huntington’s disease (Figure 6.2F)
The HD brain SEL contained very little cell body staining but there was a small amount of punctate staining that had the appearance of processes that were seen in cross section. There was only a low level of neuropil staining in the SEL. The EPL contained darker neuropil staining than in the SEL but was less intense than the normal cases (see figure 6.2F). The punctate staining was less evident in the HD EPL than in the normal brain SEL. The caudate nucleus contained many large and medium sized cells that had prominent processes and stained intensely with the ChAT antibody (see arrows in figure 6.2F); the ChAT cells were randomly distributed throughout the caudate nucleus. The HD caudate nucleus had a lot of punctate staining that was homogenously distributed throughout the caudate nucleus. No fibre staining was evident in the caudate nucleus.
6.3.7 Neuropeptide Y immunoreactivity in the subependymal layer

6.3.7.1 Normal (Figure 6.2G)
In the normal brain SEL the occasional NPY positive cell could be seen. These cells were medium sized, multipolar, darkly labelled neurons (see figure 6.2G). Also within the SEL there was a lot of fine punctate staining and densely stained fibres. The SEL consisted of an area close to the EPL that was densely stained for NPY and an area close to the caudate nucleus that was very lightly stained for NPY. The caudate nucleus contained a small number of cells that were homogenously distributed throughout. These cells were mostly darkly stained, medium sized, multipolar neurons. Another group of cells were seen in the caudate nucleus that were small, round and very lightly NPY immunoreactive. Throughout the caudate nucleus light background neuropil staining and some fibre staining was demonstrated. Thus the region of the SEL close to the caudate nucleus was only distinguished by lighter immunostaining than that of the caudate nucleus.

6.3.7.2 Huntington’s disease (Figure 6.2H)
The HD SEL contained a moderate number of NPY positive neurons. These neurons had many small processes. There was very little background immunoreactivity in the SEL space immediately beneath the EPL. However, there was a very dense band of background and neuropil staining beneath the SEL space (see figure 6.2H). Within the band of staining, NPY positive cells were mostly bipolar and densely immunoreactive. In this region a small amount of fibre staining and a lot of punctate staining was evident (see figure 6.2H). In the caudate nucleus there was moderate neuropil and background staining with many NPY positive neurons that were bipolar and multipolar that were strongly NPY immunoreactive. It was characteristic of the SEL in HD cases to be strongly immunoreactive for NPY. The background immunoreactivity was denser in the HD brains compared with the normal brains. Also the SEL was considerably thicker in HD brains compared with normal brains.

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6.3.8 Glial fibrillary acidic protein immunoreactivity in the subependymal layer

6.3.8.1 Normal (Figure 6.3A)
The normal SEL contained a moderate number of GFAP immunoreactive cell bodies and processes. The SEL was indistinct with no obvious boundary between it and the caudate nucleus. However, the general area of the SEL was more densely stained than the neighbouring caudate nucleus (see figure 6.3A). Much of the GFAP staining in the SEL was punctate staining although cell bodies were also present in the SEL and their fibres radiated either toward the EPL or the caudate nucleus. Some of the GFAP positive fibres were oriented tangentially in the SEL and at higher magnification appeared to form a tubular-like structure. The caudate nucleus contained a homogeneous spread of GFAP positive cells with many fibres evident throughout the caudate nucleus. The GFAP positive cell bodies had many processes that were oriented in all directions. The neuropil was lightly stained throughout the caudate nucleus (see figure 6.3A). The EPL contained a lot of neuropil staining and also displayed a considerable amount of radially oriented, thin, lightly stained fibres (see figure 6.3A).

6.3.8.2 Huntington’s disease (Figure 6.3B)
The GFAP stained SEL in the HD brain was much thicker compared to that of the normal brain SEL. Despite the HD SEL being quite indistinct at its boundary with the caudate nucleus, there were clearly more GFAP immunoreactive cells in the HD SEL compared with the normal SEL; however this appears only to be in proportion to the additional thickness of the SEL in HD. The cells in the SEL were stained with moderate intensity and had multiple fibres arising from them that were oriented in all directions. The fibre staining was evenly distributed throughout the SEL but was not densely packed throughout (see figure 6.3B). Very little punctate staining was demonstrated in the HD SEL, however there was a low level of background staining. The caudate nucleus contained a population of lightly stained GFAP positive cells that were irregular in shape and had a few short processes. There was much less neuropil and fibre staining in the caudate nucleus in HD compared with normal brain. Within the EPL there was a moderate number of GFAP positive cell bodies demonstrated throughout the ventral to dorsal axis, many of which contacted the lateral ventricle; these cells could only be
Figure 6.3

Glial markers in the subependymal layer of normal and Huntington’s disease brains

These photomicrographs demonstrate the adult human ependymal layer (EPL), subependymal layer (SEL) and medial caudate nucleus (CN) that have been immunolabelled for two markers of glial cells. For each immunolabel used there is a normal and HD case demonstrated.

A and B show the immunoreactivity for glial fibrillary acidic protein (GFAP), which is a marker of mature astrocytes.
C and D show the immunoreactivity for vimentin, which is an immature glial cell marker. The arrows demonstrate vimentin positive cell bodies.
E and F show the immunoreactivity for ferritin, which is a marker of microglial cells. The arrows indicate ferritin stained cell bodies in the SEL.
Glial markers in the subependymal layer of normal and Huntington’s disease brains

A  Glial fibrillary acidic protein  B
EPL  SEL  CN  Normal  EPL  SEL  CN  Huntington’s disease

C  Vimentin (Nissl Counterstained)  D
EPL  SEL  CN  Normal  EPL  SEL  CN  Huntington’s disease

E  Ferritin (Nissl counterstained)  F
EPL  SEL  CN  Normal  EPL  SEL  CN  Huntington’s disease
demonstrated at high magnification. The cells in the EPL were very densely GFAP immunoreactive. GFAP positive fibre staining was evident throughout the EPL, oriented in all directions and a small amount of punctate staining was also evident (see figure 6.3B).

6.3.9 Vimentin immunoreactivity in the subependymal layer

6.3.9.1 Normal (Figure 6.3C)
The normal brain SEL contained many vimentin positive cell bodies and processes. Most of the densely stained vimentin positive cell bodies had an irregular shape with one major process and many more thin processes that were oriented in all directions. Less intensely immunoreactive round cells were also evident within the SEL (see figure 6.3C). Throughout the SEL there was a lot of small punctate staining. This was most evident on the medial side of the SEL (adjacent to the EPL) where the irregularly shaped cells resembled the glial cells previously described (see figure 6.3C). In the caudate nucleus there were a small number of moderately immunoreactive cells that had many processes and small cell bodies (see arrows in figure 6.3C) (the section has been counterstained with cresyl violet so the caudate nucleus and SEL can be more easily identified). The number of these cells decreased in the more lateral regions of the caudate nucleus such that no vimentin positive cells were demonstrated in the lateral regions of the caudate nucleus. The EPL contained a lot of distinct punctate staining that was present throughout the EPL in its dorsal to ventral extent; immunoreactivity for fibres was also evident at different dorsal to ventral levels of the EPL. Vimentin positive cell bodies were demonstrated in the EPL that had an irregular shape and resembled the cells in the SEL (see figure 6.3C).

6.3.9.2 Huntington’s disease (Figure 6.3D)
The HD SEL was strongly immunoreactive for vimentin; two distinct bands of vimentin staining could be seen. The band adjacent to the EPL had many darkly stained cell bodies with numerous processes and a lot of neuropil and punctate staining (the section has been counterstained with cresyl violet so the caudate nucleus and SEL can be more easily identified; see figure 6.3D). The small space beneath the EPL was seen on vimentin
stained HD sections but was seldom seen in normal brain sections. The second band was present beneath the SEL and was situated in the medial caudate nucleus (see arrows in figure 6.3D). There was a lot of dense cellular staining in the second band, however there was very little background or punctate staining in this layer. The cells in the second band had many short processes and resembled glial cells. In the caudate nucleus, there was a gradient of glial-like cells from densely populated cells close by the SEL to no vimentin positive cells in the lateral part of the caudate nucleus. The distinct appearance of two separate bands of vimentin immunoreactivity was much less obvious in the normal brains, probably because the two bands were closer together in the normal brain thus making the two bands indistinguishable from one another (see figures 6.3C and D).

