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Defining the NG2-expressing cell of the adult CNS

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Abstract

The NG2 proteoglycan is believed to be an *in vivo* marker for oligodendrocyte progenitors found in the developing brain. The prevalence of NG2-expressing cells that remain in the adult CNS following the end of gliogenesis is significant. Current research is focused on how this cell participates in the normal function of the adult CNS and whether it may be activated by injury and/or contribute to repair. Despite substantial evidence for a sub-population of NG2-expressing cells playing a glial progenitor role in the adult CNS, there is much to be learned. Specifically, the heterogeneity of this population has not been adequately addressed for the adult CNS and while NG2 cells continue to divide in the adult CNS it is not clear what function they serve once myelination is complete. Future studies should elucidate the functional importance of NG2 in a variety of cell functions and shed light on the role NG2-expressing cells play in the intact and diseased CNS.

NG2 is a highly conserved molecule that is expressed in a variety of tissues during development and adulthood (Levine *et al*., 1986). The adult central nervous system (CNS) is no exception with widespread expression of NG2 by cells in all regions examined. The ubiquitous and highly conserved nature of NG2 expression has drawn warranted investigation into its role in a variety of processes including cytokine signaling, mitogenesis and cell migration. One common thread that ties NG2 expression with cell function has been the observation that a large majority of NG2-expressing cells retain the ability to divide. This property alone suggests that NG2-expressing cells exhibit a progenitor quality (Levine & Nishiyama, 1996). In addition, in the developing CNS there is a close association between PDGFα-receptor and NG2-expressing glial progenitor cells. Indeed, immunohistochemical detection of this proteoglycan has become an acceptable method for identifying glial progenitor cells in pre- and postnatal CNS. However, caution must be raised when it is assumed that the adult NG2-expressing progenitor has the same multipotency and cytokine responsiveness as an NG2-expressing progenitor found in embryonic or early postnatal development. Clear differences in migration, cell-cycle length, and lineage restriction exist between progenitors derived from early postnatal brain or embryonic brain as compared with those derived from the adult. This has been illustrated in studies of optic nerve-derived glial progenitor cells (Raff *et al*., 1983; Temple & Raff, 1986; Wolswijk *et al*., 1990).

In this review we consider the evidence that NG2-expressing cells function as glial progenitor cells in the adult CNS with emphasis on the adult spinal cord. We discuss our observations of NG2 in the adult spinal cord and adult-derived neural stem cells and compare these findings with generally accepted principles of the NG2-expressing cell in the adult and developing brain. Finally, we explore potential methods to further delineate the functional heterogeneity and plasticity of NG2-expressing cells in the adult.

Distribution and morphology of NG2 expressing cells in the adult spinal cord

One striking observation about NG2 distribution in the adult CNS is the frequency of cells that are associated with this proteoglycan (Levine & Card, 1987). The number of NG2-expressing cells in the spinal cord makes this population intriguing indeed and compelling for further study. We conducted cell counts by stereological

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methods in the adult spinal cord and in Figure 1A the number and distribution based on morphology of these cells is presented. It is important to note that the number of NG2-expressing cells represents about 2% of all white matter glial in the adult mouse spinal cord (Bjugn, 1993; Bjugn & Gundersen, 1993). This is significantly less than reported by Chang and colleagues where a qualitative description of NG2 in human tissue suggested that these cells may have a similar frequency as astrocytes (Chang *et al*., 2000).

As previously described for the brain, spinal cord cells that express NG2 have two distinct morphologies. The first is a cell with a small, oblong nucleus, limited cytoplasm and short unipolar or bipolar processes. These cells morphologically resemble protoplasmic astrocytes but do not colocalize with markers of immature or mature astrocytes (Levine *et al*., 1993; Nishiyama *et al*., 1996b). NG2-expressing cells with bipolar morphologies are a prominent feature of gray and white matter. Bipolar or unipolar NG2 cells in the white matter are predominantly located near radial elements of the spinal cord and are the most common phenotype that divides (Horner *et al*., 2000). During development, NG2-immunoreactive cells appear to associate with radial glial cells (Diers-Fenger *et al*., 2001). In the adult spinal cord it remains to be determined whether NG2 cells align with radial glia, vascular profiles or axon bundles. The second major morphotype is that of a stellate, multipolar cell that is also distributed in gray and white matter. These cells resemble microglia or pre-myelinating oligodendrocytes with a small cell soma and lacy, ramified processes. Interestingly, NG2-expressing cells have been shown to contain processes that make contacts at the nodes of Ranvier (Butt *et al*., 1999) and associate with synapses (Ong & Levine, 1999). The stellate NG2 morphotype does not co-express markers of microglia (OX-42 or F4/80) or mature oligodendroglia markers (MBP or RIP; Levine & Nishiyama, 1996; Levine & Stallcup, 1987). In addition, the stellate morphotype does not appear to produce myelin.

In the adult spinal cord, NG2-expressing cells appear to be evenly distributed in both gray and white matter compartments (Fig. 1B). This is true even in the myelin-free zone of the substantia gelatinosa indicating that NG2 phenotype is not restricted to areas containing myelin. There does not appear to be a correlation between NG2-cell density and myelin rich regions of the adult CNS. NG2 cells are also located in the region of the central canal (unpublished observations). While the ependymal cells do not express NG2 themselves, however, cells that have interdigitating processes within the central canal do abundantly express NG2.

