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SYMPOSIUM: Neural Stem Cells

Human Neural Stem Cells: Isolation, Expansion and Transplantation

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Neural stem cells, with the capacity to self renew and produce the major cell types of the brain, exist in the developing and adult rodent central nervous system (CNS). Their exact function and distribution is currently being assessed, but they represent an interesting cell population, which may be used to study factors important for the differentiation of neurons, astrocytes and oligodendrocytes. Recent evidence suggests that neural stem cells may also exist in both the developing and adult human CNS. These cells can be grown *in vitro* for long periods of time while retaining the potential to differentiate into nervous tissue. Significantly, many neurons can be produced from a limited number of starting cells, raising the possibility of cell replacement therapy for a wide range of neurological disorders. This review summarises this fascinating and growing field of neurobiology, with a particular focus on human tissues.

Introduction

Work on flies, worms and amphibians has provided enormous insight into the molecular mechanisms underlying neural development. This is a result both of the ability to genetically manipulate these animals in large numbers and the accessibility of the developing embryo to observation (18, 34). However, in mammals it is more difficult to assess the intrinsic fate of related neural cells, or the effects of other cells on them. During development, mitotic regions adjacent to the ventricle are composed of neuroepithelial germinal cells which may best be described as neural stem cells (33). These proliferate within the ventricular zone, and give rise to progenitors for both neurons and glia. Many of these migrate and mature into the various regions of the CNS, while a small population may reside within this region as stem cells, possibly into adulthood (for an overview of this process see Figure 1).

Recently, cells have been isolated from the rodent CNS which can divide in culture while retaining the capacity to differentiate into neurons, astrocytes and oligodendrocytes, and as such may represent an "*in vitro*" source of neural stem cells (23, 38, 41, 44, 46, 70, 74, 88). This opens up a new way in which some aspects of neural development can be studied *in vitro*, particularly with regard to the underlying mechanisms controlling the lineage and fate commitment of different groups of mammalian precursor cells. In addition, if neural stem cells can be isolated from human tissues they may represent a rich source for a range of cell therapy programmes aimed at treating neurological disease, many of which are currently dependant on primary fetal tissues (22). But can neural stem cells be isolated from human tissues? How do they compare with their rodent counterparts and how long can they really be propagated in culture? Do they survive transplantation into the adult brain? These are the main topics of this review.

Dividing neural precursor cells in cultures of the embryonic rodent CNS

It was the pioneering work of Harrison at the start of this century, which opened the way to an alternative method of studying the developing nervous system (31). He showed that cells isolated from the embryonic CNS could be maintained outside of the body using specialised nutrient broths. Within these cultures, developing neurites were found to emerge from isolated nerve cells, thus confirming Ramón y Cajal's earlier observations of a growth cone within fixed tissue sections (58). Since these early experiments, cell culture has played a

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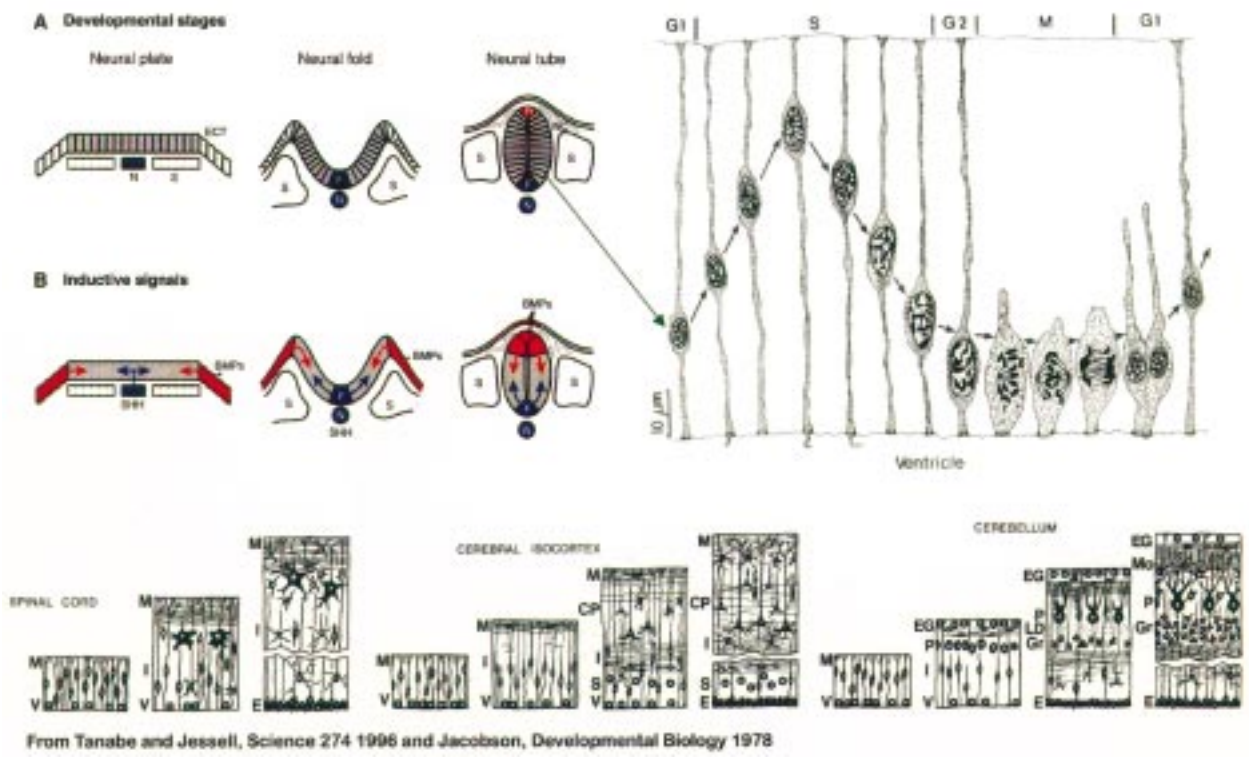


Figure 1. Schematic showing early developmental stages. (**A**) Exemplified here for the spinal cord, the neural plate folds to produce the neural tube which is lined with dividing neuroepthileal cells and a specialised floor plate region (F) below which lies the notochord (N). (**B**) Inductive signals from the floor plate (Sonic Hedgehog, shh) and roof plate area (bone morphogenic proteins, BMPs) control some aspects of differentiation (adapted from (83)). Within the neural tube, cell division occurs which is characterised by interkinetic nuclear migration between the ventricular and outer surface, with cell division actually occurring on the ventricular side (68). Through continual division followed by migration, the layers of the CNS are built up over time as shown for the spinal cord, cerebral isocortex and cerebellum. Early to late development are shown from left to right for each region. CP, Cortical plate; E, ependymal layer; EG, external granule layer; Gr, granule layer; I, intermediate zone; LD, lamina dissecans; M, marginal zone; Mo, molecular layer; P, Purkinge cell layer; S, subventricular zone; V, ventricular germinal zone. Reproduced from (33) with permission.

major role in understanding some of the basic mechanisms of neuronal maturation and axon outgrowth. The discovery that fibroblast growth factor-2 (FGF-2) could induce the proliferation of neural precursors within embryonic hippocampal cultures opened up a new avenue of investigation, where proliferation of neural precursors could also be studied *in vitro* (25). Later, a second mitogen, epidermal growth factor (EGF), was found to stimulate the division of embryonic striatal precursor cells which retained the ability to differentiate into neurons, astrocytes and oligodendrocytes (60). In addition, the ventricular zone and hippocampal regions of the adult rodent brain were also found to harbour cells which proliferated in response to FGF-2 and EGF and in many ways appeared similar to their embryonic counterparts (61, 63). Surprisingly, regions of the brain not normally associated with neurogenesis in the adult rodent also contain cells which divide in response to FGF-2, suggesting that quiescent precursor cells may be scattered throughout the entire CNS (49).

