Regulatory genes controlling cell fate choice in embryonic and adult neural stem cells

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Abstract

Neural stem cells are the most immature progenitor cells in the nervous system and are defined by their ability to selfrenew by symmetric division as well as to give rise to more mature progenitors of all neural lineages by asymmetric division (multipotentiality). The interest in neural stem cells has been growing in the past few years following the demonstration of their presence also in the adult nervous system of several mammals, including humans. This observation implies that the brain, once thought to be entirely post-mitotic, must have at least a limited capacity for self-renewal. This raises the possibility that the adult nervous system may still have the necessary plasticity to undergo repair of inborn defects and

Neural stem cells (NSCs) are the most immature progenitor cells in the nervous system and are defined by their ability to give rise to more stem cells by symmetric division (selfrenewal) as well as to more mature progenitors of all neural lineages by asymmetric division (multipotentiality). They have been described in a variety of areas in the developing nervous system, as well as in the subventricular zone (SVZ) and the hippocampus of the adult brain (Gage 2000; Anderson 2001; Alvarez-Buylla and Garcia-Verdugo 2002), although their presence in the hippocampus has been recently challenged (Seaberg and van der Kooy 2002). However, their frequency drops rapidly during development, from over 50% in the neural tube at E8.5 to less than 1% in the SVZ at P1 (Temple 2001). Owing to the lack of suitable markers, NSCs cannot be identified in vivo and are defined by their behaviour in culture. In vitro, NSCs exposed to high concentrations of mitogens such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) proliferate to form floating neurospheres (Reynolds and Weiss 1992; Davis and Temple 1994). Upon dissociation into single cells they give rise to new neurospheres (self-renewal) and following withdrawal of the growth factors can differentiate

acquired injuries, if ways can be found to exploit the potential of neural stem cells (either endogenous or derived from other sources) to replace damaged or defective cells. A full understanding of the molecular mechanisms regulating generation and maintenance of neural stem cells, their choice between different differentiation programmes and their migration properties is essential if these cells are to be used for therapeutic applications. Here, we summarize what is currently known of the genes and the signalling pathways involved in these mechanisms.

Keywords: asymmetric division, cell fate, neurogenesis, neural stem cells, transcription factors.

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into neurones and glia (multipotentiality) (Lois and Alvarez-Buylla 1993; reviewed in Gage 2000; Rietze *et al.* 2001).

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Abbreviations used: bFGF, fibroblast growth factor; bHLH, basichelix loop helix; BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; C/EBP, CCAAT/enhancer-binding protein; CNTF, ciliary neurotrophic factor; EGF, epidermal growth factor; ESC, embryonic stem cells; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; GRP, glial restricted precursor; Id, inhibitor of differentiation; IGF, insulin-like growth factor; LIF, leukaemia inhibitory factor; MBP, myelin basic protein; N-Cor, nuclear receptor co-repressor; Ngn, neurogenin; NRP, neuronal restricted precursor; NSC, neural stem cell; NT, neurotrophin; OLP, oligodendrocyte precursor; PDGF, plateletderived growth factor; RMS, rostral migratory stream; SGL, subgranular layer; Shh, sonic hedgehog; SVZ, subventricular zone; TAP, transit amplifying population; TH, thyroid hormone; VZ, ventricular zone.

(Carpenter et al. 1999; Villa et al. 2002; Nunes et al. 2003). This observation implies that the brain, once thought to be entirely post-mitotic, must have at least a limited capacity for self-renewal, possibly related to the formation of local circuits involved in memory and learning. Stimuli external to the nervous system, such as changes in the environment, appear to regulate adult neurogenesis (Gould et al. 1999; Kemperman et al. 2002), further supporting a mutual relationship between neurogenesis and behaviour. NSCs have also been shown to spontaneously migrate to areas of lesion, most notably ischaemic (Veizovic et al. 2001; Kokaia and Lindvall 2003) and neoplastic (Aboody et al. 2000), where they actively participate in repair. These findings raise the possibility that the adult nervous system may still have the necessary plasticity to undergo repair of inborn defects and acquired injuries, if ways can be found to exploit the potential of NSCs (either endogenous or derived from other sources) to replace damaged or defective cells.

It has indeed been shown that embryonic or adult NSCs survive well and can migrate over long distances when transplanted in developing or adult brain in several mammalian species (Suhonen et al. 1996; Brustle et al. 1997; Flax et al. 1998; Vescovi et al. 1999; Wichterle et al. 1999; Wu et al. 2002), although whether these cells can differentiate into the correct functional cell type remains to be determined. It is known that they differentiate into different lineages when injected in different regions and that NSCs derived from different embryonic stages behave differently (Temple 2001). It appears that neurogenesis and repair depend on an interplay of endogenous genetic programmes, soluble factors and cell-cell and cell-extracellular matrix contacts. A thorough understanding of the molecular mechanisms regulating generation and maintenance of NSCs, their choice between symmetric and asymmetric division and between different cell fates, and their migration properties will be the foundation on which therapeutic strategies are built.

In this review we summarize our current knowledge of the genes and molecules involved in the control of stemness and commitment of NSCs during development and in the adult. The major issues concerning their use in the treatment of neurological diseases and cancer will also be discussed.

Stem and progenitor cells

Definition of stem cells

Discussing the genes involved in cell fate choice of NSCs, it would be desirable to first give a clear definition of the cells involved. However, as discussed by Seaberg and van der Kooy (2003) in a recent review entirely dedicated to the definition of NSCs, this is not easily done. There is a general agreement that NSCs, in a similar manner to haematopoietic stem cells, do not give rise directly to the different lineages of terminally differentiated cells, but there is still much uncertainty as to where the line separating NSCs and more mature progenitors as defined by McKay (1997) should be drawn. NSCs, as stem cells from any other tissue, are defined by two properties: perpetual self-renewal and the capacity to give rise to multiple mature cell types. The problem arises when one has to define these parameters, and has been thoroughly discussed in the aforementioned review. However, the issue is so important that it deserves to be considered briefly here. First, how long is perpetual? If we are assaying in vivo, this should mean for the lifetime of the organism. However, by this criterion, most of the NSCs present in the embryo should not be considered stem cells, as their number is greatly diminished at P0 (Temple 2001), indicating that the vast majority has either differentiated terminally or died. Even adult NSCs present in the subependyma do not meet this strict criterion, as it has been reported that their number decreases in senescent animals, although this may be due to decreased proliferation (Tropepe et al. 1997). As pointed out by Seaberg and van der Kooy (2003), the definition in vitro is even more difficult as, under the current conditions, NSCs change their properties after a prolonged time in culture, and even become tumorigenic.

Second, should these properties be spontaneously shown by the cell, or should we consider stem cells as any cell, even differentiated, that can be induced to behave as such? It has been shown for instance that progenitor cells can revert to a multipotent cell status when cultured in the presence of EGF (widely used when culturing 'stem cells') (Doetsch *et al.* 2002) and oligodendrocyte progenitor cells derived from the optic nerve have been reprogrammed to exhibit stem-like properties *in vitro* (Kondo and Raff 2000a).

It is our opinion that, although stemness may be a biological function that can be induced by appropriate reprogramming, bona fide stem cells do exist in the SVZ and should be defined by their behaviour in vivo and in vitro, and should be distinguished from progenitor cells whenever possible. In the case of NSCs, the lack of specific markers makes this distinction difficult in vivo. Culturing these cells is not of much help as, under current culture conditions, they do not divide symmetrically as a pure stem cell population. Typically, they mostly divide asymmetrically to generate neurospheres in which most of the cells are more mature progenitors, still capable of limited (perhaps not so limited in the presence of EGF; see Doetsch et al. 2002) self-renewal and often multipotent. One should then be aware of the fact that all in vitro experiments are carried out using such mixed cultures, from which it is not easy to extract information about the behaviour of the small and variable fraction of true NSCs (Galli et al. 2003). Several attempts have been made to find molecules that can distinguish NSCs from progenitor cells, but combinations of markers that can be used to separate the different subpopulations have been described only recently (Uchida et al. 2000; Rietze et al. 2001; Capela and Temple 2002) and require quite a long procedure. On the other hand, progenitor cells isolated from other regions of the brain clearly have a limited capacity for self-renewal and are not multipotent. Thus, in a recent study that challenged the presence of stem cells in the adult dentate gyrus, two different progenitors were found in the hippocampal subgranular layer (SGL), one generating neurones and the other glial cells (Seaberg and van der Kooy 2002). Dissociation of primary colonies of these two types of progenitors into single cells never produced secondary colonies (Seaberg and van der Kooy 2002). In no instance did the investigators find cells capable of generating both neural cell types and of longterm self-renewal in this area, whereas such cells could easily be isolated from the SVZ; it was therefore concluded that NSCs only persist in the subependyma and are the likely precursors of the progenitors found in the dentate gyrus (Seaberg and van der Kooy 2002). An extra-SGL origin of the SGL progenitors is also suggested by other experiments (Nakatomi et al. 2002; discussed in more detail in the section on migration and lesion repair). Notwithstanding this uncertainty, to avoid confusion, in this review we will follow the nomenclature proposed by (McKay 1997), using the term NSCs only for the multipotent self-renewing precursor, the term progenitor for cells with a more restricted potential, and the term precursor for any cell that precedes another in a developmental pathway.

