Isolation and Characterization of Stem Cells from the Adult Human Central Nervous System and Brain Tumours

By

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If the human brain were so simple that we could understand it, we would be so simple that we couldn't.

Emerson M. Pugh

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LIST OF ARTICLES

PAPER I

Development of neuronal networks from single stem cells harvested from the adult human brain. Moe MC, Westerlund U, Varghese M, Berg-Johnsen J, Svensson M, Langmoen IA. ***Neurosurgery***. *2005 56:1182-8.*

PAPER II

Multipotent progenitor cells from the adult human brain: neurophysiological differentiation to mature neurons. Moe MC*, Varghese M*, Danilov AI, Westerlund U, Ramm-Pettersen J, Brundin L, Svensson M, Berg-Johnsen J and Langmoen IA. ***Brain***. *2005 128:2189-99.* * Co-first authors

PAPER III

Neural Progenitors from Adult Filum Terminale are plastic and can develop into functional neurons. Varghese M, Olstorn H, Berg-Johnsen J, Moe MC, Murrell W and Langmoen IA. ***Stem Cells and Development*** 2008 *In press* (electronic preprint available at Pub Med).

PAPER IV

A Comparison between Stem Cells from the Adult Human Brain and Brain Tumours. Varghese M, Olstorn H, Sandberg C, Vik-Mo EO, Noordhuis P, Nistér M, Berg-Johnsen J, Moe MC and Langmoen IA. ***Neurosurgery*** 2008 *In press*.

SELECTED ABBREVIATIONS

In order of appearance

CNS: Central nervous system	AC II: low grade astrocytoma
AHNSCs: Adult human neural stem cells	GBM: Glioblastoma Multiforme
TSCs: Tumour stem cells	VEGF: Vascular derived endothelial growth factor
SVZ: Subventricular zone	EGFR: Epidermal growth factor receptor
SGZ: Subgranular zone	PDGFR: Platelet derived growth factor Receptor
ECM: Extra cellular matrix	ABCG: ATP-binding cassette transporter protein, subfamily G
GFAP: Glial fibrillary acidic protein	Sox2: Sex determining region Y-box 2
PSA–NCAM: Polysialylated neural cell adhesion molecule	MAP-2: Microtubule-associated protein 2
EGF: Epidermal growth factor	DCX: Doublecortin
bFGF: Basic fibroblast growth factor	O4: Anti-oligodendrocyte marker
TGFα: Transforming growth factor-alpha	CNPase: 2`, 3`-cyclic nucleotide 3-phosphodiesterase
Shh: Sonic hedgehog	qPCR: Quantitative polymerase chain reaction
LIF: Leukemia inhibitory factor	FACS: Fluorescence-activated cell sorting
Wnt: Wingless	SCID: Severe immunocompromised immunodeficient
BDNF: Brain-derived neurotrophic factor	FTNPs: Filum terminale neural progenitors
DNA: Deoxyribonucleic acid	iPS: Induced pluripotent stem cells
eGFP: Enhanced Green fluorescent protein	

INTRODUCTION

Stem Cells

Stem cells were first isolated from embryos in the early 80s (1, 2). A decade later, they were isolated from the adult central nervous system (CNS) of mice (3) and humans (4-8). Based on origin, stem cells can be classified as embryonic, foetal or adult (Fig. 1). Stem cells can also be classified based on their ability to generate daughter cells i.e. *pluripotent* (cells that can give rise to all the cell types of the human body e.g. embryonic stem cells) (9, 10), or *multipotent cells* (cells with a more restricted potential, giving rise to progeny characteristic of a particular organ system e.g. haematopoietic stem cells) (11-13).

Though the hallmarks of stem cells are contentious, the prevailing view is that *bona fide* stem cells are capable of (**1**) self-renewal, with an unlimited ability to produce progeny indistinguishable from themselves (14), (**2**) proliferation and (**3**) multipotent differentiation, dividing asymmetrically to generate all the cell types of the tissue from which they are derived (15, 16). By applying the aforementioned criteria to cells harvested from the adult CNS, stem cells have been prospectively isolated from the striatum (3), lateral wall of the lateral ventricles and the spinal cord of rodents (17). In adult humans, stem cells have been isolated from the ventricular wall and hippocampal zone (4, 7, 8, 18), and are called adult human neural stem cells (AHNSCs). Similar neural stem cells have been isolated from other mammalian CNS regions including cortex (5, 19), substantia nigra (20), white matter (6) and spinal cord (21, 22).

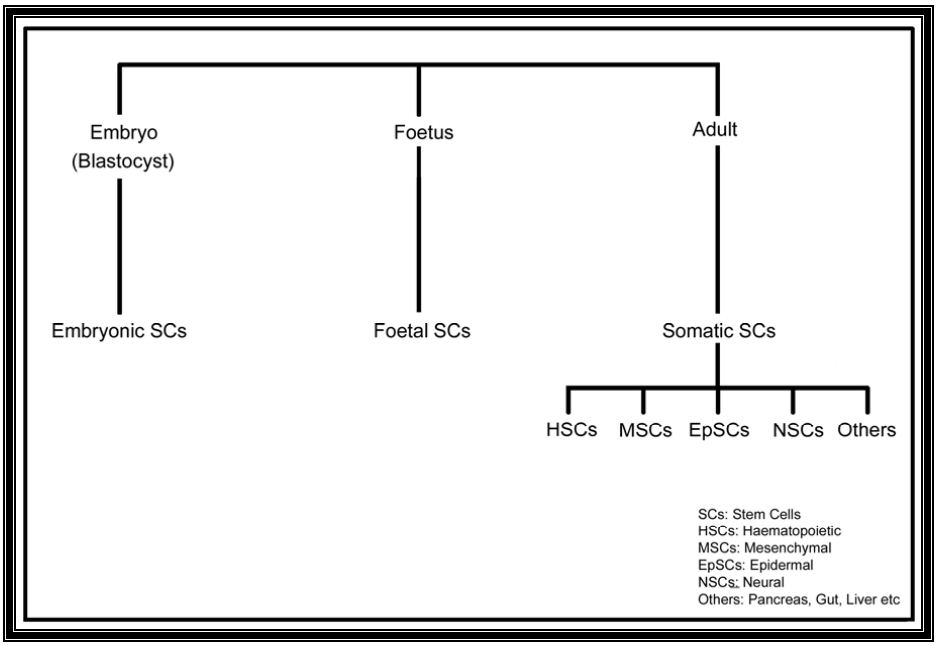

Figure 1. Classification of Stem Cells

Recently, stem cells have also been isolated from solid tumours, including brain tumours (23-25). These cells are called tumour stem cells (TSCs) and are similar to AHNSCs. The operational definition of a TSC is the ability to self-renew, proliferate and divide asymmetrically giving rise to progeny with the varied morphological features, cell specific antigens and functional properties characteristic for the tumour (23, 25-28). Implicit in this definition, TSCs can recapitulate the parent tumour upon transplantation (27, 29, 30).

The emerging hypothesis is that TSCs from brain tumours may be derived from neural stem cells in the subventricular zone (SVZ) (29, 31). This intimate association between the two populations underscores the importance of studying the two populations.

Identity of the Adult Mammalian Neural Stem Cell

The prevailing view regarding the identity of the adult mammalian neural stem cell is that either (**1**) astrocytes, including radial glia (32, 33) or (**2**) multi-ciliated ependymal cells (17, 34) are the putative resident neural stem cells. Presently, there are two centres in the adult human CNS that generate new neurons; the subgranular zone (SGZ) of the dentate gyrus of the adult hippocampus and the SVZ (35, 36). In the SVZ, there are three different cell types; the migrating neuroblasts (type A cells), the slowly proliferating SVZ astrocytes (type B cells) and the clusters of rapidly dividing immature precursors (type C cells) (32). Based on elegant experiments using pulse labelling with ^{3}H-thymidine it was shown that type B cells are the resident neural stem cells (37); Type B cells incorporated ^{3}H-thymidine (labels proliferating cells) and gave rise to both type C and A cells (Fig. 2). Type B cells are identified as SVZ astrocytes (38) based on their antigenic marker expression and ultrastructural characteristics, namely bundles of intermediate filaments, gap junctions and multiple processes (32, 39).