Stem cells, progenitors and precursors

There is still much debate over how to classify these germinal cells, particularly when they are removed from the developing or adult CNS and grown *in vitro*. A somewhat liberal approach to labelling a range of dividing cells as "stem cells" has been suggested recently, in an attempt to form a concept of where this very new field of neurobiology currently stands (1). Previous attempts have referred to other tissue systems where stem cells are classically defined as self-renewing, often for the lifetime of the organism, multipotent and able to regenerate damaged tissue (29). Thus, neural stem cells should be able to self-renew, retain the capacity to generate neurons, astrocytes and oligodendrocytes and be capable of replacing these cells when damaged.

Progenitors are similar, but normally restricted to either one or two lineage's and capable of fewer divisions. Precursors are simply the ancestors of subsequent generations and are taken simply to mean dividing cells - they could be either stem cells or progenitors (23). How can these criteria be met in practical terms? The benchmark method for defining a stem cell is clonal analysis where a single cell from a population of cells is plated in isolation and induced to produce daughters through division. These can later be assessed for their potential to generate neurons, astrocytes and oligodendrocytes. This method is enormously powerful when a positive result is seen, i.e. all three phenotypes are generated from a single clone, as one can say conclusively that this particular cell was multipotent and likely to be a stem cell.

Clonal experiments have shown that single cells derived from the embryonic cortex can divide a small number of times in culture and give rise to neurons, astrocytes and oligodendrocytes (17, 55). FGF-2 responsive precursors isolated from the embryonic hippocampus or spinal cord (43) or adult hippocampus (35) and grown as a monolayer culture, have also been shown to be stem cells (50). In some cases, these respond in a specific manner to exogenous factors; platelet-derived growth factor inducing neuronal differentiation and ciliary neurotrophic factor inducing astrocytic differentiation (35). A similar FGF-2 responsive cell, shown to be multipotent using clonal analysis, has been isolated from the adult striatum but this time grown as a free floating suspension or "neurosphere" culture (28). EGF can also drive a multipotent stem cell within neurosphere cultures generated from the embryonic or adult striatum (61, 62), at least at early passages, although the numbers of neurons spontaneously differentiating from these cells is very small (2). This may relate to the fact that under certain circumstances EGF drives a unipotent glial precursor while FGF drives a bipotent neuronal/glial precursor (37). Thus within EGF responsive mouse neurospheres a small number of pluripotent stem cells may be surrounded by other more restricted glial progenitor cell populations.

When a multipotent stem cell has been isolated in culture and cloned, does this mean that large populations of stem cells will be generated from it when it is driven by mitogens? Two situations are possible. In the ideal world, multipotent clones would divide symmetrically in culture, producing an endless supply of new multipotent cells (Figure 2). However, based on *in vivo* data these cells are often likely to divide both symmetrically and asymmetrically (15), leading very rapidly to a heterogeneous culture containing stem cells, progenitors and even terminally differentiated cells (Figure 2). This will, to a degree, depend on the exact culture conditions, but where large numbers of cells have been propagated *in vitro*, they are likely to be heterogeneous even when arising from a single cell. Evidence for this comes from the detailed analysis of the phenotypic potential of individual clones isolated from adult hippocampal neural stem cells. These showed a large degree of intra-clonal variation with regard to the percentage of neurons, oligodendrocytes and astrocytes obtained at plating consistent with a heterogeneous cell culture (50). It is, therefore, perhaps best to describe bulk cultures as consisting of neural precursor cells (NPCs), which are a mix of stem cells and progenitors. Recent studies have show that in the adult brain the stem cell may in fact be the ependymal cell lining of the ventricle (36). These cells divide very slowy and often asymmetrically *in vivo* but give rise to more rapidly dividing progenitors or transit amplifying cells. It is likely that the majority of stem cells within neurospheres are these transit amplifying cells, and the stem cell, by nature of slow and often asymmetric divisions, is relatively rare.

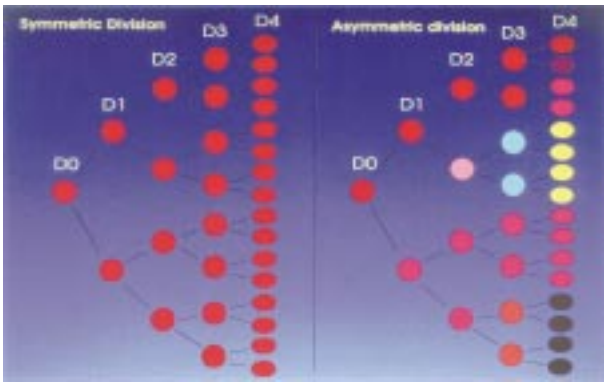
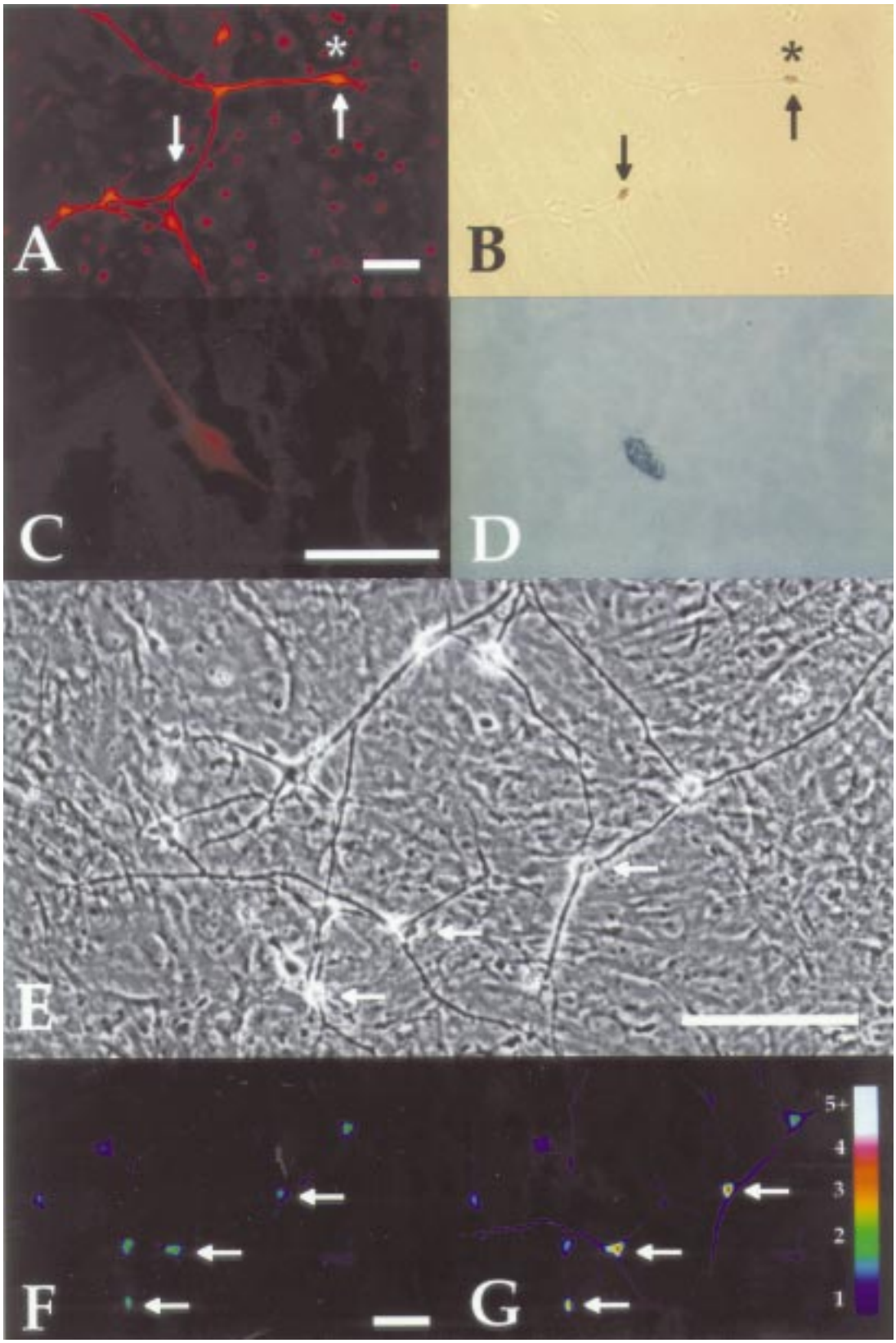