6.3.10 Ferritin immunoreactivity in the subependymal layer

6.3.10.1 Normal (Figure 6.3E)
Within the SEL ferritin stained cells were seen. The cells present were small, elongated cells located immediately beneath the EPL (see arrows in figure 6.3E). The SEL was very indistinct in normal brains and required a Nissl (cresyl violet) stain so that the SEL could be identified (see figure 6.3E). The cells in the SEL that were stained with ferritin were not typical of the ferritin positive cells from the caudate nucleus and other regions of the brain. Most of the ferritin positive cells in the SEL were observed very close to blood vessels, an area known to contain numerous glial cell types. The caudate nucleus contained a homogenous spread of very densely immunoreactive cells that were either irregularly shaped and had numerous processes or were round with a granular staining appearance (see figure 6.3E). Every ferritin immunoreactive cell had a halo of dark staining that was present around the cell body and as far as the cells processes extended. The rest of the caudate nucleus had very low background and negligible neuropil staining. The EPL was completely devoid of immunoreactivity for ferritin from its dorsal to ventral extent.

6.3.10.2 Huntington’s disease (Figure 6.3F)
The HD SEL contained ferritin immunoreactivity throughout its dorsal to ventral extent. These cells had small processes and were moderately immunoreactive for ferritin (see
arrows figure 6.3F, arrows indicate the ferritin positive cell bodies). This section was counterstained with cresyl violet so the SEL and caudate nucleus could be more easily identified. The caudate nucleus contained many small, medium and large sized cells that were moderately immunoreactive for ferritin. Some cells that were present were irregularly shaped and were densely stained for ferritin. Like the normal caudate nucleus there was no evident background punctate or neuropil staining. The EPL was completely devoid of immunoreactivity throughout its dorsal to ventral extent. The EPL contained no cells immunoreactive for ferritin and contained no background neuropil staining (see figure 6.3F).

6.3.11 GABA<sub>A</sub> α1 subunit immunoreactivity in the subependymal layer

6.3.11.1 Normal (Figure 6.4A)
In normal brains, stained for the GABA<sub>A</sub> α1 subunit with the BD24 antibody, the EPL contained a large number of short stained processes. The stained processes were oriented in all directions and were very densely immunoreactive. The SEL also contained a large number of α1 immunopositive fibres. Some of the fibres extended from the SEL out into the caudate nucleus while others were oriented tangentially, parallel with the EPL. Very few immunoreactive cell bodies could be seen in the SEL in the normal brain sections (see figure 6.4A). There was a particularly strong band of α1 immunoreactivity in the lower region of where the cell sparse space was (see arrows in figure 6.4A). In the caudate nucleus there was considerably less α1 immunoreactivity with no background staining. However, there were a number of cell bodies that had multiple processes that were densely immunoreactive for the GABA<sub>A</sub> α1 subunit. No cell bodies could be detected throughout the EPL; however, there was some fibre labelling in the EPL (see figure 6.4A).

6.3.11.2 Huntington’s disease (Figure 6.4B)
The HD brain SEL, labelled for the GABA<sub>A</sub> α1 subunit, contained many fibres and processes that stained positively for α1 but not as many fibres were immunoreactive in

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Figure 6.4

GABA_A receptor subunits in the subependymal layer of normal and Huntington’s disease brains

These photomicrographs demonstrate the adult human ependymal layer (EPL), subependymal layer (SEL) and medial caudate nucleus (CN) that have been immunolabelled for various subunits of the GABA_A receptor. For each immunolabel used there is a normal and HD case demonstrated.

A and B show the immunoreactivity for the α1 subunit of the GABA_A receptor (bd24 antibody). The arrows indicate a particularly strong band of α1 subunit staining beneath the EPL in the SEL.

C and D show the immunoreactivity for the β2, 3 subunits of the GABA_A receptor (bd17 antibody)

E and F show the immunoreactivity for the γ2 subunit of the GABA_A receptor.
GABA<sub>δ</sub> receptor subunits in the subependymal layer of normal and Huntington's disease brains

A  \( \alpha_1 \) subunit

B

C  \( \beta_{2,3} \) subunits

D

E  \( \gamma_2 \) subunit

F
the HD brain as there were in the normal brain (see figure 6.4B). The HD SEL was thicker than the normal SEL but the fibres that stained in the HD brain were not as densely packed and stained less densely compared with the normal SEL. Cell bodies were not frequently demonstrated in the HD SEL (see figure 6.4B). The caudate nucleus showed a heterogeneous pattern of \( \alpha_1 \) immunoreactivity and also had a very lightly labelled neuropil stain. There were no cell bodies demonstrated in the EPL.

6.3.12 GABA\(_{A} \) \( \beta_2, 3 \) subunit immunoreactivity in the subependymal layer

6.3.12.1 Normal (Figure 6.4C)
The SEL was predominantly devoid of neuropil immunoreactivity for the \( \beta_2, 3 \) subunits of the GABA\(_{A} \) receptor. Furthermore, no cell bodies were demonstrated in the SEL throughout the length of the SEL overlying the caudate nucleus (see figure 6.4C). The caudate nucleus contained some cell bodies in the lateral part of the caudate nucleus but \( \beta_2, 3 \) subunit immunoreactive cell bodies were seldom seen close to the SEL. The normal brain EPL contained no immunoreactivity for the \( \beta_2, 3 \) subunits of the GABA\(_{A} \) receptor.

6.3.12.2 Huntington's disease (Figure 6.4D)
There were no \( \beta_2, 3 \) immunoreactive cells in the SEL (see figure 6.4D). In the caudate nucleus however, there was intense neuropil immunoreactivity throughout and small circular shaped cells were demonstrated both close to the SEL and throughout the caudate nucleus (see figure 6.4D). The level of neuropil immunoreactivity in the caudate nucleus was higher in the HD brains compared with the normal brains. The HD brain EPL contained no immunoreactivity for the \( \beta_2, 3 \) subunits of the GABA\(_{A} \) receptor.

6.3.13 GABA\(_{A} \) \( \gamma_2 \) subunit immunoreactivity in the subependymal layer

6.3.13.1 Normal (Figure 6.4E)
In the normal brain the EPL contained some immunoreactivity for the \( \gamma_2 \) GABA\(_{A} \) receptor subunit. The ependymal staining was predominantly punctate and neuropil staining. The SEL had a densely immunoreactive neuropil and cell bodies in the SEL.
were also moderately immunoreactive for γ2. These cell bodies were small, round and displayed no immunoreactivity in their processes (see figure 6.4E). The background immunoreactivity in the SEL and caudate nucleus was very similar. However, there were fewer cells in the caudate nucleus than there were in the SEL. The caudate nucleus contained a lot of neuropil immunoreactivity and some cell bodies that were randomly distributed throughout the caudate nucleus.

6.3.13.2 Huntington’s disease (Figure 6.4F)
The SEL contained strong background immunoreactivity for the γ2 subunit throughout its dorsal to ventral extent and this labelling made it distinguishable from the caudate nucleus. However the SEL contained a lot of cell bodies that were lightly immunoreactive and were more densely packed than the cell bodies in the caudate nucleus (see figure 6.4F). The cells in the SEL were small, round and no processes were evident. The caudate nucleus had neuropil immunoreactivity and contained a small number of cell bodies throughout; these were larger and less densely stained than the cells demonstrated in the SEL. The HD brain EPL contained a lot of neuropil immunoreactivity for γ2 that formed a meshwork of background staining.
Table 6.2
Table summarising the staining demonstrated in the ependyma, subependyma and caudate nucleus in normal and Huntington’s disease brains

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Key: - no labelling, + small amount of labelling, ++ large amount of labelling
6.4 Discussion

In chapter 3 I demonstrated that the human SEL is predominantly comprised of type A, B and C cells. In chapter 4 I demonstrated that some of the SEL cells are undergoing cell division to form new neurons and glial cells in the SEL. In this chapter I have demonstrated the range of mature cell and neurochemical markers that are present in the human SEL in the normal and HD brain using antibodies to identify the various mature cell types present in the striatum.