Stem cell lineage

The two main questions about NSC lineage are the origin of the NSCs themselves and the steps leading to the generation of more differentiated cells.

As to the origin, several lines of evidence indicate that in the developing mammalian neocortex radial glia are NSCs. Thus, not only it has been shown that radial glia generate neurones in the developing embryo (Gotz *et al.* 2002; Heins *et al.* 2002; Malatesta *et al.* 2003), but that they also persist in the early postnatal ventricular zone (VZ) serving as progenitors of new neurones (Tramontin *et al.* 2003).

In the adult, a long series of experiments carried out by Alvarez-Buylla and co-workers (reviewed in Alvarez-Buylla and Garcia-Verdugo 2002) has led to the conclusion that NSCs are the SVZ astrocytes, which are functionally quite different from terminally differentiated astrocytes. On the basis of all these findings, it has been proposed that NSCs develop along a neuroepithelial–radial glia–astrocyte lineage (Tramontin *et al.* 2003).

The same group has also been able to define four types of cells in the SVZ, three of which (A, B and C cells) are actively dividing. The fourth type, the ependymal cells (E cells) do not show any division when analysed by electron microscopy (Doetsch *et al.* 1999), making it unlikely that they may function as stem cells, as suggested by Johansson *et al.* (1999). The A cells are young neurones still capable of division which form chains of migrating cells surrounded by

C cells with astrocytic characteristics. The nearby B cells are very similar to the C cells, and are the true stem cells, capable of self-renewal and giving rise to C cells, the transit amplifying population (TAP) that in turn originates the A cells, and may also be the precursors of glial cells (reviewed in Alvarez-Buylla and Garcia-Verdugo 2002). This classification is not necessarily definitive, but is useful for the purpose of defining the cells present in the SVZ.

From all these findings a model for the differentiation of NSCs emerges which is reminiscent of that proposed for haematopoietic stem cells (Fig. 1). The pattern is just beginning to emerge and the stages cannot be phenotypically defined as precisely as those in the blood stem cell lineage, but, as shown in the model, each has specific properties that can be tested empirically.

Fate choice

Neurogenesis is a complex and long-lasting process in mammals, taking place in several regions during embryonic development (Temple 2001), and in the SVZ and the dentate gyrus of the hippocampus throughout adult life (Gage 2000; Alvarez-Buylla and Garcia-Verdugo 2002). NSCs and their progeny are therefore continuously faced with the choice between asymmetric and symmetric division. The consequences of this choice are far reaching, being not only responsible for the production of the correct number of terminally differentiated cells at any given time and site, but also for controlling expansion and depletion of the reservoir of NSCs. This choice has to be carefully regulated to adapt to the hugely changing need for numbers and types of differentiated cells during development and adult life. Which are the molecular mechanisms underlying the different fate of the two daughter cells and how are they controlled? Which are the genes whose expression confers to a cell the ability to self-renew and to maintain its stem cell character?

The mechanism of asymmetric cell division during neurogenesis appears to be evolutionarily conserved (Cayouette and Raff 2002). As in several other instances during development, it relies on the asymmetric distribution of a molecule in the dividing cells, so that the two daughter cells adopt a different fate. The asymmetrically segregating molecules in mammalian neurogenesis appear to be Numb and Numb-like (here collectively called Numb), highly conserved homologues of the Drosophila Numb, which plays a similar role in the fly (Spana et al. 1995). The asymmetric localization of Numb has been studied both in vivo and in vitro and has been shown to occur not only in the mitotic divisions of early precursors but also at terminal cell division when two neurones are generated, always linked to asymmetry in cell fate (Cayoutte and Raff 2002; Shen et al. 2002). Several lines of evidence point to a role of Numb in promoting maintenance of the precursor character. Thus, in mouse cortical ventricular zone, Numb has been



Fig. 1 A tentative model for NSC differentiation in the SVZ. The persistence of radial glia has been confirmed until early postnatal life. Each cell type has its own set of testable properties. NSCs are multipotent, capable of unlimited self-renewal *in vivo* and *in vitro*, and are nestin positive, Notch positive, EGF receptor positive, FGF receptor positive and GFAP positive. Transit amplifying cells, often called progenitor cells, can be multipotent, capable of limited self-renewal *in vivo*, can revert to a NSC status upon stimulation with EGF *in vitro*, and are nestin positive, EGF receptor positive, FGF receptor positive, GFAP negative and Dlx2 positive. Each committed

found localized at the apical membrane of the cells, so that after a horizontal division it would segregate to the apical daughter, believed to remain the precursor cell (Petersen et al. 2002; Shen et al. 2002). Knockout experiments have shown that Numb null mutant mice die early at E11.5, showing precocious neurone production in the forebrain (Zhong et al. 2000) and in Numb conditional mutants in a Numb-like mutant background there is a severe loss of precursors at E10.5. Between E10 and E10.5, when most neurones are generated, in the mutant embryos both cells generated by a precursor adopt a neuronal fate, causing a transient overproduction of neurones and a subsequent depletion of stem cells, and indicating that Numb is required to keep one of the daughter cells self-renewing (Petersen et al. 2002)). Overexpression of Numb in the chick CNS has the opposite effect, enhancing proliferation of the precursor cells (Wakamatsu et al. 1999). However, there are also indications for a role of Numb in neuronal differentiation (Verdi et al. 1999; Zilian et al. 2001). This role has been recently investigated in detail using a 'cell pair assay' (Shen et al. 2002), in which E10-14 cortical cells were plated at low density and allowed to divide. After 24 h (approximately one division) the pair of daughter cells were examined for Numb and β-tubulin distribution. Some of the divisions were symmetric, but in the asymmetric divisions observed Numb was always found more often in the neuronal daughter, although a sizeable proportion of progenitors retained the

precursor has morphology and markers typical of its lineage (see text), is still proliferating, is nestin negative and unipotent. The neuroblast (A cell) can differentiate into different neuronal types, but it cannot be excluded that C cells give direct origin to precursors committed to a single neuronal type. The model is similar to that proposed for the haematopoietic system. In particular, B cells are reminiscent of long-term reconstituting haematopoietic stem cells (HSCs), C cells of short-term reconstituting MSCs, and the committed precursors correspond to those generating myeloid and lymphoid cells.

molecule. How can these controversial findings be reconciled? One explanation might be that Numb promotes asymmetric division without dictating any particular cell fate. Numb is in fact involved in asymmetric divisions of more mature progenitors, even in terminal divisions in which the choice is not between progenitor and the more differentiated state, but between two different differentiated states. In the latter case, the asymmetric division of Numb corresponds to a different morphology of the two daughter cells (Shen et al. 2002). Two sister cells, one with Numb and one without, know that they have to become different from one another and are driven by environmental cues. Numb was shown in Drosophila to antagonize Notch signalling (Spana and Doe 1996; Frise et al. 1996; Guo et al. 1996) involved in cell fate decision, and a similar type of lateral inhibition, in which the first cell to differentiate prevents the other from doing so, might also come into play. If the default fate of symmetrically dividing NSCs is differentiation, absence of Numb, depriving the system of the necessary information that one daughter is already differentiating into a neurone, would cause depletion of NSCs, without necessarily being directly involved in the maintenance of stemness.

As for the genes involved in the decision to differentiate or self-renew, the Notch signalling pathway and its downstream genes have been shown to play an important role, both in insects and in mammals. Thus, in *Drosophila*, lateral inhibition mediated by Notch controls the production of virtually all neuronal cells (Artavanis-Tsakonas *et al.* 1995, 1999). In mammals, Notch1 is specifically expressed by the SVZ cells in the CNS and in cultured neurospheres (Irvin *et al.* 2001; Chojnacki *et al.* 2003), and it has been shown that loss of function of the Notch signalling pathway causes premature appearance of neurones (Hitoshi *et al.* 2002). Conversely, expression of the active form of Notch in a loss of function of the Notch signalling pathway system suppresses the differentiation of postnatal NSC progeny both into neurones and astroglia (Hitoshi *et al.* 2002). Taken together, these findings indicate a role of the Notch pathway and its effector genes in maintaining the cells in a stem cell status (Hitoshi *et al.* 2002) (Fig. 1).