Figure 2. Schematic of the two possible types of cell division. Neural stem cells have to self renew. In symmetric divisions a single stem cell (red) is able to give rise to many other stem cells with identical properties. In reality, dividing cells in culture are more likely to undergo both symmetric and asymmetric divisions, leading to a wide variety of possible phenotypes and a heterogeneous culture after only four population doublings.

Where clonal studies show a restriction in phenotype, there is the likelihood that a progenitor has been isolated. The best studied of these is the oliogdendrocyte and type II astrocyte progenitor (O2-A) which is restricted to producing these two types of cells at the expense of neurons and has been extensively characterised (57, 85). However, it is always possible that under different culture conditions or following transplantation, progenitor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cells can express a wider range of phenotypes than seen spontaneously *in vitro* ((24) and Brüstle in this issue). Moreover, recent studies have suggested that neural stem cells derived from the CNS may also be able to give rise to cells which normally derive from the neural crest, including Schwann cells and smooth muscle (47). Experiments which have shown that quiescent nuclei from fully differentiated cells can, when transferred to an oocyte, re-express embryonic genes and generate a new organism or clone (90) suggest that our current ideas of cells with limited phenotypes may have to be radically modified in the future. Although an extreme view, it could be argued that many cell types, but in particular precursor cells capable of division, may undergo "de-differentiation" to a more primitive state given the right circumstances. The recent findings that cloned mouse neural stem cells may be able to re-populate bone marrow and transform into blood cells lends further credence to this suggestion (5). Another factor to consider when interpreting data showing restricted phenotypes arising from a given cell, is that selective cell death may eliminate a specific class of cells generated from a clone, giving the impression of uni-potency where multi-potency in fact existed (87). In summary, whether generated from a single cell or population, the phenotypic potential of precursor cells grown *en mass* will depend upon a complex interaction between both their origins, and the environment in which they are placed.

Human neural stem cells

While a large amount of evidence is accumulating to suggest that neural stem cells exist in both the developing and adult rodent CNS, what about human tissues? A cluster of recent papers show that cells derived from the embryonic human CNS can be isolated and cloned in culture ((21) and see Snyder *et al* this symposium), grown for extended periods of time *in vitro* (13, 82), and incorporate into the developing rodent CNS ((8) and see Brüstle *et al*, this symposium).

However, the first evidence that FGF-2 could induce the proliferation of human CNS precursors came over 3 years ago from the work of Buc-Caron (9). Various brain regions from embryo's between 5 and 12 weeks of age were used. Cells were expanded in the presence of FGF-2 for short periods of time as a monolayer and on a Matrigel substrate (which contains both laminin and trace amounts of growth factors) in the absence or presence of serum. Proliferation was recorded for up to six weeks although there was no quantification of population doublings. A high percentage of cells were found to express nestin, a marker of early neuroepithelial precursors (40). In addition, markers for neurons, astrocytes and oligodendrocytes were also found suggesting that a neural stem cell might be dividing in these cultures, but not ruling out the possibility that a multitude of different non multi-potent lineages were dividing along side each other. We found that cells isolated from the mesencephalon of older human embryo's (>13 weeks), but not younger ones, could be induced to divide with EGF and grew as neurospheres which appeared similar, in appearance at least, to those seen in the rodent (77). Due to problems with inducing precursors to divide from early human embryos with EGF, we began to use FGF-2 and showed that it was possible to stimulate the division of early human precursors as neurospheres with this mitogen, that it acted synergistically with EGF, and that expanded populations of cells could again give rise to neurons, astrocytes and oligodendrocytes at early passages (76). Another report has described similar problems with regard to inducing the division of human neural precursors from young cortical tissues (6-8 weeks) with EGF alone, and needed to use both 5% horse serum in combination with insulin like growth factor-I to expand the cells as neuropsheres (14).

Oligodendrocytes are often seen to differentiate from FGF-2 responsive human precursors, but have been most fully characterised by Murray *et al* (48). They showed that FGF-2 responsive neural precursors could be isolated from 8 week old human fetal CNS tissue and formed spheres in culture when seeded onto fibronectin coated flasks. Cells required brief exposure to serum to attach, but then remained in defined serum free condi-

Figure 3. (Opposing page) Serially applied FGF-2 and BDNF allow expansion and survival of neurons arising from the human subependymal zone (SZ). (**A**) Cell outgrowth from adult human SZ at 9 weeks in vitro. The explant was exposed to FGF-2 for a week in the presence of [^{3}H] thymidine, then to BDNF for 2 months, and subsequently fixed and stained for the neuronal marker microtuble associated protein-2. (**B**) Two of these MAP-2+ cells incorporated [^{3}H] thymidine during their first week in vitro (aggregations of silver grains denoted by arrows), indicating mitotic neurogenesis during the period of FGF-2 exposure. (**C**) High power of cell in A and B (asterisk), stained for MAP-2. (**D**) After [^{3}H] thymidine autoradiography. (**E**) Another outgrowth from an adult temporal SZ explant, 7 weeks in culture. This sample was also raised in FGF-2 followed by BDNF, and then subjected to confocal imaging of the fluorescent signal emitted by the calcium indicator dye fluo-3; this was perfomed to assess neuronal responses to depolarising stimuli. (**F**) Baseline fluo-3 fluorescence signal from the neurons indicated in phase in E (arrows). (**G**) Immediately after depolarization by 60mM KCL. A similar response to 10μM glutamate was observed (not shown). Bar in A-D = 25μm; bar in E-G = 75μm. From (52) with permission.