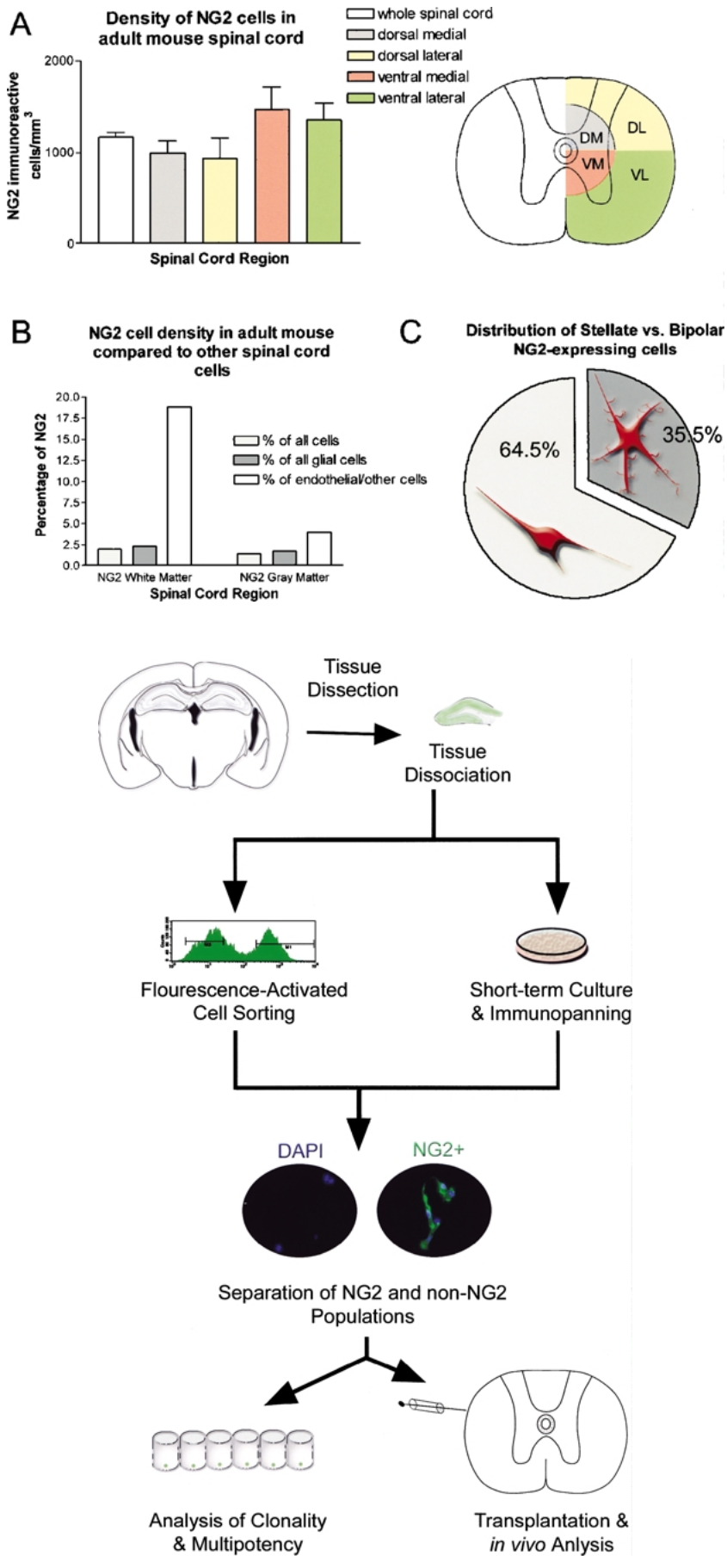
Simple conclusions cannot be drawn from the global expression of NG2. The lack of colocalization of NG2 with other markers of mature CNS glial and neuronal phenotypes argues that these cells are a unique population, possibly a unique macroglial cell population distinct from astrocytes and oligodendrocytes (Dawson *et al*., 2000). As previously mentioned, the strongest insight into a function of the NG2-expressing cells could be its association with glial progenitor receptors and progenitor physiology (Diers-Fenger *et al*., 2001; Nishiyama *et al*., 1996b). In addition, NG2-expressing cells extracted from the developing optic nerve have the capability to produce mature oligodendrocytes and type II astrocytes (Stallcup & Beasley, 1987). These findings are discussed in more detail below.

Proliferation and migration in the adult CNS

In the developing CNS and during early postnatal gliogenesis, a significant number of NG2 cells

Fig. 1. *Density and distribution of NG2-expressing cells in the adult mouse spinal cord*. The number of NG2-immunoreactive cells was quantified from 12 week old C57BL/6 mice. (A) Using the optical fractionator and the regional templates outlined in (A) NG2-immunoreactive cells that clearly contained nuclei (confirmed with DAPI labeling) were counted from 20 μm thick sections of the lumbar spinal cord (L_1–L_2). The density was approximately 1000 NG2 cells/mm^3 with a consistent dorsal-ventral and medial-lateral distribution. (B) The percentage of NG2 cells as a function of total cell number, glial cell number or endothelial/non-glial cells was estimated by dividing the density of NG2 cells by the cell densities for the mouse spinal cord reported by Bjugn, 1993. NG2 cells comprise approximately 2.5% of all glial cells in white matter and 2.3% of all glial cells in gray matter. (C) The percentage of NG2 cells that incorporated BrdU following 12 daily doses of BrdU was calculated for the entire lumbar region. Note that the bipolar morphotype was most commonly associated with BrdU incorporation.