However, Notch signalling has been found recently to induce glial differentiation instructively (Gaiano et al. 2000; Morrison et al. 2000; Lutolf et al. 2002) and to have different outcomes, according to regional and temporal differences in the isolation of progenitors, causing the cells to remain quiescent and undifferentiated or to proliferate and differentiate into glia. Again, other signals from the environment are likely to modulate the response of the precursor cells, but in no instance do they seem capable of causing neuronal differentiation of a Notch-stimulated cell. The mechanism by which Notch suppresses neurogenesis and helps maintain NSCs in an undifferentiated state is now understood in some detail. Stimulation of Notch causes cleavage of its intracellular domain, which migrates into the nucleus and associates with RBP-JK (the homologue of suppressor of hairless) (Iso et al. 2003). The complex acts as a transcriptional activator of target genes such as the Hes genes, which code for transcription factors of the basic-helix loop helix (bHLH) family. Two Hes genes are known to be expressed in the ventricular zone and in cultured neurospheres, Hes1 and Hes5, whose products have indeed been shown to be required for Notch signalling and hence essential in regulating the size of the NSC pool (Nakamura et al. 2000; Ohtsuka et al. 2001). Hes1 and Hes5 in turn repress the activity of genes required for differentiation through the N and E boxes present in the regulatory region of the target genes. One well studied example is the repression of Mash1 activity, which is achieved in two ways (Kageyama et al. 1997). Hes1 binds the co-repressor Groucho through a WRPW sequence at its carboxyl terminus (also present in Hes5) and then binds to the N box-related sequence of the Mash1 promoter repressing its transcription. Molecules of Mash1 that are already present are inactivated by a second mechanism, shutting off immediately transcription of target genes. Most bHLH transcription factors such as Mash1 bind to the E box to activate gene expression. Both Hes1 and Hes5 can form non-functional heterodimers with such factors, and it has been shown that Hes1 in a dominant negative way inhibits Mash1-dependent transcription. Hes1 sequesters E47, a common partner of bHLH factors, preventing the formation of an active E47-Mash1 complex and hence its

binding to E boxes. Through repression of *Mash1*, a proneural gene, and of other genes yet to be identified, Notch signalling is then capable of keeping progenitor cells undifferentiated both *in vivo* and *in vitro* (Kageyama *et al.* 1997; Ito *et al.* 2003). However, the story may become more complicated, if the recently discovered related but distinct bHLH protein, termed HERP (HES-related repressor protein) (Iso *et al.* 2003), is shown to be present in NSCs. HERP, whose expression is also activated by Notch, both homodimerizes and heterodimerizes with Hes proteins forming a complex with repressor activity, and so may provide a third mechanism by which to inactivate yet more genes.

The orientation of the mitotic spindle also seems to be related to the symmetric or asymmetric mode of cell division during corticogenesis. Since the pioneering studies of Chenn and McConnell (1995), it has been shown that in vertebrate corticogenesis, as well as in Drosophila, the initial symmetric division related to the increment of the progenitor pool is characterized by a vertical plane of cell division (perpendicular to the neuroepithelium). As corticogenesis progresses, the asymmetric division, yielding a high number of post-mitotic neurones, proceeds by a horizontal plane, parallel to the pseudostratified neuroepithelium. The different orientation of the mitotic spindle allows the unequal (asymmetric) partitioning of cell fate determinants into the daughter cells (Haydar et al. 2003). But what determines the orientation of the mitotic spindle and why does it switch at one point during corticogenesis? The mechanisms that regulate the cell cycle and the transition from predominantly symmetric to asymmetric divisions in the VZ are still poorly understood. Intrinsic factors and environmental changes might be involved in this process. The search for intrinsic factors involved in cell fate decisions during corticogenesis has focused recently on two genes already involved in the regional identity specification of dorsal telencephalon: *Pax6* and *Emx2*.

The paired box transcription factor Pax6 and the homeodomain transcription factor Emx2 are considered to be the regulators that control neocortical arealization (Bishop et al. 2000; Mallamaci et al. 2000b). Both genes are expressed with a characteristic gradient in the dorsal telencephalic neuroepithelium, Pax6 imparting rostral and lateral, and Emx2 caudal and medial, identity to cortical neurones. Pax6 expression persists throughout neurogenesis. Homozygous Pax6^{Sey/Sey} embryos lacking Pax6 expression fail to develop eyes and nasal cavities, exhibit brain abnormalities and die soon after birth (Hill et al. 1991; Grindley et al. 1997). Interestingly, Heins et al. (2002) found that the neurogenic potential of radial glia cells was reduced in the Pax6 mutant cortex, whereas Pax6 overexpression not only enhanced the neuronal lineage but instructed astrocytes towards neurogenesis. A role of Pax6 has recently been proposed in controlling cell cycle duration and the rate of progression from symmetrical to asymmetrical division in developing cortical progenitors (Estivill-Torrus et al. 2002). The analysis of cell

cleavage orientation revealed that in Pax6 mutants the proportion of cells undergoing asymmetrical division progressed to a higher level than in wild-type cortex. Moreover, the time taken for the onset of expression of neural-specific markers was shorter than in wild type. This was also true in dissociated cultures of cortical cells, suggesting that the differences were not influenced by environmental cues. It appears then, that Pax6 influences the cell cycle duration and mode of cell division of cortical precursors. Strikingly, there is no evidence so far for a role of Pax6 in cultured neurospheres.

Emx2, related to the fly gap gene empty spiracles, is specifically expressed in the diencephalon as well as in the dorsal telencephalon (Brunelli et al. 1996), during embryonal morphogenesis and later (Simeone et al. 1992). Emx2 protein is detectable in the pseudostratified ventricular epithelium (Mallamaci et al. 1998), as well as in cortical pioneer Cajal-Retzius neurones, which guide the proper radial migration of later born neurones, through the secretory product of the gene Reelin (Mallamaci et al. 1998). At later stages, Emx2 is confined to the ventricular VZ, and persists in the adult in neurogenic areas, namely the dentate gyrus of the hippocampus and SVZ Gangemi et al. 2001). Accurate analysis of Emx2 null mutants (Pellegrini et al. 1996; Mallamaci et al. 2000a, 2000b; Muzio et al. 2002) showed two major cortical phenotypic traits, i.e. abnormal lamination and dramatic size reduction of caudal-medial areas, including occipital cortex and hippocampus. Recently, the finding that Pax6/Emx2 double knockout mice convert their cerebral cortex in basal ganglia, with the cortical primordium acquiring morphological and molecular features similar to those of the striatum, led to the proposal that these genes may act as proper selector genes for corticogenesis (Muzio et al. 2002). Emx2 is expressed by nearly 100% of cultured neurospheres, either embryonic or adult.

The role of Emx2 in cell fate choice has been studied recently using two different experimental approaches: one a study of neurospheres derived from the SVZ of adult mice and the other of acutely dissociated cortical precursors derived from embryonic brains (Heins et al. 2001). With the first approach, neurospheres overexpressing Emx2 showed a lower proliferation rate than control cells (Gangemi et al. 2001; Galli et al. 2002), whereas neurospheres derived from knockout mice had a consistently higher proliferation rate (Galli et al. 2002). A clonogenic assay showed that a higher level of Emx2 expression causes the cells to generate fewer secondary spheres, suggesting that Emx2 in NSCs acts to promote an asymmetric mode of division that would lead to an increase in the TAP population, concomitantly reducing their self-renewal capability. However, the final fate of the cells was never affected by the amount of Emx2 present (Gangemi et al. 2001).

Using the second experimental approach Heins *et al.* (2001) showed that the size of the clones obtained is

proportional to the amount of Emx2 expressed by the cells. A large proportion of large clones derived from cells overexpressing Emx2 was composed of neurones and glial cells, indicating that bipotential or multipotential precursors giving rise to neurones and glial cells were increased by *Emx2* transduction. Analysis of the orientation of cell division in the developing cortex of *Emx2*–/– embryos confirmed the *in vitro* results, revealing a strong reduction in symmetric division of the precursor cells, which would lead to a reduction in the TAP and might explain the lower number of mature cells observed in the mutant cortex.

Altogether these results confirm the *in vivo* observations of the effects of Emx2 deficiency: a thicker VZ owing to the accumulation of the most immature precursors (stem cells?) and the scarce development of the cortical plate owing to a severe reduction in the TAP or multipotent precursors (Mallamaci *et al.* 2000a,b). These data indicate a role for this transcription factor in controlling the mode of division both in NSCs and in more mature but still multipotent progenitors. How Emx2 influences this balance between asymmetric and symmetric division, crucial to adjusting the number of NSCs and the size of the TAP to the changing needs of a developing embryo and of an adult animal, may become clearer when we will know more of its target genes.