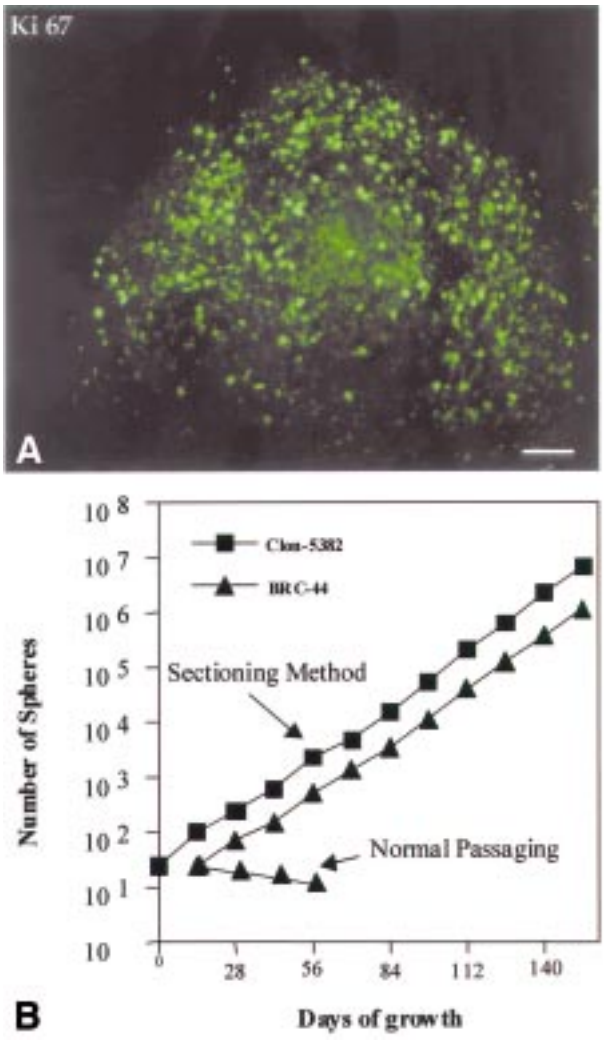

Figure 4. (**A**) Photomicrograph of a whole human neurosphere grown in FGF-2 for 100 days and stained for Ki67 which labels the nuclei of dividing cells (green). Note the many positive cells, particularly around the periphery of the sphere, suggesting extensive proliferation is occurring even at this late stage in culture. (**B**) Systematic counts of the total number of spheres at each passage (grown in FGF-2 and serum free media) was established using conventional methods where the spheres are triturated with a narrow pipette tip (normal passaging) or sectioned into quarters with a scalpel (sectioning method of passaging). Expansion of total cell number is only seen with the latter method under these conditions. Cells were derived from the cortex of an 7 week (BRC-44) or 22 week (Clon-5382) human embryo (16).

tions where active proliferation took place. Removal of FGF-2 allowed the differentiation of spheres which formed "chains" of migrating cells which were PSA-NCAM positive. The majority of these cells developed into neurons, but a small number of oligodendrocytes were also seen which could be increased in number by the addition of thyroid hormone T3.

Together, these papers show "proof of concept" that human NPCs (HNPCs) can be maintained in culture and may be similar to their rodent counterparts, but issues of regional specificity, long term growth and clonal analysis were not addressed in these papers. Interestingly, small numbers of dividing cells have also been found in cultured hippocampal slices taken from the adult human brain (39). In recent extensions to these original observations, brain derived neurotrophic factor (BDNF) has been shown to induce outgrowth from newly generated, adult derived human neurons responsive to FGF-2 [(52); Figure 3]. These studies, taken together with recent reports showing that dividing cells exist in the adult human hippocampus *in vivo* (19), raise the exciting prospect of generating neural precursor cell populations from the fully mature human CNS (27).

The Holy Grail of limitless neural tissue?

Aside from the obvious interest in HNPCs as a window on neural differentiation, they are also sought after by pharmaceutical companies and neural transplantation programmes, providing they can be significantly expanded and induced to mature into functioning neurons. A common (and incorrect) assumption, is that once a precursor is isolated and induced to divide in culture, it will continue to grow indefinitely. However, those who regularly work with dividing cells in culture know that this is not the case. Somatic, non-transformed cells are mortal. Following a set number of population doublings, ranging between 30 and 50 for most tissues, they enter replicative senescence and stop dividing (for review (32)) which may be a direct result of protective telomeres being eroded from the ends of chromosomes at each division (6). This has been well characterised in many cell types, but has not been widely discussed in the context of NPCs. There are few reports where the actual numbers of NPCs harvested at each passage have been analysed over long periods of time, from either rodent or human tissues. This is required in order to establish approximately how many doublings have occurred within a population. In one study, log growth of mouse EGF responsive precursor cells grown as neurospheres was recorded (62). We attempted to reproduce these results under identical conditions using rat in addition to mouse tissues. Although the mouse neurospheres could be grown for long periods of time, cells within the rat neurospheres consistently underwent what appeared to be replicative senescence at approximately 8 population doublings (80). This is remarkably similar to the