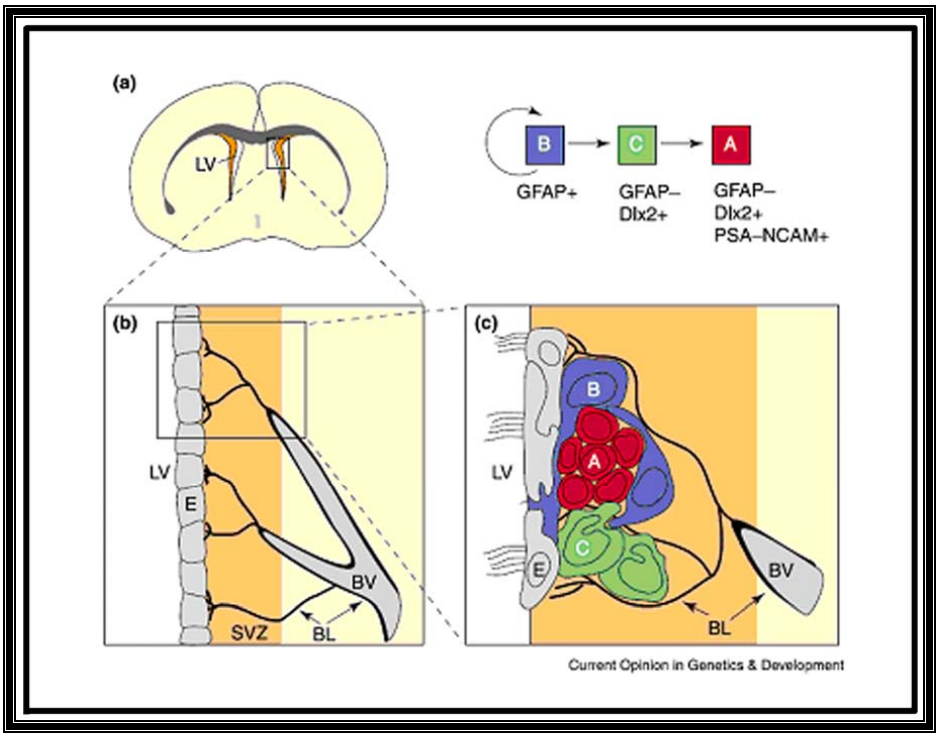

Figure 2. The SVZ niche, cell types and stem cell lineage. (a) Frontal schematic of the adult mouse brain showing the location of the SVZ in orange between the lateral ventricle (LV) and the striatum. The corpus callosum is depicted in dark grey. The box in (a) is expanded in (b) and it shows blood vessels (BV) are a likely source of signals for adult neurogenesis. A specialized basal lamina (BL) extends from blood vessels into the SVZ and terminates adjacent to the ependymal cells (E). (c) Crosssectional schematic showing the cell types and their organization in the SVZ. Multi-ciliated ependymal cells (E, grey) line the lateral ventricle; chains of neuroblasts (A, red), SVZ astrocytes (B, blue) and rapidly dividing Type C cells (C, green) are shown. An ECM-rich basal lamina (BL, black) makes extensive contact with all SVZ cell types and forms an essential part of the SVZ stem cell niche. SVZ astrocytes ($GFAP^+$) act as stem cells in this region and divide to generate transit-amplifying Type C cells ($GFAP–/Dlx2^+$), which in turn divide to generate the neuroblasts ($GFAP–Dlx2^+PSA–NCAM^+$) that migrate to the olfactory bulb. (Reproduced after permission from Doetsch *et al.,* (40))

Unlike the SVZ and SGZ, the spinal cord is not known to be a site of *de novo* neuron generation. Yet, a quiescent population of cells, fulfilling stem cell hallmarks, has been isolated from the spinal cord and cultured as neurospheres *in vitro* (22, 41, 42). The presumed location of the stem cells is around the central canal (33, 41, 43), where mitotic activity has been shown in the ependymal cells (44). Indeed, it has already been demonstrated that ependymal cells may represent putative neural stem cells (17, 45). Interestingly, the ependyma-lined central canal continues into the tail end of the spinal cord also known as filum terminale (46-50). The filum terminale also shows strong reactivity for neural cell adhesion molecule (N-CAM) (51), a neural progenitor cell marker. Given this, the filum terminale may represent a hitherto unknown source of neural stem cells.

The Stem Cell Niche and it's Factors

The stem cell niche is the microenvironment consisting of cells, extracellular substrates and vasculature that regulates stem cell maintenance, repair and tissue participation, i.e. the function of the niche is to regulate self-renewal and fate of the stem cells (52) (Fig. 2). The central element in the niche is the capillaries, and stem cells are placed in close proximity to it (Fig. 2). The niche also safeguards the stem cells from excessive cell production which may lead to cancer (12). The molecular components characterizing the neural stem cell niche include epidermal growth factor (EGF) and sonic hedgehog (Shh). Indeed, in the SVZ infusion of EGF or bFGF increases cell proliferation (53), whereas knocking out transforming growth factor-alpha (TGF α), a ligand for the EGF receptor, leads to a significant decrease in cell proliferation (54). This suggests that members of the

FGF and EGF growth factor families may play an important role in the maintenance of adult neural stem cells (55). Recently, Shh was shown to promote proliferation of adult neural stem cells both *in vitro* and *in vivo* (56-59). Several other signalling pathways are also involved in proliferation and differentiation, including LIF (60-62), Wnt (63, 64), Notch (65-67) and BDNF (68, 69). Understanding the factors that regulate neural stem cells and their niche will help lead to insights into brain repair and maybe even the origin of pathological processes e.g. cancer.

Neurogenesis

Neurogenesis is defined as the ability to produce new neurons from stem cells. Evidence for this is unambiguous for non-mammalian vertebrates; examples include, (**1**) regeneration of the medial cerebral cortex (which resembles the mammalian hippocampal dentate gyrus) in lizards in response to injury (70) and (**2**) regeneration of newts' tails, limbs, jaws, and ocular tissues including neurons of these regions (71). In mammals, however, evidence for neurogenesis was ambiguous (72). In fact, in 1913 Ramon y Cajal stated "in the adult centres, nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated" (73).

It was in 1961 that adult mammalian neurogenesis was first reported in rodents by Smart *et al.,* (74). Later, Altman *et al.,* combined light microscopy with thymidine labelling (used to mark cells that are synthesizing DNA in preparation for division), and reported ongoing neurogenesis (75) in the hippocampus (76), neocortex (77) and olfactory bulb

(78) of rodents. These results were corroborated by Kaplan *et al.,* in the 1980s (79, 80). Rakic, however, did not find any such evidence for neurogenesis in the adult brain (81).

New momentum in the field of neurogenesis came with advances that allowed identification of specific cell types by immunocytochemical methods. Using such techniques, Nottebohm and his colleagues demonstrated adult neurogenesis in the lining of ventricles of songbirds (82, 83). Evidence for adult neurogenesis in the hippocampus of the tree shrew, marmoset and macaque (84-87) followed. The most convincing evidence, however, was provided using combined retroviral-based lineage tracing and electrophysiological studies (88, 89). These studies showed that the newly generated neurons were functional i.e. they fired action potentials and exhibited well developed voltage-gated sodium and potassium channels. In addition, the neurons exhibited functional synaptic contacts; the first synapses were GABAergic and these were followed weeks later by glutamatergic synapses (90-92). Such evidence regarding the functional phenotype of new neurons was restricted to neural stem cells isolated from rodents.

The first report of neurogenesis in humans was demonstrated by Eriksson and colleagues (93). They examined brains of cancer patients who had received BrdU (used to label proliferating cells in tumours). Combining BrdU labelling with neuron-specific markers, Eriksson *et al.,* demonstrated that new neurons (positive for BrdU and neuronal marker NeuN) were generated from dividing progenitor cells in the dentate gyrus. Recently, this group has also demonstrated robust neurogenesis in the olfactory bulb of humans, comparable in magnitude to that of rodents (94). The above findings of neurogenesis in

humans are supported by *in vivo* brain imaging studies by Manganas *et al.,* (95), who have demonstrated the presence of AHNSCs in the human hippocampus using magnetic resonance imaging.

Following the initial report of neurogenesis in humans (93), several groups have isolated and cultured AHNSCs *in vitro* (4-8, 18, 96, 97). However, the ability of AHNSCs to differentiate into mature, functional neurons capable of firing action potentials and synaptic communication (98) has not been tested.

Implications of Neurogenesis

After the initial discovery of neurogenesis in the postnatal rat hippocampus, several studies linked adult-generated neurons in the rodent hippocampus to learning and memory (99-101). Van Praag and colleagues showed that mice that run several kilometers daily showed a significantly higher percentage of BrdU-positive cells that co-labelled for the neuronal marker NeuN in the dentate gyrus of the hippocampus (102), demonstrating that exercise increases cell proliferation and neuronal differentiation in the hippocampus of adult mice. In fact, it has been shown that exercise facilitates recovery from brain injury such as stroke (103) and enhances cognitive function in patients (104, 105). At the cellular level, the newly generated neurons have a striking ability to migrate, integrate (106) and extend axons and dendrites in an environment which is largely inhibitory for mature neurons (107). This may be due to the exclusively depolarizing GABAergic synaptic input (108) the early neurons receive, akin to early neuronal development (109, 110).

Aberrant neurogenesis in the hippocampus is postulated to be linked to the pathophysiology of a variety of CNS-related diseases including mood disorders e.g. depression. As the major pharmacological and non-pharmacological treatments for depression increase hippocampal neurogenesis, it is proposed that antidepressant treatments may exert their therapeutic effects by reversing or blocking the reduced hippocampal neurogenesis (111, 112). Similarly, impaired hippocampal neurogenesis has also been implicated in rodent models of Alzheimer's disease (113, 114). Whether this is true in humans is not clear, though it has been shown that factors that positively affect hippocampal neurogenesis, including stimulating activities such as reading books and playing games, such as crosswords, may reduce the risk of Alzheimer's disease (115).