6.4.1 The adult normal and Huntington’s disease subependymal layer contain some projection neurons

The normal striatum is comprised mainly of medium spiny neurons that project from the striatum to the globus pallidus and substantia nigra. The medium spiny neurons in the striatum contain GABA, which is the major inhibitory neurotransmitter in the basal ganglia (DiFiglia et al., 1989; Holt et al., 1997; Hontanilla et al., 1998; Oertel and Mugnaini, 1984; Oertel et al., 1984; Preston et al., 1980). Calbindin is a calcium binding protein that is specifically located in medium spiny projection neurons in the striatum; thus it is useful as a marker of this population of cells in the striatum and SEL (DiFiglia et al., 1989; Hontanilla et al., 1998; Kiyama et al., 1990). The presence of particular co-transmitters have been identified that label individual subpopulations of medium spiny projection neurons depending on their projection. In the striatum, enkephalin is a marker of GABAergic neurons that project to the globus pallidus (externus) (Cuello et al., 1981; Emson et al., 1980; Graybiel and Chesselet, 1984; Haber and Elde, 1981; Holt et al., 1997) and substance P is a marker of GABAergic neurons that project to the globus pallidus (internus) and to the substantia nigra (Cuello et al., 1981; Emson et al., 1980; Graybiel and Chesselet, 1984; Haber and Elde, 1981; Holt et al., 1997).

Within the normal SEL there were a small number of calbindin positive cells, but there were less calbindin positive cells in the SEL than in the striatum. In the HD SEL there were very few calbindin positive cells, much fewer than in the normal brain SEL. Also, in
the HD caudate nucleus there was a massive reduction in the number of calbindin positive neurons. The finding of reduced numbers of calbindin positive neurons in the striatum is in keeping with numerous previous studies that have demonstrated a loss of GABAergic medium spiny striatal projection neurons from the striatum in HD (Ferrante et al., 1991; Ferrante et al., 1986; Glass et al., 2000; Kiyama et al., 1990; Reiner et al., 1988; Vonsattel and DiFiglia, 1998; Vonsattel et al., 1997; Vonsattel et al., 1985). One proposed mechanism that may account for the death of projection neurons in HD is excitotoxicity. The excitotoxic mechanism of cell death in animal studies has demonstrated that lesioning the striatum with an infusion of glutamate causes a loss of striatal projection neurons, much the same as in HD. The ionotropic glutamate receptors consist of cation specific ion channels, one of which is N-methyl-D-aspartate (NMDA). These receptors mediate a rapid excitation of neurons, which is typical of glutamate neurotransmission. The NMDA receptor acts as a Na/Ca channel that is regulated by glutamate; an influx of intracellular calcium would force the ion pump to work harder to maintain an ionic equilibrium. The increased ion pump activity may deplete adenosine triphosphate (ATP) levels, which leads to cell death. Because the corticostriate neurons are the major excitatory glutamatergic input to the medium spiny neurons in the striatum, the excitotoxicity may be the cause of striatal cell death and may also be responsible for the death of calbindin positive neurons in the SEL (Coyle and Schwarcz, 1976; McGeer et al., 1978). Metabolic stress in striatal neurons caused by the disruption of energy metabolism by mitochondrial toxins, oxidative stress caused by excessive excitatory stimulation with subsequent disruption of the electron transport chain and apoptotic events may all predispose the medium spiny projection neurons to excitotoxic events (Beal et al., 1993a; Beal et al., 1993b; Beal et al., 1991; Beal et al., 1986; Beal et al., 1988; Butterworth et al., 1998; Dragunow et al., 1995; Schulz and Beal, 1995; Schulz et al., 1995). Thus it appears that the calbindin positive projection neurons in the SEL succumb to HD just as do the projection neurons in the adjacent caudate nucleus; this finding suggests that the calbindin positive cells in the SEL behave similarly to those calbindin positive cells in the caudate nucleus.
The normal and HD SEL demonstrated virtually no immunoreactivity for enkephalin or substance P (see figure 6.1 C-F and table 6.2). However, in the HD caudate nucleus there was a dramatic reduction in the level of neuropil and fibre staining. This loss demonstrates the reduction in the number of enkephalin containing neurons that project to the globus pallidus (externus) and a reduction in the number of substance P containing neurons that project to the globus pallidus (internus) and substantia nigra. The loss of the medium spiny neurons that contain enkephalin and substance P in HD has been well documented (Emson et al., 1980; Ferrante et al., 1986; Glass et al., 2000; Marshall et al., 1983; Reiner et al., 1988). Reiner et al (1988) demonstrated that there is a pattern of cell degeneration in HD such that early in HD the enkephalinergic projections to the globus pallidus (externus) are preferentially affected whereas later in the disease the substance P containing projections to the globus pallidus (internus) and substantia nigra are affected (Reiner et al., 1988). Furthermore, Glass et al (2000) demonstrated that in HD pathological grade 0, the enkephalinergic projections are lost, in grade 1 the substance P containing striatonigral projections are lost and in grade 3 the substance P containing striatopallidal (internus) projections are lost (Glass et al., 2000). Because the HD cases I have examined are grades 2 and 3, my results demonstrating a massive loss of enkephalin and substance P are in keeping with all previous studies investigating this aspect of HD.

6.4.2 The adult normal and Huntington’s disease subependymal layer contain some interneurons

The normal brain has 4 main types of striatal interneurons: 1, GABAergic parvalbumin positive interneurons; 2, GABAergic calretinin positive interneurons; 3, cholinergic choline-acetyl transferase positive interneurons; and 4, GABAergic neuropeptide Y/somatostatin positive interneurons (Kawaguchi et al., 1995). The striatal interneurons make up about 5% of the neurons in the striatum.

The normal and HD brain SEL had no parvalbumin immunoreactivity in cell bodies, fibres or neuropil (see figure 6.2 A, B and table 6.2). However, the normal brain caudate nucleus had some fibre staining. In the HD caudate nucleus there was a marked reduction
in the numbers of parvalbumin positive interneurons. This result is in keeping with other studies that have demonstrated a reduction in parvalbumin positive interneurons in HD (Cicchetti et al., 2000; Ferrer et al., 1994).

The normal and HD SEL had no calretinin positive cell bodies but did have some calretinin positive fibres present in the deep region of the SEL. The HD SEL contained lower levels of neuropil staining compared with the normal SEL. In the HD caudate nucleus there was a reduction in the amount of fibre and neuropil staining (see figure 6.2 C, D and table 6.2).

The normal SEL had a small amount of ChAT positive cells that were much smaller than those present in the caudate nucleus. In the HD SEL there was a marked reduction in the number of ChAT positive interneurons compared with the normal brains (see figure 6.2 E, F and table 6.2). The size of the ChAT positive cells was much smaller in the SEL compared with the caudate nucleus. The cells in the caudate nucleus in both the normal and HD brains were large and pyramidal in shape.