Fig. 2. *Strategies to explore the plasticity of NG2-expressing cells in the adult CNS*. This diagram outlines a strategy to isolate NG2 cells acutely from the mature CNS. The potential benefit of rapid isolation and direct analysis is the ability to determine regional variation in NG2-expressing cells as well as the homogeneity that the NG2 population exhibits in terms of cellular plasticity. Specific regions of the brain can be micro-dissected and dissociated by enzymatic and/or mechanical dissociation. Single cell suspensions can be directly labeled by fluorescent conjugated antibodies to NG2 and sorted using FACS. Alternatively, dissociated cells may need to be plated for short periods of time to allow for the re-expression of NG2 on the cell surface at which time the cells can subsequently labeled and sorted by immunopanning or FACS. Separated populations of NG2-immunoreactive and NG2 negative cells can be analyzed by clonal dilution and brief expansion *in vitro* to determine the differentiation capacity of individual cells. Finally, direct isolation followed by transplantation back into the region of origin or alternative region of the intact CNS could be performed to determine the role of adult microenvironments on NG2 cell proliferation, migration and differentiation.


A
Density of NG2 cells in adult mouse spinal cord
whole spinal cord
dorsal medial
dorsal lateral
ventral medial
ventral lateral
NG2 immunoreactive cells/mm3
2000
1000
0
Spinal Cord Region
DM
DL
VM
VL
B
NG2 cell density in adult mouse compared to other spinal cord cells
Percentage of NG2
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
% of all cells
% of all glial cells
% of endothelial/other cells
NG2 White Matter
NG2 Gray Matter
Spinal Cord Region
C
Distribution of Stellate vs. Bipolar NG2-expressing cells
64.5%
35.5%
Tissue Dissection
Tissue Dissociation
Flourescence-Activated Cell Sorting
Short-term Culture & Immunopanning
DAPI
NG2+
Separation of NG2 and non-NG2 Populations
Analysis of Clonality & Multipotency
Transplantation & in vivo Anlysis

have been shown to divide and express the PDGFα-receptor (PDGFαR) indicating they are glial progenitors (Nishiyama *et al*., 1996b; Reynolds & Hardy, 1997; Warrington & Pfeiffer, 1992). We have extended these studies to the adult by examining cell proliferation in the rat spinal cord beyond the period of postnatal gliogenesis. We utilized intra-peritoneal injection of bromodeoxyuridine (BrdU; a thymidine analogue) to label dividing cells in adult rats and mice. Using a repeated dosage regimen of BrdU (50 mg/kg; 12 daily doses), we discovered that up to 70% of all dividing cells within the adult rat spinal cord co-labeled with NG2, which is impressive since the incidence of cell division was high and the persistence of dividing cells was significant. In previous work, Adrian and Walker (1962) reported that cell division occurred in the adult spinal cord but that the dividing population only transiently persisted (Adrian & Walker, 1962). Indeed, ^{3}H-thymidine labeled cells were no longer detectable by 7 days post-injection of this mitotic marker. In contrast, our BrdU-studies demonstrated a similar incidence of labeling initially and dividing cells were found to be consistently labeled as long as 6 weeks after the initial BrdU injection. Levine and colleagues have reported that 0.14 to 0.38% of NG2 cells labeled with ^{3}H-thymidine in the adult cerebellum (Levine *et al*., 1993). Our data indicates that over a two week period approximately 3% of all NG2 immunoreactive cells are undergoing cell division. If adjusted for the time period the mitotic marker was bio-available, these studies yield similar results and indicate that NG2 cells divide at a slower rate than during the early postnatal period of gliogenesis. In our experiments, we performed a stereological analysis of BrdU-incorporation to determine where cells divided and to assess post-division cell migration. Our approach demonstrated that in the outer aspect of the adult spinal cord as many as 6% of all nuclei entered cell-cycle within a 2 week period. Initially, this rate of cell division led us to believe that these cells were most likely migrating microglia/macrophages or vascular cells. However, double labeling with NG2 demonstrated that BrdU and NG2 were consistently associated while microglia cells marked by OX-42 comprised less than one percent of BrdU labeled cells. No evidence for migration was found in either single pulse or repeated BrdU pulse experiments for up to four weeks following the last BrdU injection. This indicates that adult NG2 cells are different from NG2 cells in the perinatal period in that they are not migratory in the intact spinal cord. Similar findings have been reported for glial progenitors in the adult forebrain (Gensert & Goldman, 1996, 1997).

A common theory reported for NG2 is that this molecule is expressed by proliferating progenitor cells in a variety of tissues during development and by glial progenitor cells of the CNS. The concept of adult-derived NG2 cells in the CNS being composed of a component of actively proliferating progenitor population fits with several observations from the injured CNS (discussed below).

Plasticity of the NG2-expressing progenitor

In the intact adult spinal cord there are no cells that routinely generate neurons but the formation of mature oligodendrocytes and astrocytes does occur (Horner *et al*., 2000). What role does the NG2 cell play in the process of adult gliogenesis? It has not been determined whether a dividing NG2 cell serves as a unipotent oligodendrocyte progenitor, a bipotent glial progenitor or a multipotent neural progenitor that is restricted to a glial fate by endogenous factors. There are several criteria that must be met in order to conclude that a cell, in this case defined by NG2 expression, is a progenitor cell *in vivo*. Defining the plasticity of the NG2 cell will have important ramifications for how we view its ability to repair the adult CNS. The mere fact that NG2-expressing cells continue to divide at a significant rate in the intact adult implies that at the very least a portion of this population is plastic and may serve as a transient amplifying system for progenitor expansion and glial cell replacement.