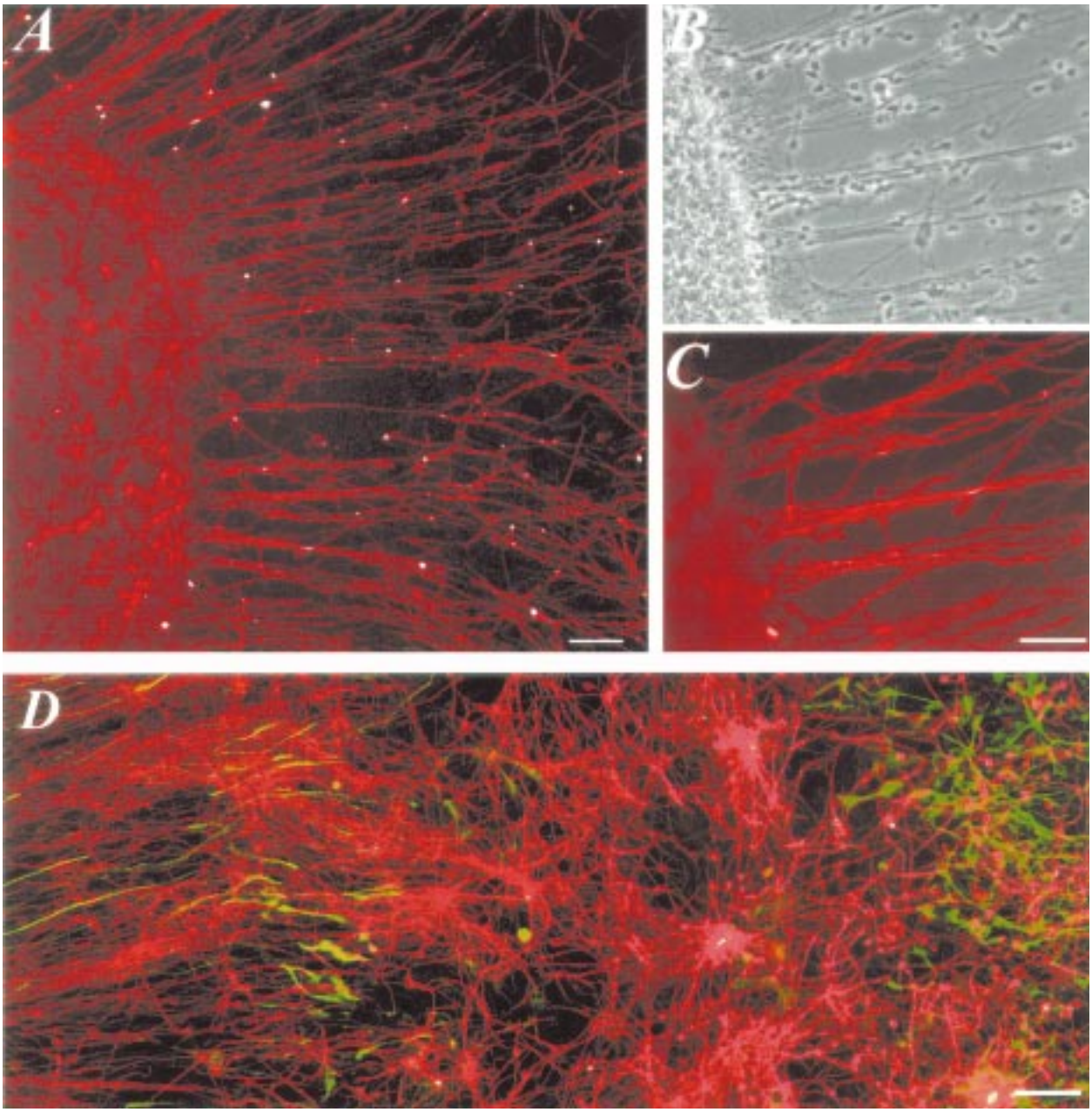

Figure 5. Whole spheres (grown for 150 days in FGF-2) were plated onto lamanin coated coverslips in the absence of FGF-2 and left for 14 days. (**A**) Radial processes could be seen to have extended from the core of some spheres along which extensive migration of neuronal precursors could be seen. Stained for TuJ1 (red) , a specific neuronal marker (45). Scale bar = 70μm. (**B**) and (**C**) show the same field under phase or stained for TuJ1 respectively from a similar culture. Note the small neuronal cells along the radial processes. Scale bar = 25μm (**D**) Lower power image showing the region of a similar human neurosphere (plated to the left and out of view) and demonstrating the large number of neurons and astocytes generated by a single sphere. Glial fibrilary acidic protein (GFAP), an astrocyte marker, in green and TuJ1 in red. Scale bar = 100μm.

number of divisions which O-2A progenitor cells are able to undergo before spontaneously differentiating in culture (56), and may be due to an intrinsic timing mechanism which limits their number of divisions. Interestingly, similar studies using rat FGF-2 responsive precursors grown attached to a substrate appear in some cases to grow for much longer periods (49, 59) although cumulative cell counts are not given for these cultures

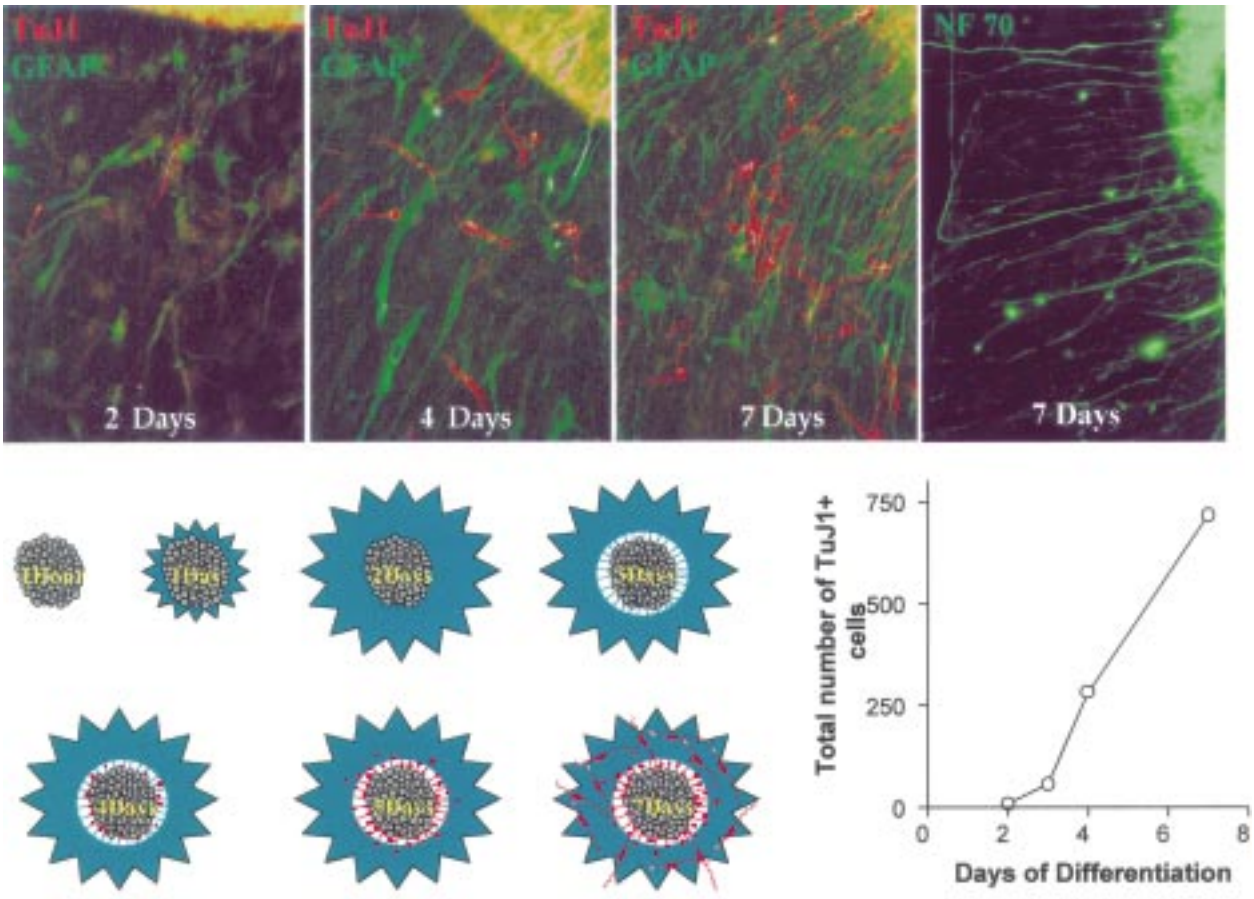

Figure 6. Photomicrographs show the sequential appearance of astrocytes (green) followed by neurons (red) when whole human neurospheres are plated and allowed to differentiate as described in Figure 5. The first cells to emerge from the sphere are almost exclusively astrocytes but are followed after a latency of 24 to 48 hours by migrating neurons. The last panel shows staining of the same sphere for neurofilament 70, another specific neuronal marker. The schematic shows this process in a stylised form and the graph shows cell neuronal cell counts from around the sphere at increasing stages of differentiation.