The normal SEL had only moderate numbers of NPY interneurons. These cells were darkly stained, multipolar neurons that were surrounded by only a small amount of neuropil staining (see figure 6.2G and table 6.2). In the HD brain SEL there were many more NPY positive cells than in the normal SEL (see figure 6.2H and table 6.2). The NPY neuropil staining was more intense and there was more fibre staining and punctate staining present in the HD SEL compared with the normal SEL. These results could be interpreted in two ways. First, dividing cells in the HD SEL may give rise to increasing numbers of NPY positive neurons; or second, because NPY cells are relatively spared in HD, the atrophy in the caudate nucleus increases the number of NPY cells/mm. However, the SEL hypertrophies in HD (see chapter 3) and there are more NPY neurons in the SEL. This suggests that the first explanation is the most likely. In studies performed by Hansel et al (2001) a neuroproliferative role for NPY was demonstrated. In their study, NPY-deficient knock out mice were generated by targeted deletion of the NPY gene; the effect this had on the rapidly dividing olfactory epithelium was examined
using wild-type littermates as controls (Hansel et al., 2001). Both the knock out and wild-type littermates were administered injections of BrdU which demonstrated an approximate 50% decrease in the number of dividing cells in the NPY deficient mice (Hansel et al., 2001). To confirm the results demonstrated with BrdU, Hansel et al (2001) used Ki67 (a cell cycle marker) and again demonstrated a significant reduction in neuronal precursor proliferation with the loss of NPY. To determine whether NPY could bring about the proliferation of cells in the nervous system, Hansel et al (2001) incubated primary olfactory cells with increasing concentrations of NPY and determined that NPY increased the numbers of tubulin positive neurons but the glial cell numbers were unchanged. The rapid rate at which cultured cells proliferated after NPY was applied suggests that the NPY may also promote the basal cells to enter the S-phase of the cell cycle (Hansel et al., 2001). NPY administration did not alter the number of TUNEL stained cells present in the cultures (Hansel et al., 2001). These collective findings together with the results in the present study suggest that NPY cells may be vital for increasing the number of progenitor cells that undergo cell division in order to produce more new cells that may replace degenerating neurons in the caudate nucleus in HD.

6.4.3 The adult normal and Huntington’s disease subependymal layer contain some glial cells

The normal brain contains a number of glial cell types. In this study I have used immunostains to GFAP, which labels fibrillary astrocytes; vimentin, which labels immature astrocytes; and ferritin, which labels microglial cells. The results of these studies will be discussed below.

The normal SEL contained large numbers of GFAP positive cells that were homogenously distributed throughout the SEL beneath the superficial cell sparse region. These cells were small-medium sized, had many processes and were quite close together. The HD SEL had more GFAP positive cells than the normal brain but the density of GFAP positive cells in the HD SEL was similar because the SEL in the HD brain was much thicker than in the normal brain. Both the normal and HD brains had many fibres.
and a lot of punctate staining. Astrocytosis has been well documented in the HD caudate nucleus and some of the increase in GFAP positive cells in the SEL may be a result of astrocytosis. However, there is also accumulating evidence that the glial cells may themselves have the potential to form new neurons or may provide trophic support for new neurons (Doetsch et al., 1999; Doetsch et al., 1997; Sanai et al., 2004). One recent study by Sanai et al (2004) demonstrated that single isolated human astrocytes had the ability to produce neurospheres that contained astrocytes, neurons and oligodendrocytes in vitro. However, it remains unclear how these data relate to in vivo conditions (Sanai et al., 2004).

In the normal vimentin stained SEL and caudate nucleus there was a moderate number of immature glial cells that had stained for vimentin. These cells were predominantly small-medium in size, had multiple processes and were located in the superficial part of the SEL. There were a number of vimentin positive cells in the caudate nucleus that were less intensely stained (see figure 6.3C and table 6.2). The HD SEL had a thicker SEL that had more vimentin positive cells than the normal brain. The band of cells in the caudate nucleus was thicker and more intensely labelled than in the normal brains (see figure 6.3D). The vimentin positive cells were all immature thus they may be a subset of GFAP positive cells.

In the normal brain SEL there was a moderate number of ferritin immunolabelled cells. These cells were small, elongated cells located immediately beneath the EPL. Ferritin stained microglial cells were also demonstrated in the caudate nucleus and these were larger cells that had processes. In the HD brain there were more ferritin positive microglial cells than there were in the normal SEL but the density of cells did not appear to change because the thickness of the HD SEL was increased. The presence of microglial cells and their function in the brain is not yet entirely clear, however, it has been suggested that they deliver trophic factors to sites where axonal or dendritic damage has occurred to help repair the damaged cell. Although the direct evidence for this hypothesis is weak, in experiments performed by Tseng et al (1996) they demonstrated that in central nervous system neurons that have a poor capacity to regenerate, only a
minimal amount of microgliosis was present (Tseng et al., 1996). Also, studies performed by Aarum et al (2003) have demonstrated that embryonic precursor cells migrate toward an increased gradient of microglial-conditioned media. Furthermore, the conditioned media increased the number of differentiated cells in the embryonic cell cultures (Aarum et al., 2003). These results suggest that the increased numbers of microglia in the HD SEL may assist in providing trophic factors for cells in the SEL that may improve the cells ability to differentiate and become a new neuron that helps replace dying neurons in the HD caudate nucleus. In contrast, there is also evidence demonstrating that inflammation, which is mediated by microglia, reduces hippocampal neurogenesis. In one such study Monje et al (2003) induced systemic inflammation by administering bacterial lipopolysacharide and measured the amount of hippocampal neurogenesis by the uptake of BrdU into progenitor cells of the hippocampus. They demonstrated that under inflammatory conditions there was reduced hippocampal neurogenesis (Ekdahl et al., 2003; Monje et al., 2003).

6.4.4 The adult normal and Huntington’s disease subependymal layer contain GABA<sub>A</sub> receptor subunits

The GABA<sub>A</sub> receptor is the most ubiquitous inhibitory receptor in the mammalian brain and has a pentameric structure that gates a chloride ion channel. The GABA<sub>A</sub> receptor is a heteropentameric structure that is made up from a range of 19 known subunits (α<sub>1-6</sub>, β<sub>1-3</sub>, γ<sub>1-3</sub>, δ, ε, π, θ, ρ<sub>1-3</sub>). In the human brain the most common GABA<sub>A</sub> receptor subunits are α1, β2, 3 and γ2; the results of immunostaining the human brain SEL and caudate nucleus will be discussed below.

α<sub>1</sub> subunit

The normal SEL had large numbers of α1 subunit staining. The staining was located on fibres that were oriented in all directions but there was a band of particularly intense α1 labelling (see arrows on figure 6.4A) that was located immediately beneath the cell sparse region of the SEL. In the HD SEL there was a reduction in the number of α1 stained fibres such that the band of staining that was evident at the cell sparse region in the
normal brain was absent. The caudate nucleus had a low level of α1 staining throughout. The general α1 staining appearance was consistent with astrocyte staining in the SEL (see figure 6.4A).

β2, 3 subunits
The normal brain SEL demonstrated no evidence of β2, 3 immunoreactivity. Both the EPL and SEL were completely devoid of immunostaining (see figure 6.4C, D and table 6.2). However, the caudate nucleus had homogenous neuropil and fibre staining throughout.

γ2 subunit
The normal brain SEL was darkly stained and had many small γ2 positive structures that were small and round. The normal caudate nucleus had low levels of neuropil immunoreactivity. In the HD SEL there were many more γ2 positive structures compared with the normal SEL but the neuropil immunoreactivity was less dense. The caudate nucleus was unremarkable from the normal caudate nucleus.