In order to evaluate the plasticity of the adult NG2 cell we must define the characteristics of a progenitor cell and then evaluate the existing data as it pertains to these basic tenets. During development, the process of cell maturation from a progenitor stage to a differentiated cell involves: (1) cell division to expand to an appropriate sized population, (2) migration through the CNS to the site of cell differentiation and (3) functional differentiation (Rogister *et al*., 1999). This latter process involves the downregulation of progenitor genes with concomitant upregulation of genes expressed in the differentiated phenotype. Thus, a cell that has been derived from an NG2 progenitor stage might possibly no longer express NG2. The technique for following an NG2 cell through its life span beyond the progenitor stage *in vivo* has not yet been reported. As a result, the preponderance of evidence for NG2 cells being multipotent progenitor cells is derived from *in vitro* expansion and analysis and from temporal studies in the intact and injured CNS. This associative data strongly suggests that a population of NG2 cells has the capability to differentiate into oligodendrocytes but there is limited data to indicate if this is a common feature of all adult NG2 cells. In our own work in the adult CNS we have found that the NG2-expressing cells are the earliest cells to divide using a single injection of BrdU and a one hour survival time in adult rats. Using a 12 day repeated BrdU dosing regimen we found that over a 6 week period following BrdU administration, the number of NG2 cells that colocalized with the mitotic marker BrdU decreased by 20–30%. Mature glial markers did not colocalize with BrdU either at one hour after BrdU labeling or as long

as 2 weeks after repeated pulsing of BrdU. A decrease in the incidence of NG2/BrdU co-expression occurred during a time frame where the number of mature glial markers colocalizing with BrdU was increasing. This suggests that NG2 cells may downregulate their expression of NG2 and upregulate mature glial markers even in the intact adult. However, it is not clear from this data whether NG2 progenitor cells produced both astrocytes and oligodendrocytes. In addition, it is not possible to determine how homogenous the NG2 population is with regards to their ability to divide and to differentiate. Interestingly, there are many more NG2 cells generated by cell division than mature glial cells being produced (Horner *et al*., 2000). One explanation is that a majority of NG2 cells undergo apoptosis although a study of spinal cord apoptosis has not been conducted and evidence for NG2-cell death in the adult CNS is lacking. However, there is evidence for apoptosis of neural progenitors during development (Morshead *et al*., 1998) and in the adult brain (Biebl *et al*., 2000).

In the intact spinal cord, approximately 3% of NG2 cells are dividing over a 12 day BrdU dosing period. Thus, the majority of NG2 cells are not in cell cycle. Given the total density of NG2 cells in the adult spinal cord this indicates that the non-dividing population represents a significant pool of cells. The high number of non-dividing NG2-expressing cells and the lack of evidence for their migration indicate a portion of the NG2 cells may be quiescent progenitor cells or an as yet unidentified differentiated phenotype.

Overall it is clear that the CNS retains a population of cells that are capable of extensive remyelination (Franklin *et al*., 1997; Zhang *et al*., 1999). The factors that stimulate this process are still being examined (Hinks & Franklin, 1999). Our data are only a limited example among other studies that suggest the adult NG2-expressing cell has progenitor qualities. Other *in vivo* evidence of NG2 expressing cells undergoing differentiation is derived from injury paradigms. For example, Levine has shown that following a stab lesion of the rat cortex, there is an increase in the number of cells that express NG2, the intensity of NG2 immunoreactivity and levels of NG2 RNA (Levine, 1994). In these experiments NG2-expressing cells are the first to incorporate ^{3}H-thymidine and are the primary responding cell. Between 2 and 7 days after the injury the number of dividing astrocytes and microglial cells increases and the percentage of NG2 cells that incorporate ^{3}H-thymidine gradually decreases. Similar to the data from the intact spinal cord, this indicates that NG2 cell may be transitioning from a progenitor phenotype to a differentiated phenotype; the astrocyte. Levine found limited evidence for co-localization between NG2 and markers of either astrocytes or microglia. Tuszynski and colleagues found similar results with an experimental spinal cord lesion with NG2 cells incorporating BrdU early after injury (Jones *et al*., 2002). Interestingly, in these experiments, NG2 was found immunohistochemically localized to microglial cells near the lesion. These experiments suggest NG2 cells may participate in scar formation. While upregulation of NG2 protein may be an indication for cellular plasticity, increased production of this proteoglycan may actually have a detrimental effect on regeneration (see Levine, this volume).

In similar experiments, NG2-expressing cells also proliferate following a contusion injury (McTigue *et al*., 2001). These cells represent a relatively small fraction of the total number of dividing cells in which microglia and macrophages predominate. Thus, in the injured spinal cord, remyelination occurs as well as the production of new astrocytes. However, it should be kept in mind that while the presumption is that many of these NG2 cells represent glial progenitor cells that differentiate into mature oligodendrocytes and astrocytes, these data are based on associative and not direct observations.