beyond 6 weeks of growth. In our hands, human neurospheres only underwent approximately 4 to 6 population doublings using standard passaging techniques (76). Murray et al (48) indicate that cultures of human neurospheres could be expanded for up to 7 months through 8 passages but provide no information on either the growth rates during this time, or the differentiation potential of the cells following extended expansion.

As the amounts of growth that we, and others, were achieving in culture appeared to be well below the Hayflick limit of 50 population doublings (32), we set out to investigate factors which might be responsible for the lack of division at later time points. After attempting a number of modifications to the culture medium we did discover that heparin sulphate was crucial for the actions of FGF-2 on free floating neurospheres (but interestingly not so important for the growth of plated precursor cells) (10). However, this, or other modifications, had no effect on the senescence seen at later stages of growth. We next turned to the way in which the cells were passaged. As the spheres become larger they begin to lose the ability to pass nutrients to their cores and as such begin to slow their growth rates. At this stage the conventional method for keeping the culture going is to break the sphere down to single cells and then re-plate into new flasks. If enzymes such as trypsin are used during this process, the continuation of growth is often very slow following passaging, perhaps due to the removal of vital receptors. Furthermore, many cells appear to spontaneously differentiate following trypsinisation, further slowing the rate of growth. If no enzymes are used new spheres will form slowly over time but there is generally over 50% cell death and so half the culture is lost, significantly reducing its overall expansion rate.

There are some reports where cells in clusters of freshly plated rodent CNS precursors remain mitotically active, whereas isolated cells within the same cultures do not often divide (26). This, and the fact that membrane associated factors can stimulate division of NPC's (84) suggested that cell/cell contacts and the extracellular matrix are vital for efficient cell division *in vitro*. In light of these studies, and our previous observations that cell density is crucial to efficient proliferation of NPC's (78), we developed a novel passaging method in which whole human neurospheres, grown from 8 or 20 week old cortex in a combination of EGF, FGF-2 and heparin, were sectioned into quarters rather than mechanically dissociated. Cells within each quarter maintained contact with each other following passaging, and each quarter rapidly rounded and began to grow, generally reaching the size of the mother sphere within 14 days (82). Using this simple method, long term exponential expansion could be achieved for up to 200 days and spheres contained large numbers of dividing cells (Figure 4). The overall growth rate was slow compared to rodent cells with a population doubling period of approximately 4-5 days. At later passages, cells divided in response to either EGF or FGF-2 alone, although in FGF-2 the neurospheres often formed disc structures which adhered to the culture dishes (82). All cultures stopped dividing between 250 and 300 days and appeared to either undergo senescence and remain undifferentiated, or spontaneously differentiated into either a neuron or an astrocyte. At this stage, most cultures had undergone approximately 30 population doublings (a theoretical increase of 1 billion fold) and it is possible that the natural Hayflick limit for these cells had been reached leading to cessation of division. This data is similar to work on propagating human astrocytes, which have been shown to achieve 40 population doublings before reaching replicative senescence (53). The fact the cells stopped dividing is important as it shows that the cells have not oncogenically transformed in culture.

The remarkable aspect of these human cells was that they continued to produce over 40% neurons when allowed do differentiate at either 50 or 150 days of expansion (Figures 5, 6). The majority of these were GABAergic, a topic discussed further in a later section This is in direct contrast to mouse neurospheres grown under very similar conditions which generate less than 5% neurons even at early passages (2), and rat neurospheres in which the percentage of neurons decreases with each passage to less than 1%, up until senescence at around 5 weeks (Rosser and Svendsen, in preparation). Furthermore, whereas human FGF-2 responsive

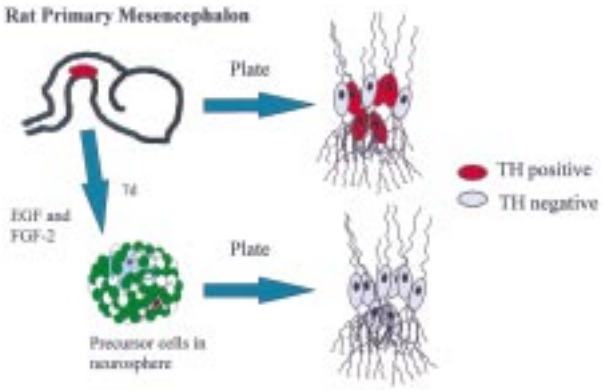

Figure 7. Rat primary mesencephalon contains many dopaminergic neurons, which express tryrosine hydroxylase when plated and cultured for 7 days (TH; red cells). However, if the same cells are expanded for 7 days and then plated, the number of TH positive neurons reduces dramatically, suggesting that the dividing cells lose their capacity to generate TH neurons spontaneously.

neurospheres grown for short periods of time gave rise to some oligodendrocytes, very few were found to differentiate from long term human neurospheres (82). This was again in contrast to mouse and rat studies where large numbers of oligodendrocytes can be found at all passage times and are able to re-myelinate lesions of the spinal cord (30). Efforts to generate more oligodendrocytes from human precursor cells are currently underway. Interestingly, canine and rodent "oligospheres" have been described recently using a method where the culture is supplemented with B104 conditioned medium (3, 92, 93). Although it is not clear whether this medium will also be able to support human neurospheres with the capacity to differentiate into large numbers of oligodendrocytes, there is some evidence that conditioned medium from other cell lines may support such cultures (Zhang and Duncan, personal communication). It is of interest that following immortalization with v-*myc*, human neural precursors have been cloned and shown to generate either neurons alone, or neurons and astrocytes but not oligodendrocytes (66), which would appear to be very similar to the long term human neursophere cultures described here.