Sox2 is a transcription factor of the HMG box family first expressed in the totipotent cells of the inner mass, in the embryonic ectoderm and in germ cells of mice (Zappone et al. 2000). Uniformly expressed in the undifferentiated neuroepithelium, Sox2 becomes restricted to the VZ upon neural tube differentiation. Recent data show that Sox2 is expressed in the NSC-containing population in vivo and in cultured neurospheres derived from the periventricular zone of the telencephalon (Zappone et al. 2000). Very recently, Graham et al. (2003) found that Sox2 activity is essential for the maintenance of neural precursor cells. Thus, inhibition of Sox2 results in premature differentiation of the precursor cells, which stop dividing and acquire early neuronal markers, whereas its constitutive expression has the opposite effect, inhibiting neuronal differentiation (Graham et al. 2003). Two closely related genes, Sox1 and Sox3, have a similar expression pattern in neural precursors (Wood and Episkopou 1999) and may have a redundant function, as expression of Sox1 can rescue the phenotype observed after Sox2 inhibition (Graham et al. 2003).

A number of genes expressed in NSCs, either during embryonic development or in cultured neurospheres, are likely to play a part in controlling their fate, but the existing functional data do not allow a precise role to be assigned as yet.

A role in negative regulation of NSC/progenitor cell proliferation has been recently attributed to the tumour suppressor Pten, a lipid phosphatase (Groszer *et al.* 2001; Li *et al.* 2002, 2003; Morrison 2002;). Deletion of the gene leads to hyperphosphorylation of Akt and S6 kinase,

downstream effectors of phosphatidylinositol 3-kinase that have been implicated in neuronal survival and cell cycle regulation. *Pten* mutant mice have enlarged and abnormal brains, and neurospheres derived from mutant mice are significantly greater in number than in wild-type mice indicating that mutant animals have more stem/progenitor cells. The greater proliferation is attributed to a shortened cell cycle whereas the cell fate commitment is unchanged (Groszer *et al.* 2001).

A gene likely to have an important role in NSCs is Zic1, which encodes a zinc finger protein, homologue of the Drosophila pair-rule gene odd-paired. The gene is first expressed at gastrulation in the ectoderm and in the mesoderm (Nagai et al. 1997), and subsequently in the dorsal neural tube. At later stages, Zic1 is found in the outer granular layer of the cerebellum, where it persists throughout adult life (Ebert et al. 2003). Its high level of expression in medulloblastoma led to the suggestion that it may have a role in controlling cell proliferation (Michiels et al. 1999). Recently Jiang et al. (2002) reported that Zic1 is a highly expressed gene in multipotent adult precursor cells derived from brain. Zic1 is also highly expressed in neurospheres derived from E18.5 embryos and down-regulated when Emx2 is overexpressed (Gangemi et al., unpublished data). Although its function has not been directly studied in NSCs, the analysis of Zic1-overexpressing and Zic1-deficient mice led to the conclusion that Zic1 is responsible for the expansion of neural precursors through inhibition of neuronal differentiation in the dorsal spinal cord. This effect may be mediated by the Notch signalling pathway as Notch1 and Hes1 appear up-regulated in Zic1-overexpressing spinal cords in mice (Aruga et al. 2002).

An entirely different series of experiments has aimed to identify genes specific for stem cells, with the goal of identifying sets of genes responsible for stemness and of true markers of stem cells in general. Do stem cells have a similar transcriptional profile? Do 'stemness' genes really exist? In an attempt to answer these questions several investigators have used DNA microarrays (Ivanova et al. 2002; Jiang et al. 2002; Ramalho-Santos et al. 2002), subtractive hybridization or both (Geschwind et al. 2001; Terskikh et al. 2001; Karsten et al. 2003), comparing stem cells containing populations of various origin, haematopoietic stem cells, NSCs and embryonic stem cells (ESC), with each other and with differentiated tissues. As expected, all studies showed that the three cell lineages have sets of expressed genes that are peculiar to their particular origin. Thus, NSCs express 'neural genes', haematopoietic stem cells 'blood cell' genes and ESCs 'embryonic' genes. Strikingly, it also turned out that NSCs are more similar to ESCs than to cells from the lateral ventricular region of the brain (Ramalho-Santos et al. 2002), with an overall 61.6% overlap between NSCs and ESCs. This global similarity led the authors to hypothesize a default model for neural development from ESCs. An

analysis of all the comparisons has demonstrated that a common pool of genes (216) is present in the different stem cells. A high proportion of these genes are expression sequence tagged (EST) sequences, and their analysis may reveal hidden self-renewal genes. Twelve of the 60 mapped genes are on chromosome 17, suggesting an ancestral clustering of stemness genes (Ramalho-Santos *et al.* 2002). It seems then that the answer to the first question should be yes; however, there is no compelling evidence as yet for genes exclusively expressed in stem cells.

Cell fate specification

Neuronal differentiation

The multipotent progenitor cell generated by the asymmetric division of the NSC has now to choose among the differentiation pathways leading to the production of neurones, astrocytes or oligodendrocytes. Although genes have been identified that seem specifically involved in this step, asymmetric division and fate choice may not be entirely separated, as it appears that some of the mechanisms active in deciding between asymmetric and symmetric division have a part also in the determination of a particular fate. It is quite possible that the progenitor emerges from the asymmetric division already biased toward a particular differentiation pathway.

Thus, as mentioned in the previous section, stimulation of the Notch pathway, through activation of Hes1 and Hes5 and inactivation of Mash1, forbids neuronal differentiation, thereby favouring production of glial cells (Fig. 2).

Sonic hedgehog (Shh) signalling, required for oligodendrocyte development (see below), has recently been found to be essential for the maintenance of early precursors in the postnatal telencephalon (Machold *et al.* 2003). Expression of some genes involved in progenitor maintenance continues



Fig. 2 Interplay between gliogenic and neurotrophic transcription factors in the decision to become a neurone or glia. Gliogenesis and neurogenesis occur by stimulation of one specific pathway and simultaneous suppression of the alternative pathway. The two pathways compete for p300, required for turning on both gliogenic and neurogenic genes, and cannot be active at the same time inside the cell.

after differentiation, suggesting a role in cell fate specification. For instance, expression of the Emx2 protein is maintained in maturing neurones, but ceases altogether in maturing cells with a glial character, both *in vivo* and *in vitro* (Mallamaci *et al.* 1998; Gangemi *et al.* 2001). However, there are no clues at present as to how this transcription factor might be involved in neuronal differentiation.

As to the genes specifically involved in the commitment towards a particular cell lineage, a group of genes encoding transcription factors of the bHLH family are considered to be equivalent to *Drosophila* proneural genes and to promote a generic neuronal fate by different mechanisms: activating a cascade of neuronal genes, promoting exit from the cell cycle and inhibiting gliogenesis (Morrison 2001; Panchision and McKay 2002; Shuurmans and Guillemot 2002; Ross *et al.* 2003).

Mash1, homologue of the proneural proteins of the *Drosophila* achaete-scute complex (AS-C), and its downstream genes appear to be especially important. Mash1 is expressed from E8.5 in restricted domains in the neuroepithelium of the midbrain and ventral forebrain, and by E12.5 expands to most of the SVZ and part of the VZ cells in all regions of the brain, marking a transient population of committed neural precursors (Porteus *et al.* 1994; Torii *et al.* 1999).

Mice lacking Mash1 have severe anomalies in the basal ganglia and cerebral cortex (Casarosa *et al.* 1999; Horton *et al.* 1999). The defects in neurogenesis in mutant brains are due to a severe loss of neuronal progenitor cells in the medial ganglionic eminence, indicating that Mash1 has a real determinant function in the specification of neuronal progenitors in this area. In contrast, its function is apparently redundant in the lateral ganglionic eminence as neuronal progenitors are still present there in *Mash1* mutant mice. Mash1 is necessary also for the generation of neuronal progenitors in the olfactory bulb (Guillemot *et al.* 1993).

Mash1 is not expressed in neurospheres maintained in aggregation culture, but is transiently expressed when they are transferred to monolayer culture conditions and then decreases when microtubule-associated protein-2, a neuronal marker, starts to be expressed (Torii *et al.* 1999). These findings confirm that Mash1 expression coincides with the time at which stem cells cease to self-renew and begin to differentiate into neurones (Torii *et al.* 1999; Lo *et al.* 2002; Ito *et al.* 2003).

Neurogenins (Ngns) are a family of three genes related to the *Drosophila atonal*, which define distinct progenitor populations in the developing CNS and PNS, exhibiting nonoverlapping expression in some areas and partial overlap in others (Sommer *et al.* 1996). In particular, in the telencephalon Ngn1 and Ngn2 are expressed exclusively in cells of the dorsal VZ, complementary to the ventral expression domain of Mash1 (Gradwohl *et al.* 1996; Ma *et al.* 1997; Fode *et al.* 2000). The defects in neurogenesis in *Ngn* mutant cortices are milder than those in *Mash1* mutants, although there is a mispecification of the dorsal cells, which acquire a more ventral identity, owing to the expansion of Mash1 expression into the dorsal domain in the absence of Ngn.

As a result of this partial compensation by up-regulation of Mash1, the function of Ngns, in particular Ngn2, as proneural genes is clearer in Mash1/Ngn2 double mutants, in which cytoarchitecture is strongly affected. The deficit of neurones in the preplate is severe and NeuroD, which is normally activated in immature CNS neurones, fails to be expressed (Fode *et al.* 2000).