Here I have examined the SEL for the most common GABA_A receptor subunits found in the brain, α1, β2, 3 and γ2 (Backus et al., 1993; Fritschy et al., 1994; Mertens et al., 1993; Mohler et al., 1995). The presence of α1 and γ2 but not β2, 3 subunits suggests that an alternative combination of GABA_A receptor subunits exists in the SEL. It is impossible to deduce from my studies whether the α1 and γ2 GABA_A receptor subunits form a functional receptor; or whether there were other subunits present in the human SEL. However, GABA_A receptor subunits do form functional receptors in the ventricular zone region of the rodent brain during development. GABA_A receptors in the normal brain are vital for inhibitory neurotransmission and have action sites for various neuroactive substance i.e. barbiturates and benzodiazepines. In experiments designed to examine the level of GABA_A receptor subunit expression, Fritschy et al (1994) stained rat brain sections taken from rats at different stages of development from birth (p0) to adulthood (p20) and examined the α1 and β2, 3 subunit expression. Although they did
not specifically examine the SEL/ventricular zone, they did demonstrate a shift in the expression of various subunits in different brain regions during different stages of development (Fritschy et al., 1994). For example, they demonstrated virtually no immunoreactivity for the $\alpha_1$ subunit in the striatum at p0 whereas by p20 there was dense $\alpha_1$ labelling in the rat striatum. These results demonstrate that the GABA$_A$ receptor subunits change as the brain develops. The $\gamma_2$ subunit is partly responsible for desensitisation of the receptor complex to GABA (Saxena and Macdonald, 1994). In the cortical plate, which is derived from the ventricular zone, there was lower expression of $\gamma_2$ perinatally than there was in embryonic stages of development. This finding suggests an abundance of GABA during embryonic development; embryonic brain development predominantly occurs in the ventricular zone in mammals (Laurie et al., 1992; Saxena and Macdonald, 1994). The role of GABA as a trophic factor during development has also been examined. In one study performed by LoTurco et al (1995), the addition of GABA to explant cultures of embryonic cortical progenitor cells led to a decrease in DNA synthesis as measured by reduced tritiated thymidine and BrdU incorporation (LoTurco et al., 1995). However, very early on in embryonic development, before the GABA$_A$ receptors have developed, GABA had no effect on DNA synthesis. The reduction in DNA synthesis in the presence of GABA could be blocked by the action of a GABA$_A$ receptor antagonist (LoTurco et al., 1995). Meier et al (1987) demonstrated the effect of GABA on rodent neuronal development by administering a GABA agonist and then following the expression of neuron-specific neural cell adhesion molecules (NCAM) that indicate the maturity of neurons. Their results demonstrated that the GABA agonist had the ability to accelerate and enhance neuronal development during the early postnatal period (Meier et al., 1987). Taken together, these two studies suggest that GABA inhibits cell division and accelerates neuronal maturation, thus cells are forced out of a cycling state to mature as post-mitotic neurons. Thus my findings of larger amounts of $\gamma_2$ immunoreactivity in the HD SEL suggests that GABA may be very abundant in the SEL and may be encouraging the development of new neurons to be formed as they do in early postnatal periods.
6.5 Summary

The normal brain SEL contains some calbindin positive cells. However, there was a reduction of calbindin positive immunostaining, which demonstrated a loss of GABAergic medium spiny neurons in the HD SEL. There was no substantial immunoreactivity in the normal or HD brain SEL for enkephalin or substance P. With respect to striatal interneuron markers, there was no evidence of parvalbumin positive interneurons. There was calretinin positive SEL fibre staining in both the normal and HD SEL, but there was a reduction in neuropil labelling in the HD SEL. The normal SEL had cell body labelling for ChAT but these cells were not commonly encountered in the HD SEL and yet there was similar amounts of neuropil labelling in the normal and HD brain SEL. One of the most notable features of the SEL was the increase in the number of NPY positive cells in the HD SEL compared with the normal SEL. The increase in NPY positive interneurons may reflect the important role of NPY in promoting neuroproliferation of cells in vivo. In particular, this may be important in HD for the upregulation of progenitor cell proliferation in the SEL that may provide new cells for cell replacement and repair in the degenerating caudate nucleus in HD.

Within the SEL there was also glial cell labelling such that GFAP cell bodies, fibres and neuropil staining were distributed throughout the normal and HD SEL. There were more GFAP positive cell bodies in the HD SEL probably by virtue of the SEL being thicker than in the normal SEL. Furthermore, the normal and HD brain SEL demonstrated immunoreactivity for vimentin, which labels immature astrocytes. The normal and HD brain SEL demonstrated many ferritin positive microglial cells that were distributed throughout the SEL. There were more ferritin positive cells in the HD SEL compared with the HD SEL. However, the increase in ferritin positive cells was proportionally comparable to the increase in size and number of cells in the SEL in HD.

The normal and HD SEL also showed a large amount of neuropil and fibre staining for the α1 subunit of the GABA_A receptor; however, the HD SEL had fewer immunoreactive fibres than the normal SEL. Neither the normal or HD brain SEL displayed any
immunoreactivity for the β2, 3 GABA<sub>A</sub> receptor subunits. By contrast, the γ2 GABA<sub>A</sub> receptor subunit was expressed at high levels in both the normal and HD SEL but there was more γ2 subunit staining in the HD brain. The γ2 subunit may play a role in accelerating the neural development of the immature cells that are present in increased numbers in the HD brain SEL.

6.6 Conclusion

In this chapter I have demonstrated for the first time that, in general, the adult normal brain SEL contained some projection neuron markers and interneuron markers but the distribution and staining patterns in the normal and HD SEL differ considerably compared with the caudate nucleus (see table 6.2). In the HD brain SEL there was a reduction in the level of immunoreactivity for projection neuron and interneuron markers with the exception of NPY. NPY was expressed more densely and on more cells in the HD SEL than in the normal SEL. NPY is a neuroproliferative factor in vivo that pushes cells into the S-phase of cell division and is probably one of the factors that cause increased production of progenitor cells in the HD SEL that may be involved in cell repair or replacement of degenerating cells in the caudate nucleus in HD.

The normal and HD brain SEL also had GFAP, vimentin and ferritin labelling demonstrating the presence of astrocytes and microglial cells in the SEL. However, there was more labelling (see table 6.2) in the HD SEL; this was a proportionally comparable increase to the overall increase in cell numbers in the HD SEL. Glial cells have been suggested to be vital for trophic support of young neurons in the SEL and recent evidence has demonstrated that some types of glial cells are capable of becoming neurons. Because astrocytes have the capacity to form new neurons and because they are found in large numbers in the SEL it is possible that astrocytes have the capacity to form new neurons in increased numbers in response to HD and this may be useful for neuronal replacement and repair in the caudate nucleus in HD.
Finally, the α1 subunit of the GABA_A receptor was present in both the normal and HD brain but the normal brain SEL was more densely labelled. There was no β2, 3 labelling demonstrated in the HD SEL. However, the γ2 subunit of the GABA_A receptor, which is involved with desensitisation of the receptor complex to GABA in the developing SEL/ventricular zone, was present in both the normal and HD SEL but there was more labelling in the HD SEL. In the rat brain the matured progeny of the SEL/ventricular zone express only low levels of the γ2 subunit compared with the very high levels in the SEL/ventricular zone during development. This indicates that in my studies the SEL is quite similar to the active ventricular zone during development. The neurotransmitter GABA is also an important trophic factor for the maturation of neurons during development.

Taken together, my data demonstrate that the SEL does appear to contain the same type of mature cells in HD that are susceptible to degeneration just as in the caudate nucleus. My results also suggest that the SEL in HD has many of the characteristics of the developing ventricular zone such that it may be capable of providing trophic support to immature cells destined to become neurons and glial cells. It is likely that this is brought about by the presence of NPY neurons, astrocytes, microglial cells and possibly the action of GABA itself. The SEL is therefore capable of producing progenitor cells that may migrate and differentiate into cells for cell replacement in the degenerating caudate nucleus in HD.
Chapter 7

GENERAL DISCUSSION

7.1 Introduction

This thesis is the first detailed study of the subependymal layer (SEL) that overlies the caudate nucleus in the human brain. In this thesis the SEL of both the normal and Huntington’s disease (HD) brains have been examined in detail. HD is a neurodegenerative, autosomal dominant disorder that has an expanded ‘CAG’ trinucleotide repeat sequence in exon 1 of the IT15 gene. Pathologically, HD predominantly affects the basal ganglia and, in particular, the projection neurons of the caudate nucleus and putamen. The adult SEL directly overlies the caudate nucleus in the wall of the lateral ventricle. It is particularly active during development where it is the origin of most of the cortical and striatal neurons. It has been a long held dogma that the SEL becomes inactive in adulthood and thus no new neurons are produced after development. In this thesis I have examined the distribution of progenitor cells and mature cell types in the normal and HD SEL. Particular attention has been directed towards investigating the capacity of the adult human SEL to produce new progenitor cells in the normal and HD SEL that may form new neurons and glial cells for cell replacement in the diseased caudate nucleus and putamen.