Our attempts at clonal analysis using human NPC's have so far been unsuccessful, and we have therefore been unable to determine the potential of individual cells. This possibly reflects an extreme sensitivity of single HNPCs in a culture dish, which may require specific additions to the medium in order to survive and divide. Until we perform these analyses it is possible that we are growing either (i) a bi-potent precursor capa-

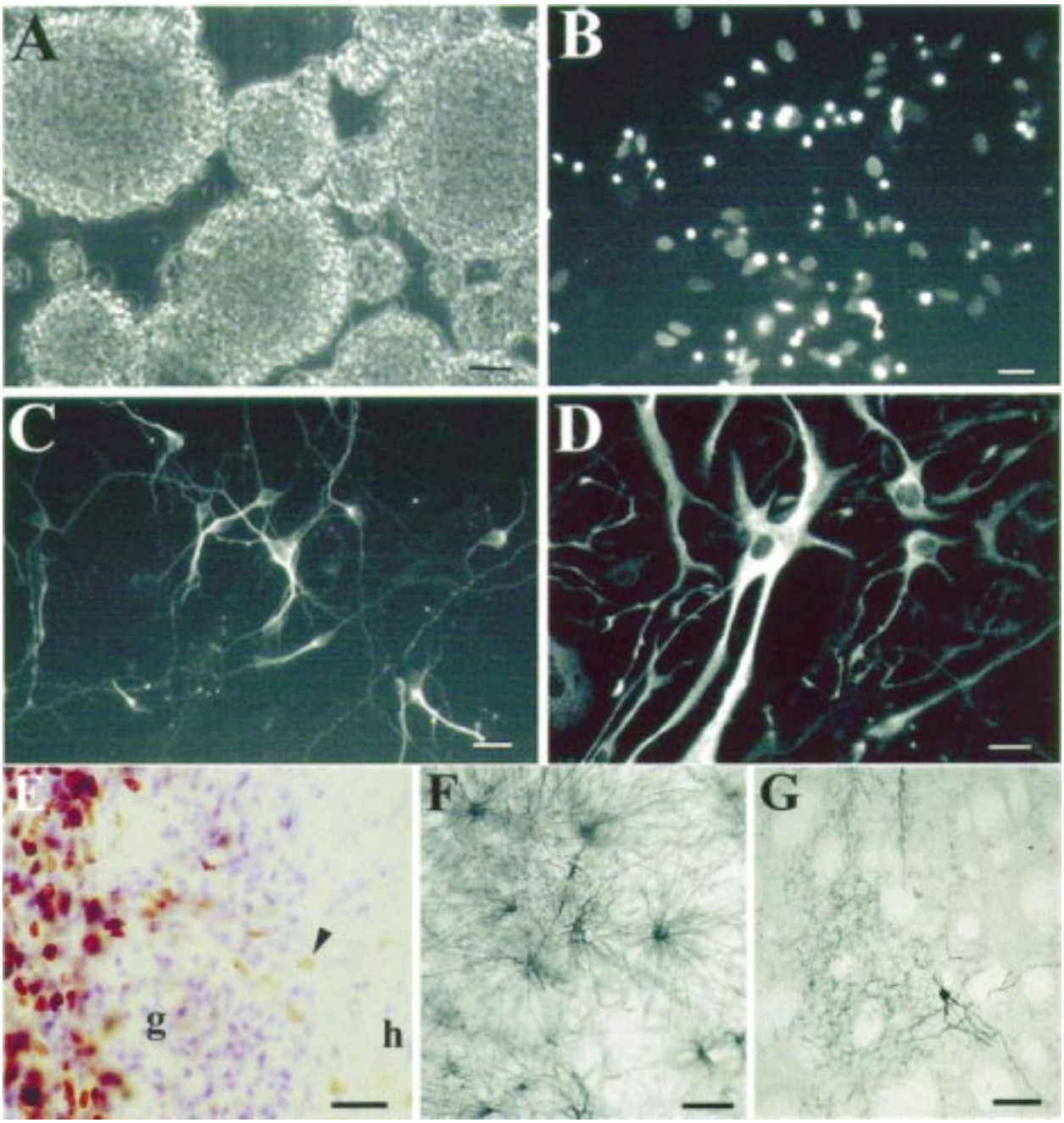

Figure 8. Human NPCs isolated from a 22 week old cortex were expanded *in vitro* for 21 days as neurospheres (**A**) with EGF and FGF-2. (**B**) Following pulsing with BrdU, dividing cells were plated and stained at 7 days with antibodies against BrdU (**B**) TuJ1 (**C**) or GFAP (**D**). Note the large number of BrdU positive cells and mixture of neurons and astrocytes labelled. (**E**) BrdU labelled cells injected into the 6-OHDA lesioned striatum began to migrate out from the core of the graft (g) at two weeks post transplantation. As they enter the host tissue (h) they have a weaker stain (arrowhead) suggesting that they are actively proliferating while migrating. (**F**) Over the next 20 weeks many cells migrated from the graft core and matured into astrocytes labelled here with human GFAP, a specific marker for human astrocytes. This human GFAP antibody will label highly reactive rodent astrocytes following a lesion, but not 20 weeks after the lesion. All sham grafted animals were negative for human GFAP staining at 20 weeks. (**G**) Staining for TH which labels dopaminergic neurons revealed a number of positive cells in two animals which had extensive ramifications into the striatum. These cells were found around the graft site in close proximity to human Tau stained neurons (not shown) and were not seen in any of the sham grafted animals. Taken from (76) with permission.

ble of making neurons and astroctyes, (ii) two unipotent cells, one able to produce neurons and the other astrocytes or (iii) a multpotent precursor which would produce oligodendrocytes under different culture conditions.

We were also interested in establishing whether rat neurospheres could be expanded beyond 5 weeks using this new sectioning method. However, in all cases the rat neurospheres underwent senescence irrespective of the passaging technique. Furthermore, they often appeared to adhere more readily to the surface of the culture dishes, and undergo spontaneous differentiation at later passages (Svendsen *et al*, unpublished observations). These studies highlight the differences between human and rodent neural precursor cell growth in culture and emphasise the need for detailed characterisation of culture systems for each species.

Very recently, another group have shown a similar problem maintaining dividing HNPCs for long periods of time under normal culture conditions, but have shown that leukaemia inhibitory factor (LIF) added to the medium will allow continual cell growth to occur, albeit at a slower rate than in our system (13). LIF acts through the gp130 signal transducing subunit and is also required for the continual growth of embryonic stem cells, and appears to maintain these cultures in a proliferative state by preventing differentiation (73). Although best characterised as a growth factor of the immune system, it is also now seen as having a wide range of effects on the CNS (51) most of which have focussed on its ability to promote differentiation rather than prevent it (64). Whether the inclusion of LIF in the medium and our method of sectioning share a common mechanism with regard to extended growth of human NPC's, and whether the type of cell propagated in both cases is the same, is presently unclear. Interestingly, using LIF in the medium results in the continual generation of small numbers of oligodendrocytes, in contrast to the sectioning method where none can be found. Continual modifications to culture mediums and passaging techniques will, no doubt, result in many other methods of long term growth for HNPCs over the coming years.

Induction of neural phenotypes.

Establishing that neural precursors can be expanded for long periods of time *in vitro* is exciting. However, what phenotype do neurons arising from such cells express? The consensus for rodent precursors, generated in many different ways from a variety of embryonic or adult brain regions, is that they appear to be extremely plastic at early passages, differentiating into neurons, astrocytes and oligodendrocytes in response to growth factors and other additions to the medium and express a variety of developmentally regulated genes (for thorough reviews see 11, 41). There is also general agreement from the majority of reports on this subject, that newly generated neurons derived from expanded populations of precursor cells are to a large degree GABAergic. However, following transplantation back into the adult rodent CNS these cells appear to respond to the local environment and produce a wider range of phenotypes, as discussed below.