Studies *in vitro* confirm the role of *Mash1* and *Ngns* as proneural genes. Transient expression of these genes is sufficient to initiate a programme of neuronal differentiation in non-neural cells such as pluripotent mouse P19 embryonic cells, which stop dividing and differentiate into specific neuronal subtypes (Farah *et al.* 2000).

Both Mash1 and Ngns seem to initiate neuronal differentiation by several mechanisms. Not only do they directly activate the expression of neuronal genes, again of the bHLH type, such as NeuroD and Math3 (Fig. 2) (Cau et al. 1997, 2002; Fode et al. 1998; Ma et al. 1998), which are considered neuronal differentiation genes and whose discussion is beyond the scope of this review, but they also promote exit from the cell cycle and inhibit gliogenesis. Inhibition of the cell cycle has been shown in vitro in P19 embryonic cells, where transient expression of any of these genes induces expression of cyclin-dependent kinase inhibitors, including p27 (Farah et al. 2000). Activation of NeuroD also contributes to inhibition of cell cycle progression through activation of p21 (Mutoh et al. 1998). The inhibition of gliogenesis by Mash1 and Ngns has been indicated by the findings that in knockout mice there is a compensatory premature generation of astrocytic progenitors in different brain regions (Tomita et al. 2000) especially in double-mutant mice, and confirmed in in vitro experiments (Nieto et al. 2001). As to the possible mechanism, Ngn1 has been shown to suppress leukaemia inhibitory factor (LIF)-induced astrocyte differentiation in cultured neural stem/progenitor cells, by sequestering the Smad1-p300 (see below) transcription complex to neuronal promoters away from astrocyte differentiation genes (Sun et al. 2001) (Fig. 2). Interestingly, it has been recently found that Ngn1 expression is increased by LIF creating a sort of autoregulatory loop, antagonizing the down-regulation of Ngn1 promoted by the growth hormone (Turnley et al. 2002).

Although bHLH factors appear necessary for the determination of neuronal fate, environmental cues are important as well, as shown by the role played by CCAAT/enhancerbinding protein (C/EBP). The C/EBP family is composed of basic leucin zipper DNA-binding proteins (C-EBPs $\alpha,\beta,\gamma,\delta,\epsilon$ and ζ), expressed in several tissues including developing and adult brain (Sterneck and Johnson 1998), which function as regulators of differentiation of several cell types (Cao et al. 1991; Muller et al. 1995; Tanaka et al. 1995; Yeh et al. 1995). In cortical precursors, activated platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors act through the MAP-ERK Kinase (MEK)-C/EBP pathway to enhance neurogenesis (Davis 1995). Thus, precursor cells from E12-E13 cortex infected with an inhibitory form of C/EBP, cultured with FGF2 and/or PDGF, fail to differentiate into neurones and remain in an undifferentiated state. Conversely, overexpression of an active form of C/EBPB enhances neurogenesis, directly activating early neuronal genes, including the α 1-tubulin gene, and at the same time prevents the cells from responding to gliogenic growth factors, such as ciliary neurotrophic factor (CNTF) (Menard et al. 2002). Menard et al. (2002) have proposed that C/EBP acts on cells committed to the neuronal fate by the expression of a repertoire of bHLH factors, causing their differentiation into neurones by activating early neuronal genes in response to growth factors present in their environment.

Members of another class of genes, the Dlx homeobox genes, are expressed in the adult SVZ (Panganiban and Rubenstein 2002). In particular, Dlx2 is expressed in the TAP and in the neuroblasts (Doetsch et al. 2002) and has been involved in the generation of GABAergic neurones (Anderson et al. 1997; Letinic et al. 2002). Interestingly, upon stimulation with EGF in vitro, the TAP down-regulates expression of Dlx2 and reverts to a stem cell status (Doetsch et al. 2002) (see Fig. 1). Dlx5 has been shown to have a role in the production of interneurones of the olfactory bulb (Levi et al. 2003; Long et al. 2003). We have found that Dlx5 is expressed in over 60% of cells dissociated from cultured neurospheres and that the number of neurones obtained from neurospheres derived from E18 Dlx5 knockout mice is greatly reduced, whereas E12 neurospheres produce normal amounts of neurones (Perera et al. 2004), suggesting that Dlx5 is indeed required for the generation of interneurones from NSCs. Figure 3 summarizes the early steps of neuronal differentiation and the genes involved.

Glial differentiation

During development, astrocytes are produced in a second wave of differentiation, once most neurones have already differentiated and migrated to their correct destination. Formation of oligodendrocytes occurs later still, when neurones are surrounded by the supportive glia and synaptic connections have been established. However, the creation of both types of glial cells proceeds through a sequence of common progenitors that become progressively more restricted. Thus, a recently identified population, called tripotential glial-restricted precursor (GRP) cells, that can generate oligodendrocytes and two types of astrocytes (Rao *et al.* 1998; Herrera *et al.* 2001), arises from totipotent NSCs (Gregori *et al.* 2002). This population in turn differentiates into the more restricted oligodendrocyte-type 2 astrocyte



Fig. 3 Some of the genes and soluble factors involved in neuronal differentiation. Increasing commitment of the NSC through the TAP and neuroblast (NB) to neurones is shown from left to right. Shh, Sox1, 2 and 3, and Notch/Hes are responsible for the maintenance of stem cell status. The role of Pax6, Zic1 and Emx2 is not yet clearly determined (see text). C/EBP, Mash1 and Ngn1/2 activate neuronal genes of cells committed to a neuronal fate, in the presence of environmental factors (PDGF, bFGF).

progenitor cells (O-2A) that can generate oligodendrocytes and only one type of astrocyte (Raff *et al.* 1983; Skoff and Knapp 1991) (Fig. 4).

Given this temporally separated production, it may not be surprising that, so far, no genes equivalent to the proneural genes *Mash1* and neurogenins have been found that commit a NSC to glial differentiation. Rather, it seems that this pathway is chosen in response to environmental cues that directly activate glial genes, while inhibiting neuronal differentiation at the same time (Fig. 4).

Thus, several soluble factors bFGF, insulin-like growth factor (IGF)-1, brain-derived neurotrophic factor (BDNF), PDGF, thyroid hormone (TH), CNTF, LIF, bone morphogenetic protein (BMP)2, Shh (Jiang *et al.* 2001; Morrow *et al.* 2001; Billon *et al.* 2002; Miller 2002) and the Notch receptor (Furukawa *et al.* 2000; Gaiano *et al.* 2000; Morrison *et al.* 2000; Tanigaki *et al.* 2001) have been shown to influence glial fate decisions in the developing telencephalon, and in NSCs and more mature progenitors *in vitro* (Fig. 4).

For some of these factors (Notch, CNTF, LIF and BMP2) the pathways are known in some detail. BMP2 and LIF act through different receptors and transcription factors, Smad1/4 and STAT1/3 respectively, to synergistically induce cultured fetal telencephalic neuroepithelial cells to differentiate into astrocytes (Fig. 4) (Bonni *et al.* 1997; Heldin *et al.* 1997; Koblar *et al.* 1998; Rajan and McKay 1998; Nakashima *et al.* 2001). As shown in Fig. 2, the two pathways collaborate through a complex between STAT3 and Smad1 bridged by the transcriptional co-activator p300 that can directly activate glial genes, such as glial fibrillary acidic protein (GFAP). This collaboration appears to be necessary as neither factor alone is able to induce expression of this astrocytic marker (Fig. 1) (Nakashima *et al.* 1999).

In addition, the BMP2–Smad pathway induces expression of Hes5 and HLH proteins of the inhibitor of differentiation



Fig. 4 Schematic model of glial differentiation. Some of the genes involved in the negative (Ngn1, Ids) and positive (Sox9,10; Ngn3; Nkx2,2; Olig1,2) regulation of astrocyte and oligodendrocyte differentiation are shown in the lower part of the figure (see text). Soluble factors required for differentiation are shown in the upper part. CNTF/LIF and BMP2 promote astrocyte commitment through the activation of Smad1 and STAT1/3. PDGF and TH are required to generate a more restricted bipotential cell population (GRP

(Id) family, which are known to interefere with the action of proneural proteins (Nakashima *et al.* 2001; Wang *et al.* 2001) (Fig. 2). The Ids act by sequestering the E proteins that are necessary for bHLH factors to form active dimers capable of binding to an E box regulating transcription (Norton 2000; Ross *et al.* 2003). As already mentioned, most proneural proteins are members of the bHLH family (Mash, neurogenins, NeuroD), but so are the *olig* genes, which promote oligodendrogenesis. Accordingly, Ids prevent premature generation of neurones (Lyden *et al.* 1999) and oligodendrocytes (Wang *et al.* 2001), but not of astrocytes, which apparently do not require the activity of bHLH factors, or at least of bHLH factors that sequester E proteins for their differentiation (Ross *et al.* 2003 and references therein).