Aberrant neurogenesis is also seen in patients with seizures. It has been shown that seizures increase hippocampal neurogenesis in children under the age of 4 years (116). This suggests that seizure-induced neurogenesis in children may contribute to aberrant network integration and seizure progression (116). In contrast, older patients with chronic seizures do not show similar increases in neurogenesis (117). This may reflect either a depletion or exhaustion of the precursor cell pool (116), a phenomenon seen with increasing age (53, 118). Imaging studies using magnetic spectroscopy have for the first time confirmed the above-mentioned phenomenon, namely that in humans neurogenesis decreases with age (95). Such imaging techniques may be used to monitor neurogenesis in humans in a wide range of neuro-psychiatric disorders. Also, the efficiency of therapeutic interventions may be assessed.

Stem Cell Therapy

The aim of stem cell therapy is to treat neurodegenerative diseases including Parkinson's disease. The clinical usefulness of neural stem cells will be determined by their ability to provide patients suffering from neurological disorders with safe, long-lasting and substantial improvements to their quality of life. Neural stem cells may improve and restore function by differentiating and integrating appropriately into regions where neurological function is lost. Alternatively, they may exert a neuroprotective effect by releasing neurotransmitters or neurotrophic factors that could improve function by supporting the survival or regeneration of the existing neurons (119-121).

Understanding the cellular and molecular cues exerted by the microenvironment over stem cell differentiation in the adult CNS is important. Transplanting stem cells into neurogenic regions (regions that produce or recruit new neurons under normal conditions) results in differentiation in a region-specific manner (122, 123); for instance, SVZ precursors generate hippocampal neurons when transplanted into the hippocampus (124), and SGZ precursors generate olfactory interneurons when transplanted into the rostral migratory stream (122). Similarly, multipotent neural precursors from the spinal cord (22) produce neurons after implantation into the hippocampus (21). In contrast, transplantations into the spinal cord, a non-neurogenic region (a region that does not produce or recruit new neurons) (125), only generate glia (21). This highlights the significance of the milieu as well as the transplanted cells in determining the outcome of transplantations.

The traditional concept of stem cell therapy involves isolation of stem cells, propagation *in vitro* and subsequent re-injection into the patient. Human embryonic and foetal stem cell use is restricted due to political and ethical controversies. Moreover, large amounts of human foetal tissue are needed for therapeutic effects (two to five aborted foetuses per side per Parkinson's patient) (126) and clinical benefit is equivocal (127, 128). An alternative is to use immortalized cells. However, as many of these are transformed using oncogenes, tumour formation upon transplantation is a genuine risk. Consequently, AHNSCs present an attractive alternative to the aforementioned sources; they can be obtained from the patient herself, either from ventricular wall biopsies obtained endoscopically (129) or from temporal lobe surgeries due to refractory epilepsy (4, 18). Recently, it was shown that AHNSCs may also be isolated from the olfactory mucosa (130). The AHNSCs can then be propagated *in vitro* to sufficient numbers and subsequently transplanted autologously into the patient (96). To achieve clinical effect in Parkinson's disease, it is estimated that approximately 80,000 cells are needed (131). As the total number of AHNSCs that can be produced from one biopsy is far beyond this number (18, 96, 129), it seems likely that sufficient AHNSCs for treating neurodegenerative diseases may be obtained by *in vitro* propagation.

Attracting endogenous neural stem cells and precursor cells to the site of injury may represent an appealing alternative to the above-mentioned transplantation paradigm (132), as it bypasses *in vitro* cell propagation and the complications associated with it. However, the effect of endogenous precursors in brain repair is extremely limited (133). This probably reflects a limited number of neural stem cells or an unfavourable

microenvironment (133). Several factors can increase recruitment of endogenous stem cells to the site of injury including, neurotrophins BDNF (134) and stromal cell-derived factor-1 (SDF-1) (134, 135). More research, however, is needed before such therapy becomes clinically available.

Recently, stem cells have also been shown to play an important role in brain tumours (28, 31). These so called TSCs, have been shown to be brain tumour-initiating cells (23, 25, 26, 136) and are similar to neural stem cells. If TSCs are crucial for tumour initiation and propagation, then treatments designed to target them could prove highly effective. Thus, given the intimate association between neural stem cells and TSCs, the second part of my thesis describes brain tumours and the role TSCs play in such tumours.

Hallmarks of Cancer

There are several hallmarks that characterize the cells that develop cancer. Cancer cells disregard internal and external growth-inhibition signals and display unlimited mitosis; they are self-sufficient and have limitless replicative potential; they bypass mechanisms that detect mutation and apoptosis, allowing accumulation of mutations and genetic instability; angiogenesis and the ability to invade surrounding normal tissue as well as to metastasize to distant sites are also characteristic (137, 138).

The Stochastic Model of Cancer

According to the stochastic model of cancer, any somatic cell can give rise to tumours when exposed to the right conditions, though the probability of this is very low (30). Thus, serial acquisition of genetic alterations in somatic cells (139), results in induction of genes promoting proliferation, silencing of genes inhibiting proliferation and bypassing of genes involved in programmed cell death. Based on this model, glioma heterogeneity may result from continuing mutations in cells of different phenotypes or

from cells being trapped within the tumour matrix. Unfortunately, the model fails to adequately explain the origin of some gliomas, such as the mixed oligoastrocytoma.

Classification of Gliomas

The term glioma was first coined by Virchow in 1846, and he postulated that the tumours arose from glial cells (140). Then in 1875, Golgi showed that some brain tumours contained distinctive star-shaped neuroglial cells (141). The basis of modern-day neuro-oncology, however, is attributed to Bailey and Cushing who in 1926 wrote the book, *A Classification of the Tumours of the Glioma Group on a Histogenetic Basis with a Correlated Study of Prognosis* (142). They classified brain tumours based on the predominant cell type and its similarity to the normal brain cell type i.e. neuron, astrocyte or oligodendrocyte. Thus, the classification of primary brain tumours is based on cellular origin as well as histological appearance (143, 144). Hence, astrocytomas, the most common type of glioma, are so called as they resemble astrocytes. Further, based on grade of differentiation, astrocytomas are divided into four groups; low grade pilocytic astrocytomas (Grade I), well differentiated diffuse astrocytomas (AC II), malignant anaplastic astrocytomas (Grade III) and high grade glioblastoma multiforme (Grade IV, GBM) (145). The hallmark of malignant astrocytic gliomas is invasion into brain tissue (146). The classification of tumours predicts malignancy, invasive potential and thereby clinical prognosis and choice of therapy; low grade astrocytomas have a better prognosis and are more likely to respond to present treatment modalities compared to high grade astrocytomas (147).

Epidemiology of Gliomas

Malignant gliomas are the most common primary CNS tumours in adults, accounting for 78% of all primary malignant CNS tumours (148). The overall incidence of brain tumours in Norway has recently been reported to be ranging from 7.2 to 13.8 per 100,000 person-years (149). These rates are on the rise both in children (150-152) and adults (153-155). Whilst the increase in tumour incidence is attributed mainly to improved diagnosis (156, 157), including an increasing willingness on the part of the physician to pursue a diagnosis for older patients (158), some reports ascribe the change in incidence to be a real increase (152, 159).

Treatment Options for Gliomas

The current standard treatment includes surgical resection, radiation therapy and chemotherapy. The role of surgery is two-fold; firstly, it helps in establishing a diagnosis (160, 161) and secondly, it allows for the removal of as much tumour tissue as is possible while preserving the patients' neurological functions. Extensive tumour resection leads to increased survival (162-164). Regarding low grade gliomas, the role of surgery is controversial (165-168), however, a recent study by Sanai *et al.,* suggests that more extensive surgical resection is associated with longer life expectancy also for these gliomas (162).

Adjuvant postoperative radiation therapy is a standard-of-care for the management of high grade gliomas (169). Prospective clinical trials have shown that survival increases linearly as radiation dose is increased (170), though the use of such treatment is

controversial regarding low grade gliomas (171). Adjuvant chemotherapy is also well established in glioma therapy especially for GBMs. Studies have shown a survival benefit for patients receiving chemotherapy and radiation therapy when compared with patients receiving radiation therapy alone (172, 173). In fact, recently it was shown that when chemotherapy with Temozolomide was combined with radiotherapy, it resulted in a statistically significant survival benefit for GBM patients with a median survival of 14.6 months (174). However, not all patients benefited (175).

Despite the aforementioned therapeutic paradigms, the prognosis for GBMs has remained unchanged for three decades (147, 148). Given the dismal prognosis of high grade astrocytomas, several novel drugs are presently being clinically tested; (**1**) A humanized neutralizing monoclonal antibody *Bevacizumab* (Avastin; Genentech, South San Francisco, Calif) against VEGF, a growth factor needed for new blood vessel formation in gliomas (148). Bevacizumab has been shown to have significant anti-tumour effect in a phase 2 trial (176, 177). (**2**) A monoclonal antibody against EGFR *cetuximab* (Erbitux; ImClone Systems, New York, NY) is being clinically evaluated (178) as it has been shown to exhibit anti-tumour effect (179). EGFR amplification is seen in around 50 % of GBMs (180). (**3**) A kinase inhibitor of PDGFR *imatinib mesylate* (Gleevec; Novartis) (181) has been shown to be important in high-grade glioma growth and angiogenesis (148) is also being tested.