Overall, the results of this thesis provide evidence for the ongoing production of new neurons and glial cells in the normal brain, and, most importantly demonstrate that in the HD brain there is an increase in the number of new neurons formed. There is variation in the number of progenitor cells present in different regions and areas of the SEL overlying the caudate nucleus (in particular the central and ventral regions) that may be able to be manipulated to enhance the cell replacement of degenerating cells in the caudate nucleus.
7.2 Summary of major results

The results in this thesis are presented in chapters 3-6. The general anatomical organisation and the cell types present, based on histochemical staining, in the normal and HD SEL is discussed in chapter 3. Chapter 4 demonstrates increased cell proliferation in the HD brain (in response to cell death in the caudate nucleus and putamen) compared with the normal brain and also demonstrates the co-localisation of neuronal and glial markers with the proliferative marker PCNA. In chapter 5 the distribution of progenitor cells in the SEL overlying the caudate nucleus is investigated; and, in chapter 6 the mature cell types present in the normal and HD SEL are demonstrated. The principal findings in each of these studies are detailed below.

The results from chapter 3 demonstrate that the human SEL is a heterogeneous region mostly comprised of type A, B and C cells. This is in good agreement with the results from studies of the mouse SEL performed by Doetsch et al (1997). Also, my results demonstrate for the first time, that there is a compartmental organisation of the human SEL. Beneath the EPL there is a small area predominantly devoid of cell body staining. The type A cells are present in a layer beneath the cell-devoid area; based on morphology these cells appear to be migrating neuroblasts. Type B cells are evenly spread throughout the SEL, but their location is not confined to any particular part of the SEL. The type C cells are located in the lower part of the SEL, above the myelin layer superficial to the caudate nucleus. Beneath the type C cells a layer of myelin separates the SEL from the caudate nucleus. In HD, the SEL was on average 2.8 times thicker than that of normals. Also, there were 2.8 times more cells in the SEL of the HD brain compared with the normal brain. The increase in cell numbers was a result of a significant increase in the number of type B cells in HD compared with normal SEL; however there was also an increase in the number of type A and C cells in the HD SEL. As the HD grade increased so did the thickness of the SEL and number of cells in the SEL; also, as the grade of HD increased so did the ratio of type B cells to type A and C cells.
In chapter 4, the proliferative capacity of the human SEL has been studied with the use of the proliferative cell marker PCNA. In particular, this study has demonstrated that there is a significant increase in the number of proliferating progenitor cells in the HD SEL compared with the normal brain SEL. Furthermore, this study has shown that as the pathological grade of HD increases so does the number of PCNA positive cells in the HD SEL; that is, there is a very strong correlation between the pathological grade in HD and progenitor cell proliferation in the HD SEL. Also, with an increase in the number of CAG trinucleotide repeats in the IT15 gene, there is also an increase in the number of PCNA positive cells in the HD SEL. In order to determine the fate of progenitor cells in the SEL triple-immunofluorescence laser scanning confocal microscopy was undertaken on sections containing the SEL that were fluorescently labelled for PCNA and either the immature neuronal marker βIII-tubulin or the glial marker GFAP; these studies were directed at determining whether proliferating SEL progenitor cells become neurons or glial cells. My results demonstrated that βIII-tubulin co-localised with PCNA positive cells indicating that neurogenesis had occurred in the HD SEL. Also, GFAP was co-localised with some PCNA positive cells, demonstrating that gliogenesis had occurred in the HD SEL. This finding demonstrates that neurogenesis occurs in the human SEL in normal and HD brains. This chapter has demonstrated that the newly formed neurons were generally located in the deeper part of the SEL immediately adjacent to the caudate nucleus, whereas the newly produced glial cells were almost exclusively located in the superficial part of the SEL close to the EPL. These results may indicate that replacement neurons are migrating toward the damaged caudate nucleus. In this study, the number of PCNA/βIII-tubulin double-labelled cells were counted in the normal SEL and compared with the HD SEL using fluorescently stained sections imaged with the laser scanning confocal microscope. The results demonstrated that there was on average a 2.6 fold increase in the number of newly formed neurons in the HD SEL compared with the normal brain SEL. This represents a significant increase in the number of new neurons in the HD SEL.

The findings in this chapter demonstrate a high level of plasticity in the HD SEL to respond to degeneration of the caudate nucleus in HD by producing increased numbers of
progenitor cells that become neurons and glial cells. It is clear that the amount of neurogenesis that occurs in the SEL in HD is insufficient to replace all the degenerating cells in the caudate nucleus and to alleviate the symptoms of HD. Since previous studies have shown that growth factors and pharmacological agents can increase the rate of neurogenesis in vivo, this endogenous increase in neurogenesis and the replacement of neurons in the HD affected areas could be augmented by the use of pharmacological agents. If this is possible, then the findings in this thesis may be of major importance for the development of novel therapeutic strategies for the treatment of neurodegenerative diseases.

The experiments performed in chapter 5 demonstrate the distribution of proliferating cells throughout the SEL overlying the caudate nucleus. In this study a very detailed regional analysis of the number of PCNA positive cells was performed in the different parts of the SEL. The results have shown that there are a similar number of PCNA positive cells in the normal brain throughout the SEL overlying the caudate nucleus except in the ventral area where there was approximately twice as many proliferating cells present compared with the middle and dorsal areas. In the HD brains there was, on average, 2.8 times more PCNA positive cells in the SEL compared with the normal brain SEL. In particular, in the HD brain SEL there was a gradient of PCNA positive cell numbers from the lowest in the dorsal area to the highest in the ventral area. The ventral area had the greatest number of PCNA positive cells of any area examined. In the rostral region, there was no significant difference between the number of PCNA positive cells in the normal and HD brain SEL, whereas in the central and caudal regions there was as much as a 3.6 fold increase in the number of PCNA positive cells in the HD brains compared with normal brains. The areas within regions (locations) demonstrated significant increases in PCNA positive cell numbers in the HD SEL compared with the normal SEL except in the rostral region. However, even in the rostral region there was a trend of more PCNA positive cells present in the HD SEL.

These results demonstrate the plasticity of the SEL in that it can respond to the extensive degeneration of the caudate nucleus by producing new cells throughout the SEL that
overlies the caudate nucleus. Because the proliferation of PCNA positive cells occurs in all locations of the SEL, these results highlight the potential use of these progenitor cells in the development of a cell replacement and cell repair therapy that targets the various areas and regions of the degenerating caudate nucleus in HD.

In the 6th and final results chapter, the mature cell types present in the normal and HD SEL were investigated. The normal brain SEL contains some calbindin positive cells. However, there was a reduction of calbindin positive immunostaining, which demonstrated a loss of GABAergic medium spiny neurons in the HD SEL. There was no substantial immunoreactivity in the normal or HD brain SEL for enkephalin or substance P. With respect to striatal interneuron markers, there was no evidence of parvalbumin positive interneurons. There was calretinin positive SEL fibre staining in both the normal and HD SEL, but there was a reduction in neuropil labelling in the HD SEL. The normal SEL had cell body labelling for ChAT but these cells were not commonly encountered in the HD SEL and yet there were approximately similar amounts of neuropil labelling in the normal and HD brain SEL. One of the most notable features of the SEL was the increase in the number of NPY positive cells in the HD SEL compared with the normal SEL. The increase in NPY positive interneurons may reflect the important role of NPY in promoting neuroproliferation of cells in vivo. In particular, this may be important in HD for the upregulation of progenitor cell proliferation in the SEL that may provide new cells for cell replacement and repair in the degenerating caudate nucleus in HD.