How homogenous is the response of the adult NG2-expressing cell to injury? In many of the experimental studies examining NG2 cell proliferation, not all cells enter cell cycle within the vicinity of the lesion. There may be a progenitor population of NG2-expressing cells that is separate from a non-dividing NG2 cell that is a fully differentiated phenotype in and of itself. For example Keirstead and colleagues have shown that following injury to the CNS there is a responsive and a non-responsive component of NG2-expressing cells that divide after x-irradiation injury of the adult spinal cord (Keirstead *et al*., 1998). This result may suggest that some NG2 cells are terminally differentiated or alternatively, in the process of differentiation. The Keirstead data also suggests that dividing NG2 cells have a limited number of cell divisions and are thus not self-renewing. This supports the concept that a portion of the NG2 population divides after injury and has characteristics of a glial progenitor cell.

Together, these data suggest that NG2 expression may represent a mosaic of cell phenotypes and lineages following CNS injury. Finally, NG2 expression may be an indication of a cell state in a complex or mixed population of cells. Methods to discriminate between these functions of the NG2 cell and to determine the heterogeneity of this population are described below.

Molecular profile of NG2 cells *in vivo*

Raff and colleagues described a population of glial progenitor cells isolated from rat optic nerves that are capable of differentiating into oligodendrocytes or type-2 astrocytes *in vitro* (Raff, 1989; Raff *et al*., 1983). These O2A progenitor cells have also been isolated from the adult rat spinal cord and have been characterized *in vitro* (Engel & Wolswijk, 1996). O2A progenitor cells express the PDGFαR *in vitro* (Hart *et al*., 1989; McKinnon

et al., 1990). *In vitro* studies suggest that PDGF secreted by type-1 astrocytes and neurons keeps O2A progenitor cells in a proliferative state until an intrinsic clock in these glial progenitor cells initiates the differentiation towards a mature oligodendrocyte (Hart *et al*., 1989). At this decision point the progenitor cell becomes unresponsive to PDGF although the PDGFαR is still expressed on the surface of the differentiating cells (Hart *et al*., 1989). It is important to note that while PDGFαR expression has been tightly associated with glial progenitor cells in the developing CNS, this receptor may also be expressed by neuronal progenitor cells during development (Erlandsson *et al*., 2001). In addition, there may be subclasses of glial progenitor cells during development with molecular profiles distinct from that described for the O2A progenitor (Mallon *et al*., 2002; Spassky *et al*., 2000). These observations underscore the importance of molecular characterization in conjunction with lineage analysis in order to determine whether a cell is a glial progenitor *in vivo*.

NG2 expressing cells are likely to be oligodendrocyte precursor cells as they show many characteristics of O2A-progenitor cells (Levine & Nishiyama, 1996; Stallcup & Beasley, 1987). Nishiyama and colleagues have shown that in the early post-natal CNS, NG2-positive cells in both, white and gray matter, express the PDGFαR (Nishiyama *et al*., 1999). In the developing rat CNS PDGFαR and NG2 are co-localized. Only a few cells in the subventricular zone are PDGFαR-positive, but NG2-negative, suggesting that they represent more primitive precursor cells. After immunostaining, NG2 and PDGFαR can be localized in the very same punctae on the cell surface implying a close association of the two molecules (Nishiyama *et al*., 1996b). In addition, NG2 has been co-immunoprecipitated from a neonatal O2A cell extract with an antibody to PDGFαR (Nishiyama *et al*., 1996a). This suggests that NG2 and PDGFαR form a molecular complex in the embryonic and perinatal rat CNS (Nishiyama *et al*., 1996a). Other experiments by Nishiyama and colleagues have shown that the co-expression of NG2 and PDGFαR is important for the proliferative response of glial progenitor cell to PDGF-AA (Nishiyama *et al*., 1996a). The reduced expression of NG2 by antibody patching resulted in a downregulation of PDFGαR expression. On the other hand, in aortic smooth muscle cells isolated from NG2$-/-$ mice PDFGαR is unresponsive to PDGF-AA (Grako *et al*., 1999). Thus, the absence of NG2 leads to a defect in signal transduction, most likely due to the inability of PDFGαR and NG2 to form a complex on the surface of these cells (Nishiyama *et al*., 1996a). FGF-2, another mitogenic factor for O2A cells (McKinnon *et al*., 1990), and PDGF-AA, both are able to bind to the core protein of the NG2 proteoglycan with high affinity. The NG2 molecule contains at least two binding sites for each of these growth factors, and binds not or little to other growth factors (PDGF-BB, TGFβ-1, VEGF, EGF) (Goretzki *et al*., 1999). NG2, thus, may be important for regulating the extracellular localization, levels and presentation of these ligands to their respective signaling receptors.

It is important to consider that most of these studies have been conducted during the post-natal period when gliogenesis is actively occurring. In this regard, the number of co-labeled cells as well as the level of expression for PDGFαR and NG2 peaks in the first post-natal week (Nishiyama *et al*., 1996b) and declines thereafter, although both molecules are still expressed in the adult CNS. In the adult rat brain the co-localization of NG2 and the PDGFαR is not as perfect as in the developing and perinatal brain. NG2 immunostaining covers the cell body and the processes whereas PDGFαR immunolabeling is confined to the cell body and proximal processes (Nishiyama *et al*., 1996a). This observation suggests that NG2 and PDGFαR do not interact directly in the adult CNS, which may explain why PDGF is no longer a mitogen for adult O2A progenitor cells *in vitro*. In the adult mouse spinal cord only 50% of the AN2-positive cells, the mouse homologue to the NG2 cells, co-express PDGFαR (Diers-Fenger *et al*., 2001). Interestingly, PDGF administration to the intact adult spinal cord does increase the yield of oligodendrocytes that can be cultured from this region (Ijichi *et al*., 1996).