Despite the multi-modal treatment of gliomas available today, clinical recurrence is universal and it most often occurs within 2 cm of the resection margin (182). The

prognosis for patients with GBM has changed little in the past three decades and survival averages ~1 year (147, 148). Our paramount inability to treat this disease may reflect our lack of understanding of glioma biology.

Gliomas, the Stem Cell Model and the Tumour Niche

In contrast to the stochastic model of cancer, where any cell is capable of giving rise to a tumour, the stem cell model of cancer, states that cancer can be viewed as an abnormal organ (30), derived by a small fraction of cells capable of self-renewal and proliferation known as TSCs (30, 183, 184). Based on this model, gliomas are thought to be initiated and propagated by TSCs (27). Indeed, TSCs were first isolated from brain tumours by Ignatova *et al*., (24) and similar cells have been isolated from both paediatric GBM (23, 26) and adult GBM (25). The TSCs can generate a phenocopy of the parent tumour upon transplantation in mice and maintain the ability to produce tumours upon serial transplantation in mice – a gold standard assay for TSCs (27).

The stem cell hypothesis evolved because of the similarities between normal neural stem cells and TSCs, including nestin expression (185-187), cell signalling pathways governing self-renewal and proliferation, including Shh (56, 188) and Notch (189-192) and cell surface molecules such as CD 133 (23) and A2B5 (193). Microarray studies have revealed an overlap between the molecular signatures of GBMs and progenitors from the developing forebrain (194). These similarities suggest that TSCs may arise from neural stem cells that have acquired mutations making them tumourigenic. Indeed, upon transplacental administration of a potent neurocarcinogen N-ethyl-N-nitrosourea (ENU)

(195, 196), many gliomas started out in the SVZ, a site harbouring neural stem cells (197, 198). As the gliomas grew, however, they moved away from the site of initiation and this may explain their seeming discontinuity from the SVZ (199). Similarly, deletion of tumour suppressor gene p53 in neural stem cells initiates gliomagenesis in the SVZ (200). Recently, it was shown that even the origin of medulloblastoma, an aggressive childhood brain tumour, could be traced to neural stem cells (201, 202).

Similar to the neural stem cell niche, it has been suggested that TSCs exist in aberrant stem cell niches (203) and deregulation of extrinsic factors within the niche might lead to uncontrolled proliferation of stem cells and hence tumourigenesis. A hallmark of GBMs is the disorganized blood vessel formation (203) and it was recently shown that CD 133^{+} TSCs produced high levels of VEGF which may contribute to tumour initiation (204). Indeed, TSCs within GBMs are located in close proximity to tumour capillaries which promote the formation and maintenance of TSCs within these vascular niches (205). The tumour niche may also protect the TSCs from chemotherapy (206) and radiotherapy (207). If this is the case, targeting these microenvironments may prove a highly effective treatment of cancer.

Implications of the Stem Cell Model of Gliomas

Given the similarities between neural stem cells and TSCs, applying the principles of stem cell biology to gliomas changes the way we study, diagnose and treat such tumours.

Traditionally, treatments have focussed on removing the bulk of the tumour tissue surgically and then non-discriminately killing the proliferating cells using radiotherapy and chemotherapy. However, this does not translate into clinically significant increases in patient survival (147). The lack of effect of chemotherapy may be explained by the high levels of ATP-binding cassette transporter protein (ABCG) (208-210) and enhanced DNA repair mechanisms (211) that TSCs possess. This highlights that effective treatment may depend on differential drug sensitivity assays discriminating TSCs from the tumour bulk. Similarly, assays identifying and discriminating TSCs from neural stem cells may help in designing new therapies aimed at destroying the former and preserving the latter. Given that TSCs are tumour-initiating cells, eradicating TSCs could prove highly effective.

Current prognosis of patients with brain tumours depends on tumour characteristics (histology, grade of differentiation, extent of resection, etc.) and patient characteristics (age, etc.). As apparently identical tumours behave differently, it is difficult to predict prognosis. Interestingly, it was recently shown that the putative TSC marker CD 133 may predict patient survival and risk for tumour re-growth (212). This suggests that prognostic models based on TSC populations may be useful, provided that TSCs are specifically being identified and characterized.

Considering that cellular therapies are being developed for neurodegenerative diseases and given that activation of stem cell pathways may lead to tumour initiation, one has to be cautious before such therapies can be translated into the clinics. In fact, it was shown

that even modified embryonic stem cells-generated cells could be potentially tumourigenic (213).

The stem cell model of cancer may also help in establishing appropriate rodent cancer models which resemble human cancers more closely. Using a stem cell virus, Welm *et al*., showed that expression of c-Met and c-Myc in primitive cells produced mammary carcinomas (214) similar to those found in humans. Similarly, Kim *et al*., have developed an animal model that targets normal lung stem cells to produce adenocarcinomas that resemble those found in human lung cancers (215). Having representative rodent models that correctly reflect tumour pathogenesis gives more relevance to the results obtained from such models. This is important when new therapeutic paradigms tested in rodent models are translated into the clinics.

AIMS

1. To isolate and propagate putative neural stem cells from adult human ventricular wall biopsies and to examine the ability of the cells to develop into functional neurons, including their ability to fire action potentials as well as to communicate through synapses (98). (**Paper I**).

2. After confirming the neurogenic potential of AHNSCs, the next step was to characterize the temporal profile of the electrophysiological development of the neurons differentiated from AHNSCs. (**Paper II**).

3. To isolate putative neural stem cells from adult human filum terminale and examine the potential of the cells to differentiate into functional neurons *in vitro* and *in vivo*. (**Paper III**).

4. To systematically compare putative neural stem cells isolated from ventricular wall biopsies to TSCs isolated from low grade and high grade gliomas *in vitro* and *in vivo*. (**Paper IV**).

MATERIALS AND METHODS

In vitro

Cell Culture

Ventricular wall biopsies were obtained (**1**) from patients undergoing surgery due to intractable temporal lobe epilepsy (**papers I and II**) and (**2**) endoscopically using a Channel neuroendoscope (Medtronic PS Medical, Goleta, CA) during routine neurosurgical procedures to treat hydrocephalus (**paper I**). Filum terminale biopsies were obtained from patients undergoing surgery for spinal cord pathology (**paper III**). Biopsies from both AC II and GBM were harvested from patients undergoing tumour resections (**paper IV**). Tissue harvesting was approved by the Norwegian National Committee for Medical Research Ethics and the Ethical Committee at the Karolinska Hospital (Dnr 01-294) and written consent was obtained from each patient. Radiology and histopathology was used to confirm diagnosis in the aforementioned patient groups.

The samples were transported from the operating theatre to the lab in Leibowitz-15 medium (L15) (Invitrogen Corp., Carlsbad, CA) at 4°C. The tissue was mechanically dissociated with a scalpel and placed in 500 µl trypsin-ethylenediamine tetra-acetic acid (trypsin-EDTA; Sigma, St Louis, MO) and 200 µl deoxyribonuclease (4000 U/ml; Sigma) (**paper I**), papain 13.2 U/ml (Sigma) and deoxyribonuclease (200 U/ml; Sigma) (**paper II**) and trypsin-EDTA (×1; Invitrogen) (**papers III and IV**). The dissociated suspension was passed through a 70 µm strainer (BD Biosciences, San Jose, CA), and resuspended as single cells at clonal density in neurosphere medium (216), DMEM/F12 (Invitrogen) supplemented with B27 (x1; Invitrogen), 10 ng/ml bFGF (R&D Inc,

Minneapolis) and 20 ng/ml EGF (R&D Inc). Cells were cultured at 37°C in 6% CO_2 and 20% O_2. The cultures were supplemented with 10 ng/ml bFGF and 20 ng/ml EGF twice a week, and additional 1 % of the final volume of DMEM/F12 was added once a week. The neurospheres were enzymatically dissociated, as referred to earlier, before their centres became necrotic, and resuspended in neurosphere medium. The time taken for neurospheres to reform and the size of the neurospheres was measured. To ensure strict clonal conditions, single cells were manually isolated with a micromanipulator (Eppendorf, Westbury, NY) and cultured further. Differentiation of cells from neurospheres was induced by adding 2 % foetal calf serum (FCS, PAA Laboratories, Pasching, Austria), removal of mitogens and plating on 20 ng/ml poly-L-ornithine-coated (Sigma) or 20 ng/ml laminin-coated glass bottom dishes (WillCo Wells BV, Amsterdam, The Netherlands) or 4-well glass slides (Nunc, Roskilde, Denmark).

Co-Culture of Neural Stem Cells and Tumour Stem Cells

Spheres resulting from AHNSCs and TSCs were stained using the fluorescent Cell-Tracker dye 2μM **CFDA-SE** (carboxy fluorescein diacetate succinimidyl ester - excitation 492 nm, emission 517 nm - Molecular Probes, Eugene, OR) or the fluorescent lipophilic tracer 0.1% **DiI** (1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarboxcyanine - excitation 492 nm, emission 517 nm - Molecular Probes) respectively, according to the manufacturer's protocol. The labelled spheres were washed, centrifuged, and suspended in a 1:1 ratio, and followed on a Leica DM 4000 B fluorescence microscope or a Leica TCS SP2 confocal microscope (Leica Inc, Kista, Sweden).