Within the SEL there was also glial cell labelling such that GFAP cell bodies, fibres and neuropil staining were distributed throughout the normal and HD SEL. There were more GFAP positive cell bodies in the HD SEL resulting in a thicker SEL than in the normal brain. Furthermore, the normal and HD brain SEL demonstrated immunoreactivity for vimentin, which labels immature astrocytes. The normal and HD brain SEL demonstrated many ferritin positive microglial cells that were distributed throughout the SEL. There were more ferritin positive cells in the HD SEL compared with the HD SEL. However, the increase in ferritin positive cells was proportionally comparable to the increase in size and number of cells in the SEL in HD.
The normal and HD SEL also showed a large amount of neuropil and fibre staining for the α1 subunit of the GABA_α receptor; however, the HD SEL had fewer immunoreactive fibres than the normal SEL. Neither the normal or HD brain SEL displayed any immunoreactivity for the β2, 3 GABA_α receptor subunits. By contrast, the γ2 GABA_α receptor subunit was expressed at high levels in both the normal and HD SEL but there was more γ2 subunit staining in the HD brain SEL. The γ2 subunit may play a role in accelerating the neuronal development of the immature cells that are present in increased numbers in the HD brain SEL.

7.3 General Discussion

Worldwide attention is presently focused on the potential use of exogenous progenitor cells as a source of cells for transplantation therapy in neurodegenerative diseases such as Huntington’s and Parkinson’s disease. Because controversy has surrounded the use of human foetal and embryonic tissue for the purposes of experimentation and transplantation, new sources of cells for transplantation have been sought. An exciting development in the field of cell replacement therapy is the recent demonstration of the presence of endogenous progenitor cells in the adult human brain in both the hippocampus and the subependymal layer overlying the caudate nucleus (Eriksson et al., 1998). These observations indicate that, contrary to the well-rehearsed dogma, the adult human brain contains progenitor cells that are capable of producing new neurons. This raises the possibility that endogenous neural progenitor cells may provide an alternative strategy for cell replacement therapy through the enhancement of endogenous neural replacement mechanisms to produce new neurons that replace the cells that die in neurodegenerative diseases.

The findings in this thesis have demonstrated that in Huntington’s disease (HD) there are significantly more dividing cells in the SEL compared with the normal SEL. In this study I have shown that there is a 2.7 fold increase in the number of proliferating progenitor cells in the HD SEL compared with the normal SEL and that about 3.39% of the cells that
have recently divided have a neuronal phenotype. There is an approximate 2.6 fold increase in the number of new neurons in the HD SEL compared with the normal SEL. Approximately 50% of the dividing SEL cells become glial cells in the HD SEL. In this case, the trigger for the increased cell production and neurogenesis in the Huntington’s disease brain appears to be the death of neurons in the striatum because as cell death advances (increased HD pathological grade) in HD more dividing cells are present in the subependymal layer of the diseased brain. However, in HD it would appear that the increase in cell proliferation and neurogenesis is too little too late. If the proliferation, migration and differentiation of endogenous progenitor cells could be pharmacologically enhanced then progenitor cells may more effectively be able to replace the cells that die in neurodegenerative diseases.

Neural progenitor cells have been shown to respond to a variety of mitogenic growth/trophic factors and cytokines in vivo such as epidermal growth factor (EGF), fibroblast growth factor (FGF-2), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), transforming growth factor α (TGFα), erythropoietin and others by facilitating the proliferation, migration and differentiation of progenitor cells in the adult central nervous system (Arsenijevic and Weiss, 1998; Arsenijevic et al., 2001; Benraiss et al., 2001; Chmielnicki et al., 2004; Ciccolini, 2001; Craig et al., 1996; Fiore et al., 2003; Kuhn et al., 1997; Pencea et al., 2001; Reynolds et al., 1992; Reynolds and Weiss, 1996; Tirassa et al., 2003; Yoshimura et al., 2001; Zigova et al., 1998b). In one such in vivo study the growth factor EGF was infused into the lateral ventricle of adult mice; the results demonstrated a dramatic increase in the number of proliferating cells in the subependymal layer of the lateral ventricle (Craig et al., 1996). Immunohistochemical analysis revealed that more than 95% of the cells present in the subependymal layer were immunopositive for the EGF receptor and were also nestin positive, a well-established marker of proliferating neural progenitor cells (Craig et al., 1996). Also, in these experiments EGF induced the migration of progenitor cells into the parenchyma away from the wall of the lateral ventricle. More recent studies have demonstrated the effect of intraventricular infusion of the growth factors EGF and FGF-2. In one such study Kuhn and colleagues (1997) demonstrated a
strong mitotic effect of FGF-2 on progenitor cells in the subventricular zone throughout the period in which FGF-2 was infused; however, during this period migration of progenitor cells was diminished (Kuhn et al., 1997). Shortly after FGF-2 administration ceased the progenitor cells in the subventricular zone appeared to undergo rapid migration. Still other studies have demonstrated that the neurotrophic factor BDNF plays an important role in differentiating progenitor cells into mature neurons in both neurogenic and non-neurogenic regions of the adult brain and has neuroprotective properties (Benraiss et al., 2001; Chmielnicki et al., 2004; Pencea et al., 2001; Takahashi et al., 1998; Zigova et al., 1998b). Fallon et al. (2000) demonstrated induction of proliferation, migration and differentiation in vivo, in the rat forebrain after the substantia nigra had been lesioned with 6-hydroxy-dopamine and TGFα had been infused into the striatum (Fallon et al., 2000). Cytokines, such as erythropoietin, also enhance the proliferation and differentiation of endogenous neural progenitors (Shingo et al., 2001; Yu et al., 2002). Erythropoietin, a member of the haematopoietic cytokine superfamily, not only serves to enhance proliferation and differentiation of neural progenitor cells but also acts as an anti-apoptotic factor in the embryonic brain (Shingo et al., 2001; Yu et al., 2002).

Apart from endogenous stimulants of progenitor cells, pharmacological compounds such as lithium and fluoxetine (both commonly prescribed antidepressant drugs for recurrent mood disorders) have been shown to increase the number of proliferating cells in the brain. A study performed by Chen and colleagues (2000), in which mice were chronically administered lithium and the cell cycle marker BrdU, demonstrated a 25% increase in the number of proliferating cells in the dentate gyrus of the hippocampus compared to controls (Chen et al., 2000). Lithium has been shown to increase the levels of B-cell lymphoma protein-2 (bcl-2) that exerts a major anti-apoptotic neuroprotective effect in vitro and in vivo and may also exert a trophic effect as well (Chen et al., 2000; Malberg and Duman, 2003; Manev et al., 2001; Manji et al., 1999; Manji et al., 2000a; Manji et al., 2000b; Nonaka et al., 1998; Senatorov et al., 2004; Willing et al., 2002). Although not a lot is known about the intracellular signaling involved in adult neurogenesis it has been demonstrated that long term treatment with antidepressants leads to an up regulation of
BDNF as a result of activation of a cAMP-cAMP response element binding protein (CREB) cascade (Duman et al., 2001; Malberg et al., 2000).

The use of in vivo gene transfer techniques may provide an alternative technique for the delivery of mitogenic factors to progenitor cells in the adult brain. Intraventricular delivery of a viral vector (see figure 7.1), such as adenovirus, adeno-associated virus, herpes-simplex virus or retrovirus, encoding a mitogenic factor such as FGF-2 or BDNF would allow the ability to directly target long-term, sustained expression of exogenous mitogenic factors to neurogenic regions and neural progenitor cells in the adult brain in the absence of effects on non-targeted cells and effects in the periphery. Furthermore, the use of gene transfer techniques would provide the ability to regulate the expression of exogenous mitogenic factors in vivo through the use of regulator systems such as the tetracycline regulated system. Supporting this proposal, recent studies have demonstrated that delivery of mitogenic factors such as BDNF (adenoviral- BDNF (Benraiss et al., 2001)) or FGF-2 (adenoviral-FGF-2 (Matsuoka et al., 2003), herpes simplex virus-FGF-2 (Vicario and Schimmang, 2003)) via gene transfer techniques can evoke significant proliferation of progenitor cells as well as substantially augment the recruitment of new neurons into both neurogenic and non-neurogenic sites in the adult rat brain. It may be that the best regime for enhancing the proliferation, migration and differentiation of progenitor cells is with a combination of growth factors, mitogenic agents and gene therapy techniques such as have been used in gene therapy experiments. In one such study adenoviral BDNF and noggin over expression brought about the suppression of glial differentiation and potentiated the recruitment of DARP-32 neurons to the striatum in the adult rat brain (Chmielnicki et al., 2004). With the discovery of endogenous mitogenic factors and pharmacological agents that increase the proliferation, migration and differentiation of progenitor cells there is potential to develop novel therapeutic strategies that increase the brain’s ability to replace the cells that are dying in neurodegenerative diseases.