PDGF-A knockout mice have a reduced number of PDGFαR oligodendrocyte precursor cells, fewer mature oligodendrocytes and show signs of dysmyelination, although not all parts of the CNS are equally affected (Fruttiger *et al*., 1999). Whether the PDGF-A knockout mice have reduced numbers of NG2-expressing cells remains to be investigated. PDGFα receptor expression seems not to be confined to O2A progenitor cells. Although during later stages of neurogenesis the PDGFαR expression appears to be mainly restricted to O2A progenitor cells (Pringle *et al*., 1992), a few studies showed that some neuronal populations of the developing and adult CNS are able to express the PDGFαR (Oumesmar *et al*., 1997; Vignais *et al*., 1995). This suggests that PDGF may have additional functions to its mitogenic actions for glial progenitors in the adult CNS.

In situ hybridization showed that O2A progenitor cells in the adult mouse are positive for NG2, PDGFα receptor and express receptors for FGF-2 (Redwine *et al*., 1997). Double-labeling of NG2-positive cells *in situ* with a variety of antibodies specific for certain cell types was done to characterize the NG2–expressing cells in the mature rodent CNS. In accordance with their putative role as glial progenitors, NG2-positive cells are sometimes co-labeled with O4, a more mature oligodendrocyte marker (Reynolds & Hardy, 1997; for review see Dawson *et al*., 2000). Only rarely, however, NG2-expressing cells stain for more mature oligodendrocyte markers, *e.g.* MBP, RIP, GC consistent with NG2 being a oligodendrocyte precursor marker. Labeling

with the astrocyte markers S100β, GFAP (Levine *et al*., 1993; Nishiyama *et al*., 1996b) or glutamine synthase (Reynolds & Hardy, 1997) did not reveal any co-labeling with NG2. Microglial markers like OX-42 or F4/80 were never co-localized with NG2 and NG2-positive cells did not bind to GSA I-B4 lectin, which binds to microglia (Reynolds & Hardy, 1997; Nishiyama *et al*., 1997). Thus, NG2-expressing cells in the adult rodent CNS are distinct from astroglia and microglia.

It is clear that the adult NG2 cell has similarities and differences in its molecular make-up compared to the NG2 cell described in development and postnatal CNS. For example, NG2-expressing cells of the adult CNS have reduced expression and altered distribution of PDGFα receptors. There is clearly a great deal more be learned of the cytokine receptors expressed and the response to cytokines of adult NG2 cells.

Methods to define the plasticity of the NG2 cell

During development all tissues contain stem cells, which are able to give rise to all cell types in the tissue. A precursor or progenitor cell, in contrast, is a committed "stem cell" with limited self-renewal, which is able to give rise to certain lineages only and displays a restricted proliferative potential. As the most primitive stem or precursor cells are often quiescent, they may not express any specific markers and, thus, no stem cell or progenitor marker exists presently. In the CNS, progenitor cells are found throughout life in the dentate gyrus of the hippocampus (Altman & Bayer, 1990; Altman & Das, 1965b; Bayer *et al*., 1982; Cameron *et al*., 1993; Kuhn *et al*., 1996) and in the subventricular zone (Altman & Das, 1965a; Lewis, 1968). The cells proliferate and give rise to granule neurons in the dentate gyrus or olfactory bulb neurons, respectively. *In vitro*, these precursor cells are multipotent and are able to give rise to glial and neuronal cells (Palmer *et al*., 1997). Other regions of the CNS also contain progenitor cells (Palmer *et al*., 1995, 1999). Precursor cells in non-neurogenic regions are giving rise to glial cells *in vivo*, but are able to differentiate into neurons *in vitro* and when transplanted into neurogenic sites *in vivo* (Shihabuddin *et al*., 1997, 2000).

To investigate if an isolated cell is a "stem cell" or a precursor cell, the fate and potential of this cell has to be determined. The assessment of the potential, however, is a challenging task as extrinsic cues will determine the phenotype of the cell, but the intrinsic properties of the cell could allow the cell to achieve various phenotypes depending on the environmental cues. In addition, some procedures used to isolate a particular cell type result in cell loss leaving only a fraction of the original population. Thus, it cannot always be concluded whether a cell property is ubiquitous among that population or a unique property of a subset of cells. In later sections we will discuss methods that may help illuminate the lineage restriction and heterogeneity of NG2-expressing cells *in vivo*.

Three main approaches are presently used to investigate the potential of a stem or precursor cell (Gage, 2000). Stem cells can be labeled by vital dyes or a retroviral vector and the progeny of this cell can be assessed *in situ* (lineage tracing). Proliferative cells can be isolated from the tissue and the plasticity of these cells can be examined using an *in vitro* approach (differentiation analysis). Transplantation of freshly isolated or long-term cultured cells into the CNS allows to study their fate *in vivo* (transplantation analysis; Gaiano & Fishell, 1998). Lineage tracing using retroviruses, thymidine or bromodeoxyuridine (BrdU) labels dividing cells and allows following the fate of a particular cell. This procedure, however, is not very efficient and the expression of the retrovirus is often downregulated with terminal differentiation. It would be useful to produce a retroviral reporter for NG2-expressing cells but the promoter sequence for NG2 is not yet known. In addition, BrdU and thymidine signals are becoming diluted with progressing cell divisions. The *in vivo* lineage tracing is able to reveal if new cells are born and if a given cell has the potential to give rise to more than one cell type, but cannot determine if a proliferative cell is multipotent and self-renewing (for review see *e.g.*, Gage, 2000).