Immunocytochemistry

Immunostaining was performed as previously described (17, 216, 217), with the following primary antibodies and dilutions (rb: rabbit, ms: mouse, gp: guinea pig, gt: goat); *stem cell markers*, human specific nestin (**HuNest**, ms, 1:1000; R&D Inc) and **Sox2** (rb, 1:500; Chemicon, Temecula, CA); *neuronal markers*, microtubule-associated protein 2 (**MAP-2**, rb, 1:500; Chemicon), doublecortin (**DCX**, gt, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), **β-III-tubulin** (ms, 1:1000; Sigma) and neuronal nuclear marker **NeuN** (ms, 1:1000; Chemicon); *glial markers*, **GFAP** (rb, 1:1000; Dako, Carpinteria, CA), **O4** (ms, 1:100; Chemicon), 2`, 3`-cyclic nucleotide 3-phosphodiesterase (**CNPase**, ms, 1:500; Chemicon) and **RIP** (ms, 1:1000; Chemicon). Neurotransmitters were stained with glutamate receptor-1, 2 and 4 (**GluR-1**, rb, 1:100; **GluR-2 and 4**, ms, 1:100; Chemicon), vesicular glutamate transporter-1 (**VGlut-1**, gp, 1:10,000; Chemicon) and glutamic acid decarboxylase (**GAD-65,** rb, 1:1000; Chemicon). In addition, a proliferation marker **Ki-67** (rb, 1:100; Dako), human specific nuclei (**HuN**, ms, 1:200; Chemicon), inflammation/microglial marker **OX42** (ms, 1:1600; Harlan SERA-LAB, Loughborough, U.K.), **synaptophysin** (rb, 1:1000; Binding Site, Birmingham, England) and **laminin** (rb, 1:200; Sigma) were used. Either **TO-PRO-3** (1:10 000; Molecular Probes) or **Hoechst** (1:200; Invitrogen) was used for nuclear staining. For secondary antibodies the fluorescent conjugate markers **Cy3** (1:1000; Jackson, West Grove, PA), **Cy5** (1:1000; Jackson), **Alexa Fluor 488** (1:500; Molecular Probes), **Alexa Fluor 594** (1:250; Molecular Probes), **fluorescein isothiocyanate** (1:150; Jackson), or an avidin-biotin complex (**ABC-elite**, 1:50; Vector, Burlingame, CA)/diaminobenzidine (**DAB**; 50 mg/100 ml, Sigma) were used.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from tissue samples, stem cells and differentiated cells from AHNSCs and TSCs using RNAeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and subsequently treated with RNase-free DNase I (RQ1 DNase I, Promega GmbH, Mannheim, Germany). Reverse transcription and quantitative real time PCR of each sample were run in triplicates using the TaqMan PCR Core Reagent Kit and the ABI Prism 7900 Sequence Detection System and software (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The oligonucleotide primers and probes for nestin, β-III-tubulin and GFAP were bought from TaqMan Applied Biosystems. As an endogenous control, 18S rRNA (TaqMan rRNA control reagents, Applied Biosystems) was used. Data acquired were analyzed with the Sequence Detector software (version 1.6.3, Applied Biosystems). A standard curve was obtained by amplifications of cDNA obtained from serial dilutions of total RNA. Gene expression was presented relative to the levels of 18S rRNA.

Telomeric Repeat Amplification Assay

Telomerase protein was extracted from AHNSCs and TSCs in CHAPS lysis buffer and the activity was detected using PCR-based TRAPeze® XL Telomerase Detection kit (Chemicon) according to the manufacturer's instructions. The assay is based on the test samples capacity to add telomeric repeats onto the 3' end of a substrate oligonucleotide (TS). Telomerase activity was measured as TPG/μg protein (Total Product Generated/ μg protein). TPG corresponds to the number of TS primers extended with at least 3 telomeric repeats by telomerase in the extract in a 30 minute incubation at 30°C.

Retroviral Enhanced Green Fluorescent Protein Transduction

A moloney murine leukemia based retroviral system was used to generate amphotropic replication incompetent viruses. The Phoenix^{TM} amphotropic packaging cell line (218) (generously provided by Dr. M. J. T. Veuger, LIIPAT, Institute of Pathology, Rikshospitalet, Oslo) was grown in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Pailsy, UK) supplemented with 10% foetal calf serum (PAA Laboratories) and antibiotics. Helper-free recombinant retrovirus was produced after transfection with the retroviral vector pLZRS-IRES (internal ribosomal entry site)–EGFP (enhanced green fluorescent protein) (218) (generously provided by Dr. M. J. T Veuger) into the Phoenix cells using a calcium-phosphate transfection kit (Invitrogen). Positive cells were selected using 2μg/ml puromycin (Sigma), then grown in IMDM and supernatants containing viral particles were collected at 24 hours post-transfection, passed through 0.45 μm Millex GP filters (Millipore Co., Bedford, MA) and stored at −80 °C.

Untreated T25 tissue culture flasks (BD Biosciences) were coated with 10 μg/cm^2 rFN (Retronectin, Takara Shuzo Co., Shiga, Japan) according to the manufacturer's instructions. Retrofibronectin-coated flasks were pre-loaded with retroviral supernatants for 4 hrs at 37 °C. The supernatant was then removed, and the plates washed with phosphate buffered saline (PBS). Single cell suspensions, AHNSCs or TSCs, were added to the virus loaded rFN-coated flasks. The cells were then incubated at 37 °C in an atmosphere of 5% CO_2 in air for 24-48 hours before the medium was changed to fresh complete culture medium.

Fluorescence-Activated Cell Sorter Analysis

For fluorescence-activated cell sorting (FACS), a single-cell suspension of AHNSCs or TSCs, was prepared by incubating $EGFP^+$-spheres in trypsin-EDTA at 37°C for 5 minutes. The EGFP fluorescence was measured using a FACSAria Flow cytometer (Becton-Dickinson, San Jose, CA) (219) with a 488 nm argon laser and FL-1 530 nm BP emission filter. Highly fluorescent cells were sorted and thereafter transplanted into Fox Chase severe combined immunodeficient (SCID, Taconic, Lille Skensved, Denmark).

Electrophysiology

The whole-cell patch-clamp technique was used to examine the neurophysiological properties of individual cells. Cells grown in culture dishes were placed in a recording chamber on the stage of an inverted microscope (Nikon, Tokyo, Japan). The cultures were perfused with DMEM/F12 between 28 and 32°C and bubbled with 95% air and 5% CO_2. A Multiclamp 700A amplifier and pClamp 8 software (Axon Instruments, Union City, CA) was used to control pipette potentials and to inject current during recordings. Patch pipettes were pulled from thick-walled borosilicate glass capillaries to resistances of 4–6 MΩ and were filled with pipette solution containing (in mM) K-gluconate 125, HEPES 10, EGTA 10, KCl 5, Mg–ATP 2 and $CaCl_2$ 0.2 (pH = 7.3) Lucifer Yellow 0.1% (Molecular Probes) was added to the pipette solution to retrospectively identify immunocytochemical markers of the cells tested with electrophysiology. In addition, all cells tested were photographed, and the exact position in the culture dish was marked with a thin water resistant pen to facilitate cell identification. Cells tested electrophysiologically and those that were positive for the neuronal markers MAP-2 or ß-

III-tubulin were defined as neuron-like cells and included in analysis. The membrane time constant (τ_{in}) and input resistance (R_{in}) were estimated in current-clamp by the voltage responses of the cells to small injected rectangular hyperpolarizing current pulses of –10 to –30 pA depending on the R_{in} of the cell. R_{in} was derived from the linear portion of the current-voltage plot, and τ_{in} was calculated by minimizing the squared deviation between the function and the data between 5 and 25 ms of the pulse. The function used was $f(t) = V_{ss} - [V_{ss} \cdot \exp(t/\tau_{in})]$, where V_{ss} is the steady-state response (220). The voltage-clamp protocol for testing active membrane properties consisted of a 100 ms hyperpolarizing pulse from a holding potential of –70 to –90 mV that preceded each of the depolarizing steps to remove inactivation, followed by 200 ms depolarizing steps with 10 mV increments at 0.5 Hz, taking the membrane potential from –90 to 60 mV. In the current-clamp protocol, current pulses (0–0.1 nA, 0.5 Hz) were injected through the patch pipette to examine whether the cells were capable of producing action potentials.

During dual patch-clamp recordings, a whole-cell recording was first obtained from one cell with morphological and electrical membrane properties characteristic of neuron-like development. Subsequently, the second whole-cell recording was made from another neighboring and seemingly connected neuron-like cell. The presynaptic cell was recorded in current-clamp mode, and a single action potential was evoked by current injection (0.1 nA). Postsynaptic currents (PSCs) were recorded in voltage-clamp mode.