This thesis has also demonstrated the detailed anatomy of the SEL and has shown the
Figure 7.1

Possible method of targeting the subependymal layer for the delivery of substances that facilitate cell proliferation and differentiation

Diagrammatical representation of a coronal section through the striatum and lateral ventricle of the adult human brain with a delivery needle located in the lateral ventricle. The square that is enlarged to the right demonstrates a possible method of delivering growth factors or other cytokines so they can readily diffuse into the subependymal layer (SEL) to facilitate the proliferation and differentiation of progenitor cells (blue). In Huntington’s disease the principal brain region affected is the striatum; therefore the progenitor cells do not have to migrate long distances to replace the dying cells.
Lateral Ventricle

Cytokines
Viral vectors for gene transfer
Exogenous components
Growth Factors

SEL
Striatum
changes that occur in the HD SEL. Of note is the very close proximity of the PCNA/BIII-tubulin double-labelled neurons to the caudate nucleus. In particular, the SEL that has increased cell numbers in HD is very close to the degenerating caudate nucleus (see figure 7.2). The newly produced cells in the SEL have only a short distance to migrate in order to replace the dying cells in the HD caudate nucleus (see figure 7.2, the arrows indicate the possible direction of migration). Furthermore, the SEL contains glial cells that are important for the development and migration of neurons during striato- and cortico-genesis; most importantly, recent evidence suggests that glial cells may be capable of developing a neuronal phenotype and may be able to replace degenerating cells in the caudate nucleus (Doetsch et al., 1999; Sanai et al., 2004). Animal studies performed by Tattersfield et al (2004) have recently demonstrated that in rats that received a striatal quinolinic acid (QA) injection (100 nM QA), to experimentally induce a Huntington's disease-like pathology, there was an increase in the number of BrdU positive cells in the SEL that reached a maximum at 7 days after the striatal lesion; they also observed migration of neuroblasts into the lesioned striatum that contained BrdU and also expressed neuronal markers (Tattersfield et al., 2004). There were also BrdU positive cells that expressed mature neuronal markers in the striatum that were located within the area that underwent cell loss. Similar results were also demonstrated by Arvidsson et al (2002) after rats received unilateral middle cerebral artery occlusion (MCAO) induced strokes. In their study, new neurons that had migrated from the SEL to the area that was affected by the MCAO contained the proteins that are specifically found on striatal projection neurons indicating cellular proliferation had occurred in the SEL followed by migration and differentiation into regionally specific cell types (Arvidsson et al., 2002). In one very recent human study performed by Jin et al (2004), western blotting and immunohistochemistry were used to demonstrate increased expression of immature neuronal marker proteins including doublecortin, polysialylated neural cell adhesion molecules, NeuroD and TUC-4 in the hippocampus of 14 Alzheimer's brains. These results demonstrated increased proliferation and differentiation of progenitor cells in the hippocampus in response to degeneration and neuropathology in the Alzheimer's hippocampus (Jin et al., 2004).
Figure 7.2

Diagram of the anatomy of the subependymal region in the Huntington’s disease brain showing the possible path of migration for replacement cells moving from the subependymal layer to the caudate nucleus

This diagram demonstrates the general anatomical structures of the ependymal layer (EPL), subependymal layer (SEL) and caudate nucleus (CN) in the HD brain. The ependymal cells separate the lateral ventricle (LV) from the SEL. The SEL consists of βIII-tubulin positive neurons and GFAP positive glial cells. Some of the neurons and glial cells were also PCNA positive indicating that they had recently divided. Within the SEL there were many PCNA positive cells that did not stain for neuronal or glial markers, which suggests that they are undifferentiated progenitor cells. A thin layer of myelin separates the SEL from the caudate nucleus. The distance between the SEL and the caudate nucleus is small and thus it seems very likely that newly produced neurons could migrate from the SEL into the caudate nucleus which may be useful for replacing degenerating neurons in the caudate nucleus in the HD brain. The arrows indicate the close proximity of the SEL to the caudate nucleus and the pathway that new neurons may take to migrate and replace degenerating neurons in the caudate nucleus.
Neurodegenerative diseases usually progress over at least a 10-15 year period; therefore, if progenitor cells could be 'encouraged' to divide, migrate and differentiate very early on in the disease process then the rate of degeneration may be slowed, halted or indeed reversed. The production of new neurons in the diseased brain is likely to be based on the demand for replacement cells suggesting there would always be a delay in the production of new cells. Therefore encouraging progenitor cells to migrate and differentiate into the area undergoing degeneration prior to extensive cell loss would be paramount to developing a successful therapy. Migration of neural progenitor cells into the region of the brain affected by neurodegeneration very early on in the disease process would allow time for the new cells to integrate into the established circuitry.

The very close proximity of the lateral ventricle to the subependymal layer means that injection into the lateral ventricle would provide a direct access route for the delivery of mitogenic factors to progenitor cells in the subependymal layer. Indeed, in vivo experiments have demonstrated the effectiveness of intraventricular injections of growth factors (such as FGF-2, EGF and BDNF) when targeting subependymal cells (Craig et al., 1996; Martens et al., 2002; Pencea et al., 2001). In the human brain the ventricular system can be accessed with a fine needle using stereotaxic neurosurgical techniques in order to deliver a bolus injection or a continuous infusion via an indwelling catheter with negligible side effects and a high level of accuracy (see figure 7.1). Thus, in a proposed endogenous progenitor cell enhancement therapy the lateral ventricle infusion may be a very suitable and convenient delivery method (see figure 7.1).

In neurodegenerative diseases such as HD the subependymal layer progenitor cells, which also contain the HD gene, are also potentially at risk of gene-induced degeneration. In HD, aggregation of the huntingtin gene product (a protein called huntingtin) and the translocation of the N-terminal protein fragment to the nucleus in mature cells is a probable major cause of the cell dysfunction and death (Martin-Aparicio et al., 2002; Martin-Aparicio et al., 2001; Ross, 1997). However, evidence suggests that dividing and recently divided cells are less likely to be affected by the toxicity of aggregated huntingtin protein fragments, thus giving a time advantage to recently formed
neurons irrespective of the presence of the huntingtin gene in these cells (Martin-Aparicio et al., 2002; Yoshizawa et al., 2000).

Recent studies performed to determine the time course and pattern of neurodegeneration in HD have demonstrated that the rate at which subpopulations of striatal neurons die is not uniform (Glass et al., 2000). In HD the neurons that degenerate appear to function normally until around 35 years of age (the age of onset varies depending on the number of CAG repeats in the huntingtin gene (Snell et al., 1993)); therefore, because progenitor cells produce new replacement neurons in the SEL it could be speculated that these new neurons would not undergo degeneration for some time. Furthermore, within the striatum the medium-spiny neurons that project to the external segment of the globus pallidus are affected in the early stages of the disease although the medium-spiny neurons that project to the internal segment of the globus pallidus are affected much later in the disease (Cicchetti et al., 1996; Cicchetti et al., 2000; Glass et al., 2000; Reiner et al., 1988). These observations indicate that newly formed neurons may function normally for a considerable length of time.

In conclusion, the results of my studies indicate that the adult human brain has the potential to repair itself after injury or disease. Combined with the knowledge that various mitogenic growth factors and pharmacological agents can enhance the proliferation and differentiation of endogenous progenitor cells; this suggests that novel therapeutic strategies could be developed to enhance the natural repair mechanisms of the human brain in response to brain injury and disease.
Chapter 8

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