As reported above, proneural Ngn1 can inhibit gliogenesis at two different levels: by blocking STAT activation and by associating with Smad1–p300 complex and competing for the formation of the gliogenic signal Smad1–STAT–p300 complex (Sun *et al.* 2001) (Fig. 2).

As already mentioned, the Notch signalling pathway plays a major role in maintaining NSCs in an undifferentiated state. However, several reports indicate a role of Notch in promoting gliogenesis, both *in vivo* and *in vitro* (Furukawa *et al.* 2000; Gaiano *et al.* 2000; Morrison *et al.* 2000; Tanigaki *et al.* 2001). Apart from regional and temporal cues, this enhanced gliogenesis in the presence of an active Notch pathway may be explained by the fact that its main action is repression and inactivation of proneural genes. Inhibition of genes that not only commit the cell to neuronal differentiation but also prevent glial differentiation, would free the cells to respond to environmental signals promoting glial differentiation, such as BMP2, LIF or CNTF. However,

bipotential) from a tripotential GRP. The initial commitment of OLPs is established by Shh through the induction of the *olig* genes. An increasing concentration of bFGF may contribute to the induction of the oligodendrocyte fate. PDGF in combination with bFGF, IGF-1, neuregulin (NRG) and C-X-C motif ligand (CXCL1) continues to stimulate proliferation of the OLPs at different maturation stages. In the late stages of oligodendrocyte differentiation, TH coordinates the onset of myelination (see text for references).

the story may be more complicated, as it has been shown that the intracellular domain of Notch directly activates the GFAP promoter (Ge *et al.* 2002).

CNTF promotes glial fate also by relieving a different kind of repression, causing cytoplasmic translocation and phosphorylation of the nuclear receptor co-repressor (N-Cor) (see Fig. 4). N-Cor was initially identified as a factor that mediates inhibition of gene transcription by nuclear receptors such as thyroid hormone and retinoic acid receptors (Horlein et al. 1995), but it has been shown to act as a co-repressor of many other transcription factors, including homeodomain factors and C promoter binding factor/recombination signalsequence binding protein (CBF1/RBP-JK) (Kao et al. 1998; Xu et al. 1998). N-Cor has indeed been shown to bind CBF1 and to repress the GFAP promoter (Hermanson et al. 2002). The protein is highly expressed in the cortical VZ at E14.5, the site of proliferating NSCs, and is present also in the outer layers of the cortex, where there are more differentiated cells. N-Cor-/- embryos, which die by E18, present anomalies in brains from E14.5, in particular a decrease in nestin-positive cells and a widespread GFAP expression in the cortex at a time at which it is normally absent (Jepsen et al. 2000; Hermanson et al. 2002). Cortical progenitor cells from N-Cor -/- E13 embryos do not proliferate and differentiate spontaneously into a much higher number of astrocytes. Overexpression of N-Cor keeps cultured neural stem/progenitor cells undifferentiated even in the presence of CNTF, probably because of saturation of the mechanism that keeps it inactive, indicating that N-Cor is the main target of CNTF (Hermanson et al. 2002).

Until very recently, it was thought that bHLH proteins had no role in glial differentiation, apart from the *olig* genes involved in the generation of mature oligodendrocytes. However, although there is still no evidence for bHLH involvement in the early stages of glial differentiation, in a recent study (Lee et al. 2003) Ngn3 was found transiently expressed in the bipotential glial progenitor cells in the mouse spinal cord, and to have a role in the generation of both astrocytes and oligodendrocytes. Thus, Ngn3 null mice lose expression of Nkx2.2, a transcription factor required for oligodendrogenesis. Accordingly, myelin basic protein (MBP) and proteolipid protein, two mature oligodendrocyte markers, are greatly reduced in these mice, as they are in Nkx2.2 null mice. However, lack of Ngn3 also causes a reduction in GFAP, indicating that this factor is implicated in astrocyte differentiation as well (Lee et al. 2003). This finding is puzzling, as the available evidence indicates that neurogenins inhibit gliogenesis by sequestering the SMAD1-STAT-p300 complex (see above). However, as argued by the authors, Ngn3 may have a mechanism of action different from that of Ngn1 (Lee et al. 2003).

Although *Sox9* appears to be essential for glial differentiation (Stolt *et al.* 2003), this gene will be discussed in the next section, with the related genes *Sox8* and *Sox10*, which are restricted to the oligodendrocyte lineage.

Oligodendrocyte differentiation

Oligodendrocytes are among the latest cells to differentiate in the nervous system, although oligodendrocyte precursor (OLP) cells are specified very early (rat E13.5, mouse E11) during development (Pringle and Richardson 1993; Thomas *et al.* 2000). OLP cells expressing the PDGF receptor α arise from multipotent precursors from a ventral area of the medial ganglionic eminence and later migrate tangentially to the cerebral cortex (Tekki-Kessaris *et al.* 2001). OLP cells expressing this receptor are already determined to an oligodendrocyte fate, as demonstrated by experiments in which immunoselected PDGF receptor α -positive cells from late embryonic rat spinal cord and brain were shown to differentiate exclusively into oligodendrocytes in culture (Hall *et al.* 1996; Tekki-Kessaris *et al.* 2001).

Several lines of evidence have shown that development of these precursors requires Shh signalling in both spinal cord and telencephalon (Pringle *et al.* 1996; Orentas *et al.* 1999; Nery *et al.* 2001; Tekki-Kessaris *et al.* 2001). This initial commitment would be established by Shh through the induction of oligodendrocyte-specific *olig* genes (see Fig. 4) (Lu *et al.* 2000; Zhou *et al.* 2000), as expression of these genes is lost in *Shh* null mice.

The *olig* genes code for two bHLH transcription factors, Olig1 and Olig2, underscoring the role played by this class of factors in determining cell fate in the nervous system. Their involvment in oligodendrocyte differentiation, suggested by their pattern of expression, particularly in the adult white matter, the most oligodendrocyte-rich area (Lu *et al.* 2000; Zhou *et al.* 2000), has been demonstrated in several studies. Thus, forced expression of Olig1 in cortical precursor cells in vitro promotes generation of OLPs (Lu et al. 2000) and injection of a retrovirus carrying the olig1 gene promotes oligodendrocyte differentiation in telencephalon (Lu et al. 2001), whereas ectopic expression of Olig2 in the spinal cord leads to oligodendrocyte differentiation in conjunction with homebox factor Nkx2.2 (Zhou et al. 2001). Moreover, overexpression of Id2, a negative regulator of bHLH activity, inhibits oligodendrocyte development (Wang et al. 2001). How they regulate oligodendrocyte differentiation is as yet unknown. However, because their expression in the developing nervous system overlaps that of OLP cells expressing PDGF receptor α , but precedes their emergence, it has been postulated that they may cause expression of this receptor, thus enabling these cells to respond to environmental cues promoting this particular cell fate (Lu et al. 2000; Zhou et al. 2000; Tekki- Kessaris et al. 2001). Moreover, as mentioned above, overexpression of Ids, negative regulators of bHLH activity, and hence of the Oligs, inhibits oligodendrocyte development (Wang et al. 2001). Interestingly, expression of Ids is not shut off at the onset of oligodendrocyte differentiation, but the proteins translocate from the nucleus to the cytoplasm (Kondo and Raff 2000b; Toma et al. 2000; Nakashima et al. 2001; Wang et al. 2001). Ids lack both the DNA-binding region and the nuclear localization sequence, and require an interaction with other nuclear proteins, such as E proteins, to enter the nucleus. This finding suggests that this interaction may be inhibited when OLPs differentiate into mature oligodendrocytes, although it is not yet known how this inhibition is brought about. The homeobox transcription factor Nkx2.2 is also under the control of Shh (Briscoe et al. 1999; Pabst et al. 2000) and it has been shown to cooperate with Olig2 in the maturation of OLPs (Fu et al. 2002), but not in their generation (Qi et al. 2001).