Spontaneous synaptic events were recorded during single whole-cell patch-clamp experiments in voltage-clamp at different holding potentials. GABAergic currents were

blocked using 10 μM of bicuculline, while the glutamate-mediated currents were blocked by 20 μM of 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, blocks AMPA/kainate receptors) and 15 μM of D-2-amino-5-phosphonovaleric acid (MK-801, blocks NMDA receptors). Solutions were brought from Sigma. Neurons were stimulated with 1 mmol/L glutamate from a micropipette positioned close to the cells and the response was blocked by glutamate-receptor antagonists 20 μM CNQX and 15 μM of MK-801. The responses were recorded using whole-cell voltage-clamp recordings.

Spike threshold was defined as the membrane potential at which the slope of the voltage trace increased abruptly during current injection. Spike width was calculated as spike duration at 50% of maximum spike amplitude.

Confocal Microscopy

For confocal imaging an inverted microscope (Nikon) with a confocal imaging system (MRC 600, Bio-Rad, Hertfordshire, UK), equipped with an argon ion laser was used. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured using the acetoxymethyl ester of the two Ca^{2+}-sensitive fluorochromes fluo-3 (fluo-2 AM) and fura red (fura red AM) (221) (Molecular Probes). The cells were incubated in DMEM/F12 containing 0.2 μM fluo-3 and 1.8 μM fura red for 20 min before the superfluous dye was washed out. Emitted fluorescent light was detected using two separate photomultiplier tubes at the wavelengths 525–555 nm (fluo-3) and >600 nm (fura red), respectively. Neurons were stimulated by pressure puff application of 60 mM of potassium chloride (KCl) for 10 s and one image was acquired

every 2 s, and the fluorescence ratio was calculated using the Time Course/Ratiometric Software Module (TCSM, Bio-Rad).

External Test Solutions

External test solutions included 0.5 μM of tetrodotoxin (TTX) to block voltage-dependent sodium channels, 500 μM of nickel chloride (NiCl) to block the voltage-gated Ca^{2+}-channels, 500 μM of 4-aminopyridine (4-AP) and 5 mM of tetraethylammonium (TEA) to block potassium currents. All the solutions were from Sigma.

In vivo

All animal procedures were approved by the Ethical Committee of Animal Research in Northern Stockholm and the National Animal Research Authority in Norway

Transplantation of AHNSCs from Filum Terminale into Adult Rats Exposed to Global Ischemia Using a Two-vessel Occlusion Model

Global ischemia was induced in accordance with a previously published protocol (217, 222) Adult male Sprague-Dawley rats (270 ± 10 g, Taconic) were anesthetized with halothane (Fluothane; AstraZeneca, Södertälje, Sweden), endotracheally intubated, and artificially ventilated. The common carotid arteries were exposed bilaterally by means of a ventral midline incision and occluded with non-traumatic microvascular clips for 11 minutes, while keeping the blood pressure below 45 mmHg by increasing the concentration of halothane to minimize compensatory flow in the vertebral arteries.

Physiological parameters monitored throughout the procedure were rectal temperature (37.5 ± 1°C), mean arterial blood pressure, and cerebral blood flow recorded by a laser Doppler probe attached to the skull. Control rats did not undergo any surgery. Seven days post-injury, rats were anesthetized with an intramuscular injection of Hypnorm (10 mg/ml fluanisone and 0.2 mg/ml fentanyl; Janssen Pharmaceutica, Beerse, Belgium) and Dormicum (1 mg/ml midazolam; Roche, Stockholm, Sweden), and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). A 2 µl cell suspension containing 10,000 cells/µl was slowly injected, using a cannula (Plastics One, Roanoke, VA) attached to a Hamilton syringe (Hamilton Bonaduz, Bonaduz, Switzerland) into posterior periventricular region just above the hippocampus (coordinates: anteroposterior, -2.8; mediolateral, 2.0; ventrolateral, -2.6 from bregma, with the nose bar set at -2.0. The needle was left *in situ* for 2 min postinjection before being removed slowly. The Sprague-Dawley rats received immunosuppression with cyclosporine A subcutaneously (4 mg/kg every Monday and Wednesday and 8 mg/kg every Friday and the day before transplantation) (Sandimmun, Norvartis Sverige AB, Stockholm, Sweden), in accordance with a previously published protocol (223). The transplanted rats were lethally anesthetized after 10 weeks.

Transplantation of AHNSCs or TSCs into the CNS of Adult SCID Mice

Fox Chase SCID mice (7 – 9 week old, Taconic) were anesthetized subcutaneously with previously described drugs (217), and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA). Immediately prior to transplantation, suspensions of AHNSCs or TSCs, native and GFP-tagged, were prepared in L-15 medium, and a 2 µl

suspension containing 100,000 cells was injected into the right striatum just below the corpus callosum using a cannula (Plastics One, Roanoke, VA) attached to a Hamilton syringe (Hamilton Bonaduz, Bonaduz, Switzerland). The needle was left *in situ* for 2 min postinjection before being removed slowly. The implanted mice were killed after 14 weeks or sooner if symptoms including weight loss or poor appetite developed.

Brain and Tissue Processing

The transplanted rodents were killed by deep anaesthesia with Hypnorm/Dormicum and transcardial perfusion with 0.9 % NaCl followed by 4 % buffered formaldehyde. The fixed brains were cryo-protected in 17% sucrose for 48 hours, cut into 14 µm sections on a freezing microtome (Leica), thawed onto Super Frost/Plus slides (Menzel-Gläser, Braunschweig, Germany) and stored at -20˚ C.

Brain sections were stained with (**1**) haematoxylin and eosin (H & E), (**2**) immunohistochemistry and (**3**) avidin-biotin complex method. Immunohistochemistry on sections was performed as previously described (217, 224). Briefly, sections were incubated in primary antibody overnight at 4°C, rinsed 3 times with PBS, incubated in secondary antibody at 4°C for an hour, followed by rinsing thrice with PBS. After nuclear staining the sections were mounted with PBS/glycerol 1:1.

For avidin-biotin complex staining method, sections were incubated with an avidin block solution (Blocking kit, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature, followed by washing in PBS. A biotin block solution was added to the

primary antibody solution containing HuN and sections were incubated overnight at 4° C. The sections were then incubated at room temperature with biotinylated secondary antibody (1:200, horse α-mouse IgG, Vector Laboratories) for 1 hour and in avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain® Elite® ABC kit, Vector Laboratories) for 1 hour, followed by rinses. The signal was visualized using diaminobenzidine (DAB, substrate kit for peroxidase, Vector Laboratories). Double-labeled sections were treated in the same manner with OX42 and the stain was developed using the Nova red substrate kit (Vector Laboratories). Slides were air-dried and mounted with PBS/glycerol 1:1 (**paper III**).

Light microscopy slides were analyzed using a Zeiss Axioskop 2 microscope (Zeiss, Munich, Germany) and immunolabelled sections were studied using Olympus BX61W1 FluoView confocal microscope (Olympus, Hamburg, Germany).

Statistics

The results are presented as mean ± S.E.M. Differences were tested with independent-sample t-tests (Student's t-test), and considered significant when $p < 0.05$.

RESULTS

PAPER I

In **paper I** we showed that AHNSCs could be isolated from ventricular wall biopsies obtained (**1**) endoscopically during routine neurosurgical procedures to treat hydrocephalus or, (**2**) during surgery for refractory temporal lobe epilepsy. The AHNSCs developed into neurospheres when grown in a defined sphere-promoting medium. They were capable of self-renewal and proliferation even after repeated enzymatic dissociation. Under differentiating conditions, AHNSCs developed into astrocytes, neurons and oligodendrocytes proportionate to their *in vivo* organization. Importantly, the neurons matured over time and developed functional activity. Specifically, after four weeks in culture neurons fired low-threshold, overshooting, repetitive action potentials and exhibited voltage-gated sodium and potassium ion channels. Furthermore, the neurons possessed presynaptic terminals with functional glutamate receptors. Using double patch-clamp recordings, we showed that functional neurons communicated synaptically when integrated in a network.

PAPER II

Given that AHNSCs can be propagated as neurospheres *in vitro* and that they respond to differentiation cues developing into mature neurons (**paper I**), in **paper II** we characterized the stages of electrophysiological development of the neurons differentiated from AHNSCs. Early in development, neurons stained only for neuronal markers without exhibiting any functional activity. During the second week, ‘depolarizing potassium-

dependant humps' followed by calcium-dependant action potentials were seen. Next, broad high-threshold sodium-dependant action potentials which gradually developed into low-threshold repetitive action potentials by the fourth week were seen. Concomitant to electrophysiological maturity, the neurons developed a more polarized and arborized appearance with multiple dendrites (Fig. 3). Moreover, after 4 weeks the neurons communicated using GABAergic and glutamatergic synapses; post-synaptic currents were demonstrated using patch-clamp recordings.