To study cell plasticity *in vitro*, the region that has been shown to contain dividing cells *in vivo* has to be microdissected. After digesting the tissue, the dissociated cells are exposed to high concentrations of growth factors in defined or supplemented medium and grow as monolayers or neurospheres depending on the tissue source and the culturing conditions. Once the cells are proliferating in culture, they are induced to differentiate by withdrawal of the mitogens and/or addition of other factors that prompt the cells to differentiate into a certain lineage (*e.g.*, fetal bovine serum, retinoic acid and/or forskolin in the case of neural precursor cells). The differentiated cells are then analyzed by antibody staining to determine their phenotype. A clonal analysis (low density plating or labeling of individual cells using a retroviral vector) allows one to determine if a single progenitor cell is able to give rise to all three neural phenotypes, neurons, astrocytes and oligodendrocytes. This differentiation analysis takes place in an artificial environment with unphysiological concentrations of growth and/or differentiation factors. Thus, the results of *in vitro* studies have to be carefully interpreted and to be verified *in vivo*. Transplantation studies could be the next step in the analysis. Progenitors, which have been shown to be multipotential *in vitro*, can be transplanted *in vivo* to verify their potential (Gaiano & Fishell, 1998). The fate of the grafted cells appears to be mainly influenced by the microenvironment. The neural progenitor cells can be transplanted either in a neurogenic site, where the environment is providing cues for neurogenesis, *e.g.* the dentate gyrus

of the hippocampus (Palmer *et al*., 1997) or the SVZ (Morshead *et al*., 1994), or in a non-neurogenic site like the spinal cord (Shihabuddin *et al*., 1997; Weiss *et al*., 1996) or septal and striatal parenchyma (Palmer *et al*., 1995), where progenitor cells do not give rise to neurons, but glial cells.

To investigate if NG2-positive cells are stem, neural progenitor or committed glial precursor cells, and to explore the function of NG2 various approaches will be needed. Some of the strategies are outlined below.

ISOLATING NG2-EXPRESSING CELLS

To analyze NG2-positive cells *in vitro* an enriched NG2-population is needed. NG2-based fluorescent activated cell sorting (FACS) or immunopanning as direct isolation methods from the adult CNS would be useful techniques. FACS sorting has not been reported for NG2, which may reflect the difficulty in isolating these cells acutely Fig. 2. There would be distinct advantages to being able to freshly sort cells expressing NG2 with good viability. Such an approach would allow the acute engraftment into neurogenic (*e.g*., hippocampus) or stem cell regions (*e.g*., SVZ) to address the environmental influence of plasticity of the NG2 expressing cells without prior manipulation *in vitro*. In addition, acute isolation followed by an *in vitro* clonal analysis would allow to address the heterogeneity of adult-derived NG2 cells. From own experience and reports of others, there are clearly hurdles that must be overcome to make this procedure possible. For example, traditional enzymatic digestion of tissue could lead to cleavage of the NG2 proteoglycan which due to its single membrane spanning peptide and limited glycosylation is sensitive to this procedure (Stallcup & Beasley, 1987 and our own observations). This suggests that in order to isolate large numbers of NG2 cells tissues would have to be digested with non-protease treatments such as collagenase and mechanical trituration. Given the structural complexity of stellate NG2 cells, significant mechanical disruption may lead to selective cell death compared to the bipolar NG2 cells. Another potential issue is the prospect of isolating proliferating cells that may be more sensitive to isolation procedures due to the metabolic demands of the cell division. This is apparently not a concern for direct isolation of cells that are in cell cycle as reported in studies progenitor cells from the adult forebrain (Gensert & Goldman, 2001). In these studies, glial progenitors that had recently divided *in vivo* were isolated and cultured with success.

To date, enrichment of NG2-epxressing cells from the adult has been accomplished by first culturing cells freshly isolated from the CNS followed by post-hoc enrichment or analysis. Stallcup and Beasley (Stallcup & Beasley, 1987) isolated glial precursor cells from the rat postnatal optic nerve and achieved a 90–95% purified population of NG2-positive cells using immunopanning with a monoclonal antibody directed against A2B5 and complement lysis of mature oligodendrocytes. The enriched NG2-expressing cells were cultured after the panning for A2B5 and were not exposed to any growth factors, but immediately induced to differentiate. After five days in defined medium and 10% fetal bovine serum 95% of the cells were NG2-positive, more than 80% were positive for glial fibrillary acidic protein (GFAP), and fewer than 10% GC-positive. The GFAP+/GC− astrocytes continued to express NG2 for up to 10d in culture. After 5d in serum-free supplemented medium less than 15% of NG2-positive cells expressed GFAP and up to 40% expressed GC. NG2 was expressed only for a short time by GFAP− /GC+ oligodendrocytes and mature oligodendrocytes were NG2-negative. These results derived from studying postnatal NG2-positive cells suggest that a sub-population of NG2-expressing cells are O2A-progenitors.

From the adult sub-cortical white matter and neocortex, an enriched population of cycling progenitor cells has been isolated and prospectively analyzed for cell surface markers (Gensert & Goldman, 2001). Adult rats were pulsed with BrdU and cells were isolated using a cell isolation technique routine for the culture of glial progenitor. From these studies, the primary dividing cell of these regions were O4 positive with a sub-population expressing A2B5. NG2 expression only represented a minority of the cells labeled with BrdU. These findings suggest that the NG2-expressing cells are not the major phenotypic component of glial progenitor in these regions of the brain. However, for the very reasons stated above, a prospective isolation of a pure population of NG2 expressing cells may be useful to validate these findings.