Another class of transcription factors that have been implicated in oligondendrocyte differentiation are the Sox genes of the E class. Thus, three of these genes are expressed during oligodendrocyte differentiation and in mature oligodendrocytes, Sox 8, Sox 9 and Sox10. In the CNS, Sox10 is expressed exclusively in the oligodendrocyte lineage (Kuhlbrodt et al. 1998) and is essential for the generation of mature oligodendrocytes (Stolt et al. 2002). Sox10 is present in oligodendrocyte progenitors before terminal differentiation as well, but is apparently not essential at this stage (Stolt et al. 2002). As its pattern of expression is similar to that of Olig1 and Olig2, regulation of PDGF receptor α has also been proposed for this gene (Lu *et al.* 2000; Zhou et al. 2000; Tekki-Kessaris et al. 2001). The role of Sox9 is more complex. In the developing spinal cord, this gene is expressed in all the neuroepithelial stem cells and in the radial glia (Stolt et al. 2003). Later its expression is confined to all GFAP-positive cells, being absent from cells positive for neuronal markers (Stolt et al. 2003). Its expression is maintained in oligodendrocyte progenitors expressing Sox10, but disappears when they start to terminally differentiate (Stolt et al. 2003). Accordingly, specific conditional ablation of Sox9, using the nestin promoter to drive expression of the Cre transgene, leads to a severe loss of astrocytes and oligodendrocytes (Stolt et al. 2003). On the basis of these results, Stolt et al. (2003) proposed a model in which the combination of Sox9 and Olig2 would be essential for oligodendrocyte specification, with Sox9 conferring glial identity and Olig2 subtype identity. A functional redundancy between Sox 9 and Sox10 would also explain why Sox10 is essential only for the terminal differentiation of the oligodendrocytes and not at the OLP level where there is abundant Sox9 protein. Despite the broad expression of Sox8, which overlaps that of both Sox9 and Sox10, Sox8 null mice do not show any alteration in the maturation of both astrocytes and oligodendrocytes (Sock et al. 2001). Again, this lack of phenotype has been attributed to a functional redundancy between these genes (Sock et al. 2001). However, in this case, the explanation may not be so simple, as one would expect a similar lack of phenotype after disruption of Sox9 and Sox10.

Migration and lesion repair

A remarkable feature of the developing CNS is the extensive migration of cells, which end up in places often distant from where they were originally born. Four types of movement are particularly prominent, which occur along different axes of the brain: the tangential migration of interneurones from the ganglionic eminence into the neocortex (Pleasure et al. 2000; Anderson et al. 2001); the radial migration of cortical dorsal telencephalic neurones guided along the radial glia by the extracellular matrix protein Reelin secreted by the Cajal-Retzius cells (Ogawa et al. 1995); the posterior-anterior migration of progenitors from the subventricular zone through the rostral migratory stream (RMS) (Lois et al. 1996), which provides interneurones to the olfactory bulbs; and the migration of precursors of cerebellar granule cells from the germinal trigone to the external granular layer and subsequently to the internal granular layer (Komuro et al. 2001; Yacubova and Komuro 2003). Control of these movements is rather complex, involving several regulatory genes, as well as several extracellular signalling molecules. Of the many genes to which a role has been assigned, perhaps not surprisingly, some are also involved in controlling neural cell fate (see above). Thus, Emx1/2 cooperate to promote cell influx from the ganglionic eminence into the cortex (Shinozaki et al. 2002), and Pax6 is proposed to limit the invasion of the cortex by cells originating in the ganglionic eminence (Chapouton et al. 1999). The Dlx genes play a fundamental role in the control of migration of neuronal progenitors along the RMS toward the olfactory bulbs and of the tangential migration from the ganglionic eminence to the dorsal cortex (Marin and Rubenstein 2001; Panganiban and Rubenstein 2002).

In the fully developed CNS, most of these movements cease completely, the only exception being migration through the RMS, which relies on diffusible factors (slit, Netrin) (Wu et al. 1999), cell-cell adhesion polysialylated neural cell adhesion molecule (PSA-NCAM) (Hu 2000) and cell-matrix adhesion molecules of the integrin family (Murase and Horwitz 2002) and lasts throughout adult life. In primates newborn neurones migrating through the white matter to the neocortex were demonstrated by Gould et al. (1999). Neurogenesis was found to occur in the prefrontal, temporal and posterior parietal cortex, which are important for cognitive function, but not in the primary sensory area. The newborn neurones were subsequently shown to have a transient existence (Gould et al. 2001), with the number of new cells in all three areas declining within 9 weeks. Adult rats injected with bromodeoxyuridine (BrdU) (Gould et al. 2001) showed the presence of labelled neurones in the anterior neocortex as well as in the dentate gyrus. However, these results have not been confirmed and newly generated neurones are consistently found only in the olfactory bulbs and in the hippocampus (Kornack and Rakic 2001; Koketsu et al. 2003).

What is the function of this long-lasting neurogenesis and cell replacement in the adult? In mice, hippocampal neurogenesis declines with age, but short-term exposure to a stimulating enviroment leads to a striking increase in new neurones, improving learning parameters and locomotor activity (Kempermann et al. 2002). In the neocortex of adult mice endogenous neural precursors can be induced to differentiate into mature neurones in regions that do not normally undergo neurogenesis in response to the induction of targeted neuronal death (Magavi et al. 2000). NSCs also respond to hypoxia, both in vivo and in vitro, producing an increased number of neurones (Shingo et al. 2001). It is then likely that a limited neuroplasticity exists in adult individuals, which is stimulated by a sustained active life, and by neuronal death and hypoxia, conditions likely to require cell replacement.

A challenging goal in neurobiology is then to stimulate and guide the proliferation, migration and differentiation capabilities shown by NSCs in the CNS to repair and cure different types of neurological diseases. However, an effective and safe strategy for endogenous repair, if possible, will only derive from a detailed knowledge of all the factors governing the behaviour of these cells *in vivo*. It is clear that even in the adult CNS mechanisms exist to trigger neurogenesis, but they are largely unknown. Also, as pointed out by Cao *et al.* (2002), endogenous neurogenesis has been demonstrated only following focal apoptotic injury, and it remains to be established whether it can be induced by other types of injury, such as neurodegeneration, ischaemia or physical trauma. For instance, dorsal funiculus sectioning (Johansson *et al.* 1999) or traumatic injury (Kernie *et al.* 2001) produce proliferation of cells that ultimately differentiate into astrocytes. Furthermore, endogenous regeneration may not be sufficient to sustain repair of the damaged tissue in many cases, especially in areas where neurogenesis does not seem to take place (see below).

Environmental factors seem to play the major role, as shown by transplantation experiments. Rodent and human brains, both embryonic and adult, are a source of NSCs that can be extensively cultured and propagated in vitro without losing stem cell properties (self-renewal and multipotency). Transplantation experiments have shown that these cells, once grafted back into the brain, migrate extensively and differentiate into neurones and glia (Flax et al. 1998), but their behaviour depends on the site of injection. Thus, when precursor cells derived from adult hippocampus are implanted in the dentate gyrus, they generate cells similar to those normally produced at that site and, if implanted in the RMS, give rise to neurones of the olfactory bulbs with a phenotype of the neurones normally generated in the olfactory bulbs (Gage et al. 1995). However, if implanted in non-neurogenic areas, they only give rise to glia (Gage et al. 1995). It appears that local cues are present in different regions of the brain that can drive differentiation into different cell types and that cultured neural precursors maintain the ability to respond to these cues and differentiate into the appropriate cell type dictated by the instructive signals present in the environment. The importance of the environment is underscored by the fact that engraftment and differentiation are enhanced in areas of lesion. This may be due to a locally increased production of factors promoting the correct regeneration of the damaged tissue. It has been shown that in regions of synchronous targeted degeneration of cortical callosal projection neurones in the adult mouse cortex, several genes are up-regulated in the cortical interneurones, leading in particular to secretion of BDNF, neurotrophin (NT)-4/5 and NT-3 (Wang et al. 1998), factors known to influence the neurotransmitter and receptor phenotype of neurones. These and other as yet unidentified cues may be at least in part responsible for the correct differentiation of neuroblasts transplanted in such lesions (Shin et al. 2000).

However, several lines of evidence indicate that the state of commitment and the region of origin of the transplanted cells may have a critical role as well. This issue has been recently reviewed by Cao *et al.* (2002), but deserves some discussion as it is crucial for successful repair and underscores the importance of identifying the genes regulating NSC differentiation. In several instances, cultured pluripotent NSCs, when transplanted as such, are unable to differentiate into neurones in non-neurogenic areas of the adult CNS (see above) and only give rise to glia. This failure to generate the correct type of cells required to repair the lesion, probably due to lack of the signals necessary to drive neuronal differentiation of the NSCs, severely restricts the usefulness of this approach. To overcome this problem, two strategies have been used: to 'train' NSCs, trying to commit them to a neuronal fate or to transplant neuronal-restricted precursors (NRPs). Both approaches are potentially feasible. Thus, when NSCs were allowed to differentiate in vitro for 5 days before transplantation into lesioned striatum, they gave rise to both neurones and glia (Vescovi et al. 1999), in contrast to glial cells only when undifferentiated NSCs were used. However, the use of both exogenous factors (Johe et al. 1996; Whittemore et al. 1999) and expression of transcription factors driving neuronal development (Yamamoto et al. 2001) has proved only partially successful. Clearly, these strategies require a much deeper knowledge of the regulatory genes and the signalling pathways involved in NSC differentiation to obtain pure populations of the correct cell type. The alternative approach, the use of NRPs, holds some promise. NRPs can be isolated from several sources, such as embryonic CNS, embryonic stem cells (ES) and multipotential NSCs (Mujtaba et al. 1999). In contrast to NSCs, they differentiate only into neurones in vitro and, when transplanted, adopt the correct phenotype of the engraftment site (Yang et al. 2000). However, it appears that maturation of engrafted NRPs is retarded in the injured spinal cord and that survival of dopaminergic precursors in rodent models of Parkinson's disease is impaired (see Cao et al. 2002 and references therein). This approach will also benefit from a better understanding of the mechanisms underlying stem cell differentiation and survival.