Attempts to induce dopamine neurons from precursor cells have been the focus of many studies, due mainly to the interest in producing such cells as a source of tissue for transplantation therapy in PD (79). It has been shown that in the presence of FGF-2, precursor cells isolated from the developing rodent mesencephalon will undergo at least a few divisions while retaining the capacity to generate another dopamine neuron, but subsequent divisions lead to precursors which can no longer spontaneously generate dopamine neurons (7, 10) (Figure 7). This allows short, but not long term expansion of the primary dopamine neuroblasts. There appears to be evidence that Interleukin 1 is able to induce the dopamine phenotype in a large proportion of mesencephalic rodent precursors, and that this effect can be significantly enhanced by adding membrane fragments, conditioned medium, IL-11, LIF and growth factors such as GDNF (42). However, induction of the dopaminergic phenotype has been more difficult using the same method on HNPCs at later passages (13). We have assessed the effects of this cocktail and sonic hedgehog and FGF-8 on induction of dopaminergic phenotypes from the human precursors, previously shown to be involved in this process *in vivo* (91), with negative results (Svendsen *et al*, unpublished observations). Does this mean that the synchronous growth of HNPCs leads to a cell which can no longer respond to cues vital to differentiation? We think this unlikely due to recent studies where these cells have been transplanted into either the embryonic or adult rodent CNS as described in the next section. Furthermore, transcription factors such as *Nurr1* and *Ptx3*, have been discovered which may play an important role in the induction and phenotypic maturation of dopamine neurons (67, 71). Over-expression of these transcription factors in neural precursor cell populations, in combination with the appropriate growth factor treatments may yet lead to the controlled generation of dopamine neurons.

Transplantation of human NPC's into the developing or adult CNS

When primary rodent tissues, which contain a high proportion of precursor cells, are transplanted back into the developing CNS they appear to migrate widely with little preference for the location from which they originated (12, 20). In other studies hippocampal precursor cells have been expanded for significant lengths of time *in vitro* and subsequently injected back into various regions of the adult CNS. When placed in the rostral migratory stream they have been shown to migrate along with host cells and, upon arrival in the target zone, differentiate into neurons in a site-specific manner, even generating dopamine neurons in some cases (75). However, in other non-neurogenic brain regions less cells survive and differentiate (75). These, and many other rodent precursor transplantation studies, have been reviewed in detail recently (24) and are discussed further in this symposium (see Brustle chapter), but what about the transplantation of human neural precursors?

Transplantation of HNPCs into either the embryo or neonate are discussed elsewhere within this symposium (Brustle and Snyder chapters). Here transplantation into the adult CNS will be considered. In one of the first studies, cells isolated from the cortex of a 12 week old human fetus were exposed to FGF-2 for 11 days *in vitro*, infected with an adenovirus encoding the *lac*Z gene and then transplanted into the striatum of immunosupressed rats (65). Although no cell survival was found in grafts of 600,000 cells or less, three out of four animals with a million cells or more transplanted had surviving grafts at three weeks, which contained a number of beta galactosidase expressing neurons. As the original cultures had not been passaged, and the cells within the grafts were not labelled with mitotic markers to prove they had divided in culture, it is possible that these neurons may have been primary cells from the initial culture, or cells which had not undergone division. We addressed this issue directly by transplanting two populations of human precursors into the striatum of rats with 6-OHDA lesions of the dopaminergic neurons within the ventral mesencephalon. EGF responsive cells isolated from the ventral mesencephalon were grown for either 10 or 28 days and then transplanted. We found that whereas the 10 day old cultures gave rise to solid grafts which contained a number of dopaminergic neurons, cultures grown for 28 days had no discernible graft mass although there was the occasional dopaminergic neuron within the lesioned striatum (77). Thus, cells which had been passaged to remove primary neurons appeared very different to cells which still contained primary neurons, perhaps relating to the loss of appropriate differentiation signals with increasing divisions as discussed in the previous section. In a later study we showed that when expanded populations of HNPCs (between 14 and 28 days of growth, passaged every 7 days) isolated from the developing cortex were transplanted into lesioned animals, solid grafts were found at 2 weeks post transplantation, but these reduced in size over time such that by 20 weeks only a thin strip of cells remained (76). It should be noted that in this study between 200,00 and 500,000 cells were transplanted, and that higher cell densities may result in a large graft mass remaining at later survival times. Using a variety of human-specific antibodies, we were able to demonstrate that a large number of cells had migrated out and differentiated into astrocytes while a far smaller percentage had differentiated into neurons (Figure 8). Interestingly, in two animals a significant number of these neurons had become dopaminergic by 20 weeks and were able to reverse rotational deficits associated with the lesion (76).

This study provides the first evidence that HNPCs can engraft into the adult CNS, mature into dopamine neurons and restore function in a rodent model of PD. Why did this only occur in a small number of animals? This in part reflects biological variability in terms of haplotype and HLA status in a cohort of wild type rats. We are currently exploring this further by transplanting HNPCs into immunodeficient rats and assessing other types of immune suppression in addition to cyclosporin, which has normally been used in our studies. However, the fact that some of these cells can develop into mature dopaminergic neurons following transplantation into this rat model of PD lends impetus for future studies in this area, and provides proof of concept that these HNPCs are capable of maturing into functioning dopamine neurons *in vivo*.

Conclusions

This review covers the recent developments in human neural progenitor and stem research, from basic developmental biology through to possible therapeutic applications. Recent evidence suggests that a human neural stem cell does indeed exist (21), although arguments over the percentage of these cells in various model systems will no doubt persist for many years. In strict semantic terms, cells within such cultures should be referred to as neural precursors. More specifically however, the term neural stem cell is a useful description (and appears in the title of this article), as it is then possible to relate them to embryonic, blood and skin stem cells which have been well characterised as stem cells

and have a more clearly defined biology (29, 54, 89). It is a particularly exciting time for stem cell biology since recent studies have shown that human embryonic stem (ES) cells, capable of producing all tissues of the body, can be grown in culture from human fertilised eggs or the gonadal tissue of the developing fetus (69, 86). ES cells will be important for studying the molecular mechanisms of human development, and it may soon be possible to derive neural stem cells from them directly, thus greatly facilitating the possibilities for genetic engineering ((72, 81) and see Isacson et al, this symposium). The ability to grow large populations of human NPC's will provide a source of tissue for many types of applications, from developmental biology through to drug screening and cell therapy. In addition, the recent demonstration that rodent neural stem cells may be able to form blood expands the possible therapeutic horizons further still (4). However, the characterisation of human cells in culture and elucidation of those genetic and epigenetic factors which control their fate, are a prerequisite for their use in a clinical setting. This is particularly pertinent, as there are often significant differences between these human cells and their rodent counterparts. The next few years should begin to see these goals being met.

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