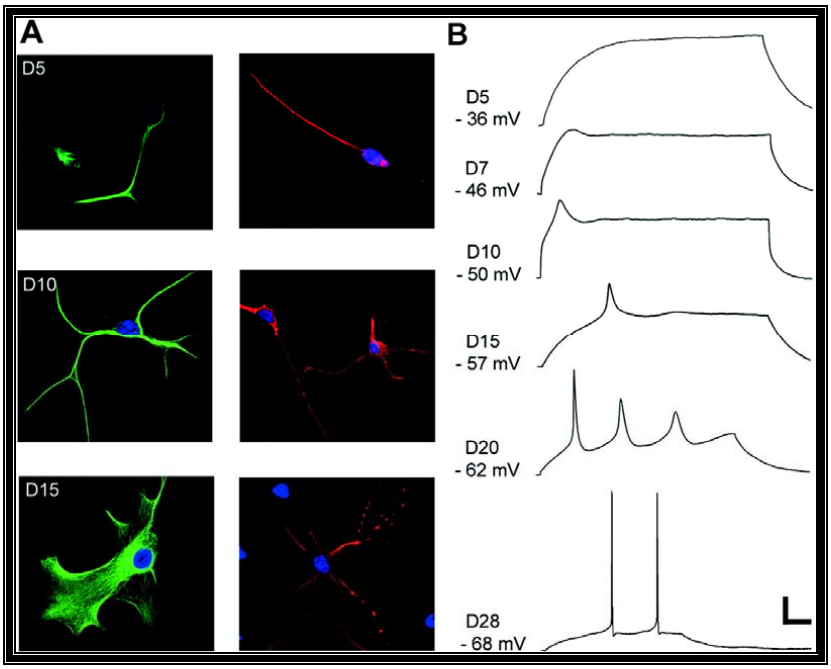

Figure 3. (A) Immunocytochemical staining for glial (GFAP, left panel) and neuronal (MAP-2, right panel) markers at different stages of development. (B) Whole-cell patch-clamp recordings showing responses to a 0.1 nA intracellular current pulse at different stages of differentiation. The pulse duration varied from 300 to 500 ms

depending on the maturation of membrane properties. Scale bars: 50 μm (A top panel); 20 μm (A lower panel); 15 mV and 50 ms (B).

PAPER III

In **paper III** using the assays already established in **papers I** and **II**, we showed that neural progenitors isolated from filum terminale (FTNPs) could self-renew and proliferate into neurospheres. This ability to develop into neurospheres was maintained even after enzymatic dissociation (passaging). Furthermore, FTNPs exhibited tripotent differentiation into neurons, astrocytes and oligodendrocytes. Using patch-clamp recordings, we demonstrated that the neurons exhibited low-threshold, overshooting action potentials, displaying both the fast inactivating TTX-sensitive sodium current as well as 4-AP and TEA sensitive potassium currents. The FTNPs survived transplantation into adult CNS exposed to ischemia and no tumour formation was observed. The FTNPs were found not only in the graft, but also in the lesioned CA1 region displaying a tendency to migrate to areas of pathology. Interestingly, despite neuronal differentiation *in vitro,* FTNPS differentiated only into astrocytes *in vivo.*

PAPER IV

In **paper IV**, we compared AHNSCs isolated from ventricular wall samples to TSCs isolated from both AC II and GBM. Though TSCs have been isolated from paediatric low grade tumours (23), this is the first report of such cells being isolated from adult AC II. Both AHNSCs and TSCs proliferated into spheres, but TSCs showed significantly higher growth rate and self-renewal capacity ($p < 0.05$). This was reflected in their telomerase

expression which was high in TSCs and correlated to the malignancy grade (GBM>AC II) ($p < 0.04$). AHNSCs, however, had low telomerase expression. To investigate the tumour-forming capacity, TSCs and AHNSCs were transplanted into SCID mice. Only the TSCs from GBM formed tumours following orthotopic transplantation and the tumour-forming ability was retained upon serial transplantations. Upon differentiation *in vitro*, (**1**) TSCs differentiated faster than AHNSCs; (**2**) There was a dramatic fall in the proliferation index (Ki-67) of TSCs ($p < 0.05$); (**3**) AHNSCs gave rise to astrocytes, oligodendrocytes and neurons, whereas morphologically aberrant bipotent cells often expressing both glial and neuronal antigens were seen in TSCs cultures. Whole-cell patch-clamp recordings of differentiated progeny uncovered distinct functional phenotypes; AHNSCs differentiated into neurons (high electrical membrane resistance, ability to generate action potentials) and astrocytes (low membrane resistance, no action potentials); in TSCs cultures, only one functional phenotype was seen - cells with high electrical resistance and active membrane properties capable of generating action potentials.

DISCUSSION

Sources of Neural Stem Cells

Adult human neural stem cells have been isolated from ventricular wall biopsies from patients undergoing surgery for intractable epilepsy (4, 18, 96, 225) or endoscopy due to hydrocephalus (129). Other CNS regions including hippocampus (7, 97), cortex (5, 19), white matter (6), spinal cord (21, 22) and olfactory bulb/mucosa (130, 226-229) also harbour AHNSCs. In fact, a recent report suggested that the CNS may be much more neurogenic than previously hypothesized (230). In keeping with this idea of a widely neurogenic CNS, we have isolated AHNSCs from a hitherto unknown source, namely filum terminale (**paper III**). Indeed, neural stem cells have been isolated and propagated from several non-neurogenic regions including the spinal cord (22), suggesting that similar cells may be isolated from different CNS regions by customizing and optimizing isolation and culture protocols (230). Despite similarities between stem cells isolated from different regions, there are distinct differences; we show in **paper III** that despite neurogenic differentiation *in vitro*, FTNPs differentiated into glia *in vivo*, in contrast to a bipotent fate of AHNSCs *in vivo* (217).

Non-neural cell types may serve as a source of neural stem cells/neurons e.g. cells from the placenta (231), mesenchymal cells (232-234) and adipocytes (231, 235). Alternatively, non-neural cells may modulate the local environment to increase neural proliferation and maturation of endogenous stem cells (236). It was recently shown that human somatic cell types e.g. fibroblasts, could be reprogrammed to undifferentiated pluripotent stem cells (237, 238). This is referred to as induced pluripotent stem cells

(iPS) (239). Similar reprogramming has been achieved in mice neural stem cells (239). Thus, using iPS technology it is possible to generate patient-specific iPS cell lines. However, the clinical applicability will depend on optimizing the protocol, eliminating the use of retroviral insertional mutagenesis and avoiding teratoma formation (239). Though the possibility of obtaining neurons from such sources is attractive, the ability of these cells to differentiate into functional neurons that communicate synaptically and integrate into a network has to be investigated before they can be considered as tools for regenerative therapy.

Are Neural Stem Cells Isolated from Ventricular Wall Biopsies Obtained During Epilepsy Surgery Normal?

Neural stem cells are usually obtained from ventricular wall biopsies from patients undergoing surgery for refractory temporal lobe epilepsy (4, 18, 96). Though epilepsy is shown to increase neurogenesis (240, 241), some reports suggest seizure activity to result from aberrant neurogenesis (242, 243), bringing into question the normality of the isolated AHNSCs. Interestingly, increased hippocampal neurogenesis has been shown in patients under the age of 4 years (116) and not in older patients (117). In our study the ventricular wall samples were obtained from patients that belonged to the latter group. Also, the karyotypic analysis of AHNSCs revealed no gross cytogenetic irregularities (18).

In **papers I**, **II** and **III** we showed that differentiated AHNSCs exhibited two functional phenotypes; neurons with a high electrical membrane resistance with the ability to

generate action potentials and astrocytes with a low membrane resistance unable to generate action potentials, in keeping with the normal CNS. Moreover, transplanting AHNSCs into adult CNS resulted in differentiation into neurons and glia with no seizure activity seen in the recipient rodents (18, 217). Though the evidence presented here is in favour of AHNSCs isolated during epilepsy surgery as being normal, more research is needed to answer the question definitively.

Bona Fide Neural Stem Cells or Neural Progenitors

Adult human neurogenesis *in vivo* was first demonstrated by Eriksson and colleagues (93). Adult human neural stem cells were first isolated by Steindler *et al.,* (4). Thus, by applying already established assays to AHNSCs from ventricular wall biopsies (**papers I and II**) and filum terminale biopsies (**paper III**), we showed that AHNSCs could proliferate and self-renew into neurospheres. Per definition, a *bona fide* stem cell is said to exhibit extended self-renewal i.e. more than five passages (14). In our hands, the cells displayed limited passage number, i.e. they exhibited limited self-renewal. Similar limited proliferation and self-renewal has been demonstrated by others (5, 6, 244), suggesting that AHNSCs are transitional cells with a limited self-renewal capacity.

Given that the CNS may be more neurogenic than postulated previously (230) and reports showing that AHNSCs can be propagated and expanded *in vitro* for prolonged periods (18, 96), suggests that culture paradigms may have to be modified to realize the full potential of AHNSCs *in vitro*. For instance, addition of Shh, a growth factor lacking in

our culture conditions, has been shown to increase proliferation and self-renewal of adult neural stem cells in rodents (56-59).

Though it would be advantageous to expand AHNSCs indefinitely and create cell banks, it has been shown that after prolonged culture *in vitro*, stem cells tend to prefer a gliogenic fate rather than a neurogenic one (245, 246). Furthermore, long-term cultures can *per se* increase the appearance of mutations and karyotypic changes (247, 248) and with it the concern for tumour formation. Given the aforementioned caveats, optimizing culture protocols as well as characterization of neural stem cells, over multiple passages with respect to tumour initiating ability, has to be documented before such cells can be used as therapeutic tools for neurodegenerative diseases.