One aspect of neural cell replacement that has not been well investigated yet, and is required for the future, is whether *ex vivo* differentiated neurones actually integrate appropriately into the neural network and are electrically active as well as expressing the appropriate phenotype.

The same problems are faced in trying to induce remyelination. Again, it appears that induction of commitment of NSCs to the oligodendrocyte lineage in vitro (Smith and Blakemore 2000) or the use of oligospheres (Zhang et al. 1998), which contain progenitor cells expressing oligodendrocyte markers, leads to a significant increase in remyelination in animals in which areas of demyelination had been chemically or physically induced. Both endogenous remyelination and remyelination by local transplantation have been extensively reviewed by Cao et al. (2002) and we will only discuss a recent new approach to this problem. Chronic inflammatory demyelinating diseases of the CNS are multifocal and therefore hard to treat by local transplantation of myelin-forming cells or their precursors. To overcome this problem, Pluchino et al. (2003) have investigated the possibility of exploiting the migration capacity of NSCs contained in undifferentiated cultured neurospheres to reach all the demyelinated sites in an experimental model of multiple sclerosis - experimental autoimmune encephalomyelitis. The initial observation was that the pattern of surface proteins expressed by these cells (CD44 positive, very late

antigen (VLA)-4 positive, p-selectin glycoprotein ligand (PSGL)-1 positive, leukocyte function-associated antigen (LFA)-1 positive, L-selectin positive) indicated that they might have the capacity to cross the blood-brain barrier. Neurospheres were then injected intracerebroventricularly or into the bloodstream (i.v) of affected and control mice. As expected from the previous observation that a lesion is necessary to attract these cells (see above), no donor cells were found in the CNS of untreated animals. On the contrary, the results in the affected animals were striking. Thirty days after injection (especially i.v.) most of the cells were present in areas of demyelination in contact with damaged axons and were shown to differentiate in PDGF receptor α -expressing OLPs, although some of them gave rise to astrocytes and neurones, or remained undifferentiated. As discussed by Cao et al. (2002), generation of astrocytes is particularly important for their trophic effect in axonal regeneration. Interestingly, transplanted NSCs acted not only by directly participating in remyelination, but also by dampening negative effects of inflammation and boosting the endogenous repair potential. Thus, mRNAs coding for factors known to promote astrogliosis were decreased, whereas other mRNAs coding for other neurotrophic factors were unchanged; the overall density of oligodendrocyte progenitors was five-fold increased, with only 20% of them being of donor origin. Accordingly, treated mice recovered from the disease and the long-lasting positive effect was confirmed by a nearly-normal central conduction time 80 days after transplantation. This study not only shows that NSCs can be used effectively to treat demyelinating diseases, but that their migratory properties may be exploited to reach multiple damaged areas, by simply injecting them intravenously. It should be noted that in this study no donor cells were found in other body organs (Pluchino et al. 2003).

The possibility that NSCs may integrate appropriately throughout the mammalian CNS following transplantation paved the way to a new approach for the therapy of neurological diseases. NSCs can be stably transduced (Aboody et al. 2000) with foreign, potentially therapeutic genes for localized delivery of discrete molecules, integrating into the host cytoarchitecture, forming essential circuits and repairing the damaged host brain. NSCs also appear to produce beneficial factors such as neurotrophic factors, cytokines and as yet undefined cancer inhibitory molecules (Benedetti et al. 2000; Klassen et al. 2003; Lu et al. 2003). Some biological properties of NSCs make them more suitable for gene/cell therapy than other cells such as bone marrow-derived cells, as NSCs are attracted to cerebral sites of neurodegeneration, ischaemia and tumour transformation in both young and aged individuals (Noble and Dietrich 2002; Park et al. 2002). Transplanted NSCs and their descendants are not only attracted toward lesions, but also appear to 'track down' isolated cancer cells (Aboody et al. 2000). NSCs may therefore be defined by another property, in addition to the traditional self-renewal and multipotency: the ability to integrate appropriately in the mammalian CNS following transplantation and to populate degenerating or injured regions.

NSCs have been tested as therapeutic tools in models of several neurodegenerative, ischaemic and neoplastic diseases. One of the first was a mutant mouse for a frameshift mutation of the β-glucuronidase gene that accumulates undegraded glycosaminoglycans, causing a fatal and progressive degenerative disease. Neurosphere-derived cells overexpressing the therapeutic gene were injected into the cerebral ventricles at birth. They delivered the gene product through the brain, cross-correcting the enzymatic defect (Snyder et al. 1995). Another was the shiverer mouse, a model of extensive dysmyelination due to defects in the gene encoding MBP. Transplantation at birth of clone C17.2 NSCs derived from neonatal cerebellum (Snyder et al. 1995) resulted in widespread engraftment with repletion of MBP. The most important observation was that the cells adapted their multipotential fate, shifting their differentiation towards an oligodendroglial fate (up to 52%) to compensate for the specific deficient cell type (Yandava et al. 1999).

Selective death of a vulnerable neuronal population caused by global cerebral ischaemia is followed by regeneration of CA1 hippocampal neurones upon intraventricular infusion of EGF and bFGF (Nakatomi et al. 2002). However, only 0.2% of the dead striatal neurones are replaced by newly formed cells, as most of the new striatal neurones die after 2-5 weeks (Arvidsson et al. 2002), showing that endogenous repair without any outside help is unable to overcome the emergency. In rodents, however, hypoxic and ischaemic brain injury may be repaired by transplanted NSCs that migrate and preferentially integrate into the ischaemic lesion (Gray et al. 2000; Toda et al. 2001; Modo et al. 2002). A NSC C17 clone transduced with neurotrophins (NT-3) gave rise to a higher percentage of neurones at the infarction site (Liu et al. 1999), showing that gene therapy and cell replacement may occur simultaneously in the same recipient. Aboody et al. (2000) demonstrated that the C17.2 NSC clone invades the tumour bed not only if transplanted within the tumour but also if the site of implantation is distant or even in the opposite hemisphere. When C17.2 clonal line cells were engineered to produce an oncolytic molecule (the enzyme cytosine deaminase) (Aboody et al. 2000), or different interleukins (Benedetti et al. 2000), they dramatically reduced the tumour size, and even led to the survival of most of the tumour-bearing mice. Survival of mice transplanted with mock transduced neurosphere-derived cells was also longer than that of control animals, suggesting that an anti-tumour factor may be produced by the neural progenitors (Benedetti et al. 2000).

In all these cases, NSCs are not simply a vehicle of a therapeutic gene product, but, being able to migrate extensively within the CNS, successfully target different types of lesion. However, virtually nothing is known about the mechanisms and the genes involved in such migration. Understanding them is a major goal in this field, and will require identification of several extracellular signals, dissection of their action on transcription factors, and the identification of the intracellular pathways ultimately responsible for the *in vivo* behaviour of the transplanted cells.

Conclusions

In this review we have attempted to integrate information derived from studies of NSC biology with that derived from neuroembryology. Integrating and comparing results from the two fields is not always easy because, as pointed out by Anderson (2001), the questions addressed and the experimental manipulations used have been rather different. However, addressing many key issues concerning these cells, such as the extent to which expression of particular transciption factor(s) commits a cell to a given fate *in vivo*, or how dissociation and expansion in culture affect regional identity and the developmental potential of NSCs, will require the tools of the neuroembryologist. For these studies, it is essential to identify better markers for NSCs, not only to isolate them directly but also to study their behaviour *in vivo* in the intact brain.

Several genes and signalling pathways governing maintenance and differentiation of NSCs have now been identified and we are beginning to link them together. However, the picture is far from complete. For many genes we know little or nothing of the mechanism(s) of action, and in some cases their role is only inferred by their expression pattern or the effect of their knockout on the developing nervous system. There are big gaps to be filled before we can learn how to manipulate these cells in vivo or in vitro to provide new therapies for neurological diseases. Transplantation experiments have shown that these cells (or their descendants) are capable of moving extensively towards areas of lesion, where they spontaneously attempt to repair the damage. It is clear that this behaviour requires extensive signalling and gene regulation to attract and guide the cells, and to drive their differentiation into the appropriate cell types. A detailed knowledge of these mechanisms would be of great help in designing strategies to boost endogenous repair or to train or engineeer exogenous NSCs. Unfortunately, these mechanisms are still largely unknown and their unravelling will be a major challenge and an exciting field of research.

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