While analysis of directly isolated NG2 cells would be ideal it may never be adequately feasible. A second approach would be to utilize organotypic slice cultures to follow NG2 cells *in situ*. Such an experimental design would be dependent upon the ability to label NG2 cells within the slice and follow their migration and differentiation with time. This technology has been applied to progenitors of the developing cortex with successful observation of cell migration and division (Kakita & Goldman, 1999).

NG2− /− MICE

Transgenic and knockout technologies would also help to understand the role of NG2 in progenitor cell function in the adult. The anatomical localization and phenotype of neural progenitor cells has not yet been determined in detail for the adult mouse spinal cord. NG2 knockout mice are available and do not have a major phenotype (Grako *et al*., 1999). The phenotype of NG2 knockout animals and neural progenitor cells may be subtle and a detailed and careful analysis will be needed. Behavioral tests of knockout mice are another

way of analyzing deficits in the CNS. Detailed analysis of proliferative cells of NG2−/− mice has not been done yet. Studying BrdU-incorporation and the phenotype of these proliferative cells in NG2 null mutants will be a first step towards understanding the role of NG2 for neural progenitor cells. It is important to determine if the NG2 null mice contain the same number of oligodendrocytes and oligodendroglial precursor cells as wildtype mice. As the majority of oligodendroglial progenitor cells expresses the PDGFαR and many of those cells also express NG2 (Diers-Fenger *et al*., 2001; Wu *et al*., 2000), immunostaining for the PDGFαR may help to reveal if the cell type that expresses NG2 in the wildtype is still present in the NG2 null mutants. To find out if NG2 is important for proliferation BrdU-incorporation in wildtype and NG2 knockout mice has to be compared. It has been shown that NG2 influences the migration of certain cell types (Fang *et al*., 1999). Thus, it will be interesting to compare the location of BrdU-positive cells and/or of PDGFαR expressing cells in wildtype and NG2 null mice at various time points after BrdU-injection.

Spinal cord progenitor cells have been isolated from rat and mouse spinal cord (Shihabuddin *et al*., 1997; Weiss *et al*., 1996). Most of these progenitor cells remained undifferentiated *in vitro*. However, a percentage of progenitor cells gave rise to glial cells or neurons (Shihabuddin *et al*., 1997) depending on the spinal cord regions the precursor cells were isolated from. Spinal cord preparations comprising all regions yielded a high percentage of RIP-positive oligodendrocytes, few GFAP-positive astrocytes and some TubβIII-neurons (Yamamoto *et al*., 2001 and our own unpublished observation). For mouse spinal cord such a detailed *in vitro* analysis has not been done yet. Thus, it will be important to perform progenitor isolation from wild type mouse spinal cord and compare this population of cells with the progenitor cells isolated from NG2−/− mice. An investigation of proliferation as well as the differentiation potential of progenitor cells from wild type and NG2 knockout brain and spinal cord may shed light on the role of NG2 in these processes. If NG2-positive cells comprise the majority of glial precursor cells, less glial differentiation has to be expected. However, if the chondroitin sulphate proteoglycan NG2 is not necessary for the cells to "function" as glial progenitor cells, no difference should occur.

So far, most of the proteoglycan knock-out animals lacked an obvious phenotype and *in vitro* studies often failed to reveal any differences between wildtype and knock-out cells (Hartmann & Maurer, 2001). Compensatory mechanisms *e.g.* by redundancy of function of different proteins may explain the lack of obvious phenotypes. Proteoglycan function may be localized in the GAG chain, in the protein or in both. Thus, if the GAG chain is important for the function of a proteoglycan, another proteoglycan with the same or similar GAG chain may compensate the function as long as the expression occurs in the same region at the same time (Hartmann & Maurer, 2001).

NG2 GAIN OF FUNCTION

Overexpression of the proteoglycan NG2 in cultured brain or spinal cord progenitor cells is another way of addressing the function of this proteoglycan. Retroviral vectors (NIT-GFP) that contain a neomycin resistance gene and a *tTA* transactivator gene regulated by the retroviral LTR and express the green fluorescent protein (*GFP*) gene as a marker are one vector system, which can be used to transfect proliferative cells. As this vector contains a tetracyclin regulatable promoter, the expression of NG2 could be turned on and off *in vitro*. Differentiation analysis with NG2 overexpressing progenitor cells could reveal insights into NG2 function.

Alternatively, using a retroviral or lentiviral approach, overexpression of NG2 *in vivo* could be achieved. Transfection of neural progenitor cells in neurogenic regions would allow to investigate if NG2-expressing cells are pushed towards a glial lineage or if multipotential progenitor cells keep their multipotentiality independent of NG2 expression.

Summary

During development a tight correlation exists between cellular expression of NG2 and PDGFαR-expressing glial progenitor cells. However, there is reason to suspect that NG2-expressing cells may not be as homogenous as their developmental counterpart. In the adult, NG2 cells have both morphological and cell cycle characteristics that differ substantially from that of embryonic and early postnatal tissue. It is clear that a molecular characterization of NG2 cells *in vivo* would further our understanding of the heterogeneity of this population and shed light on novel roles that NG2-expressing cells may play in the adult CNS. In addition, newer methods to isolate and characterize NG2-expressing cells derived from the adult will expand our understanding of the plasticity of these cells and how they may be manipulated to repair the injured or demyelinated CNS.

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