Significance of Electrically Active Neurons from Neural Stem Cells

As mentioned earlier, AHNSCs have been isolated and propagated *in vitro* by several groups (4, 7, 96, 249). However, identification of the differentiated progeny has been confirmed using antigenic markers. Evidence regarding the functional status of neurons is available primarily for rodent neural stem cells (250). As shown in **papers I** and **II** despite neurons staining for neuronal specific antigens they do not necessarily exhibit typical neuronal activity. This underscores the need to test neurons for their functional phenotype. Both Nunes *et al.,* (6) and Walton *et al*., (18) have demonstrated that AHNSCs exhibit single broad action potentials. Unfortunately, as such activity has also been demonstrated in developing glial cells (251), it is not conclusive evidence for the presence of mature functional neurons. In **papers I** and **II**, we show that AHNSCs

differentiate into cells possessing the hallmarks of neurons, including the ability to generate action potentials and communicate via synapses (98).

The functional significance of voltage-gated ion channels underlying the electrical activity expressed by the differentiated progeny of the AHNSCs is manifold. Whilst potassium channels limit excitability of mature neurons, in immature cells it is responsible for migration (252). Calcium channels on the other hand, are important for neurite growth, axonal growth and path finding, as well as cell signalling and synaptic transmission (253, 254). While sodium channels are important in generating action potentials, their overactivity has been associated with epilepsy (255). Knowing that the clinical application of neural stem cells will be limited by their ability to develop into functional neurons, research optimizing the functional characteristics of neural stem cells is necessary.

Using confocal microscopy, we showed that AHNSCs differentiated primarily into glutamatergic and GABAergic neurons. Both GABA and glutamate have important functions during development, including regulating proliferation and migration, synaptic integration and neuronal differentiation (256). Though not specifically tested in **papers I**, **II** or **III**, AHNSCs are presently being investigated for their ability to generate other neuronal phenotypes, including dopaminergic neurons. Were this to be the case, it would be possible to transplant cells autologously into patients suffering from neurodegenerative diseases, including Parkinson's disease. This would open a new avenue for the treatment of such diseases.

The Relationship Between Neural Stem Cells and Tumour Stem Cells

It has already been shown that neural stem cells and TSCs share several similarities (28, 183), including surface expression of CD 133 (23, 34). This may be a consequence of the transformation of neural stem cells into TSCs. In fact, neural stem cells are considered the most likely candidates for such a transformation due to their long life span and increased propensity to accumulate mutations (28).

Though several studies have alluded to an intimate association between neural stem cells and TSCs, studies have not compared these two populations. When conducting such a comparative study, it is important to look at cells isolated from fresh biopsies and not rely on cell lines as shown by Lee *et al.,* (257). Several clinical correlates can be drawn from the results of **paper IV**; (**1**) The role of TSCs has been shown for GBMs but not for AC II in adults (23-26). Thus, identification of TSCs from AC II may present a new promising therapeutic alternative for these tumours, given that the role of surgery is controversial (165-168); (**2**) Spheres cultured from TSCs mirror several biological features of human brain tumours (258, 259) including growth rate. Such sphere assays can thus be regarded a representative model for glioma growth *in vitro* (259, 260). It can be used to study the kinetics of normal and tumour cell movement, as well as tumour progression (261-263) and therapy outcome (264, 265); (**3**) When exposed to differentiating conditions, there was a dramatic attenuation in the proliferation of TSCs. This suggests that TSCs which escape surgical resection and chemotherapy, would be unable to proliferate and thus incapable of giving rise to relapses were they to be subjected to differentiation therapy (25).

Significance of Voltage-Gated Ion Channels in Tumour Cells

The presence of functional activity and voltage-gated ion channels has been shown in cell lines (266) as well as tumour tissue (267). **Paper IV** presents the first report of functional activity in differentiated progeny of TSCs derived from AC II and GBM. Voltage-gated ion channels influence several biological functions including, proliferation and metastasis of tumours; it is also well known that potassium channels inhibit apoptosis; in fact they may even enhance multidrug resistance in tumour cells (268, 269); indeed, sodium channels have been shown to be important for tumour invasiveness (270, 271). Moreover, the presence of seizures in patients with brain tumours (272) may be due to the action potential generating progeny of the differentiated TSCs. Given the manifold significance of voltage-gated ion channels, several channel blockers are being currently tested for their anti-tumour activity.

Neurosurgeons, the Friendly Neighbour

The studies conducted in this thesis were only possible due to a close collaboration between the neurosurgical departments and the research lab. This highlights the neurosurgeon's role as a provider of both normal and tumour tissue from which stem cells can be isolated. Equally important, it is the neurosurgeons who will ultimately translate bench work to the bedside by offering patients the latest treatments, which often stem from basic research.

CONCLUSIONS

The studies in this thesis have looked at neural stem cells isolated from adult human CNS and brain tumours. Specifically, AHNSCs were isolated from the ventricular wall and filum terminale, while TSCs were isolated from AC II and GBM.

Adult human neural stem cells can be isolated from the ventricular wall, propagated as neurospheres *in vitro* and serially passaged. Upon differentiation, AHNSCs exhibit tripotent differentiation into neurons, astrocytes and oligodendrocytes. In fact, AHNSCs can respond to differentiation cues and develop into neurons capable of generating overshooting, repetitive action potentials and communicating synaptically using functional glutamatergic and GABAergic receptors, fulfilling the hallmarks of a neuron (98). In fact, cells capable of self-renewal, proliferation and multipotent differentiation can also be isolated from hitherto unknown sources, namely filum terminale. This supports the emerging hypothesis that the CNS may be more neurogenic than previously postulated (230). Despite the similarities between AHNSCs and FTNPs, there are distinct differences between the stem cells isolated from the aforementioned sources, e.g. while the former adopts a neurogenic fate *in vivo*, the latter adopts a gliogenic fate. Our results highlight the putative neurogenic potential of regions such as filum terminale. Given that neural stem cells are potentially invaluable as tools for regenerative therapy, further research looking into long-term maintenance and manipulation of *in vivo* fate need to be addressed before clinical applications can be assessed.

Neural stem cells and TSCs share several properties, including self-renewal and proliferation (183). Given that TSCs can be isolated from low grade and high grade astrocytomas opens numerous therapeutic alternatives for the treatment of such tumours by targeting TSCs. Notwithstanding the similarities, TSCs exhibit several key differences when compared to AHNSCs including increased rate of self-renewal, proliferation as well as generation of morphologically aberrant tumour cells *in vitro* and upon transplantation. Taken together, this suggests that by conducting comparative studies one may not only elucidate the stem cell hypothesis, but identify and target TSCs while preserving AHNSCs.

FUTURE PROSPECTS

The battle for the perfect neural stem cell is still on. Research looking at standardizing and optimizing culture conditions for AHNSCs has to be intensified. Equally important, the presence of other sources of neural stem cells needs to be investigated. The potential of AHNSCs to develop into diverse phenotypes including dopaminergic neurons deserves attention. Additionally, the influence of the microenvironment on transplanted cells is a very exciting field that needs examination. The elucidation of the stem cell niche, both *in vivo* and *in vitro* is a pressing issue in basic research and learning how the niche and niche-related factors control stem cell fate and direct differentiation into particular cell types may provide new therapies in the near future. Knowledge gained may also help understand the aberrant tumour niches. Furthermore, the advent of novel technologies such as RNA interference and genomic and proteomic analyses of stem cells and their progeny, are tools that will not only help elucidate the biology and function of neural stem cells but also realize the true potential of stem cells for therapy.

Based on our studies and those of other groups, it is clear that in order to identify and target TSCs whilst preserving AHNSCs, comparative studies between the two populations are essential. Subsequently, the identified targets can be used to develop new treatments for gliomas. Comparative studies will also help elucidate the cell of origin of TSCs. Given that brain tumours are still diagnosed based on histological appearance, the stem cell model may herald in an era where diagnosis is based on the molecular signature of the TSCs. Indeed, it is possible that patient specific treatments may also be available with immunotherapy (using the body's immune system to find and destroy TSCs e.g.

vaccines) (273, 274) and gene therapy (using either suicide genes to destroy TSCs, or enhancer genes to cause an immune response against the patient's own tumour) (275-277).

Another tantalizing concept is the tumour niche, which includes the tumour vasculature (12). The niche has a protective role, harbouring the stem cells. It may also provide factors to regulate stem cell proliferation and stem cell fate (278). Thus, TSCs might be protected from conventional therapies by factors within this niche. In the future, it is highly possible that drugs that disrupt the stem cell niche may provide treatment alternatives for brain tumours, specifically GBMs.

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APPENDIX

Papers of interest:

1. Endoscopically harvested stem cells: a putative method in future autotransplantation. Westerlund U, Svensson M, Moe MC, Varghese M, Gustavsson B, Wallstedt L, Berg-Johnsen J, Langmoen IA. Neurosurgery. 2005:779-84.
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