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NG2 cell response in the CNP-EGFP mouse after contusive spinal cord injury

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Abstract

NG2⁺ cells in the adult CNS are a heterogeneous population. The extent to which the subpopulation of NG2⁺ cells that function as oligodendrocyte progenitor cells (OPCs) respond to spinal cord injury (SCI) and recapitulate their normal developmental progression remains unclear. We used the CNP-EGFP mouse, in which oligodendrocyte lineage cells express EGFP, to study NG2⁺ cells in the normal and injured spinal cord. In white matter of uninjured mice, bipolar EGFP⁺NG2⁺ cells and multipolar EGFP^{neg}NG2⁺ cells were identified. After SCI, EGFP⁺NG2⁺ cell proliferation in residual white matter peaked at 3 days post injury (DPI) rostral to the epicenter, while EGFP^{neg}NG2⁺ cell proliferation peaked at 7 DPI at the epicenter. The expression of transcription factors Olig2, Sox10 and Sox17, and the basic electrophysiological membrane parameters and potassium current phenotype of the EGFP⁺NG2⁺ population after injury were consistent with those of proliferative OPCs during development. EGFP^{neg}NG2⁺ cells did not express transcription factors involved in oligodendrogenesis. EGFP⁺CC1⁺ oligodendrocytes at 6 weeks included cells that incorporated BrdU during the peak of EGFP⁺NG2⁺ cell proliferation. EGFP^{neg}CC1⁺ oligodendrocytes were never observed. Treatment with glial growth factor 2 and fibroblast growth factor 2 enhanced oligodendrogenesis and increased the number of EGFP^{neg}NG2⁺ cells. Therefore, based on EGFP and transcription factor expression, spatio-temporal proliferation patterns, and response to growth factors, two populations of NG2⁺ cells can be identified that react to SCI. The EGFP⁺NG2⁺ cells undergo cellular and physiological changes in response to SCI that are similar to those that occur in early postnatal NG2⁺ cells during developmental oligodendrogenesis.

Keywords

Oligodendrocyte Progenitor Cells; Olig2; CNP gene; endogenous repair; cell proliferation; glial growth factor; fibroblast growth factor

INTRODUCTION

The chondroitin sulfate proteoglycan NG2 is a marker for oligodendrocyte progenitor cells (OPCs) during development (Stallcup, 1981; Levine et al., 1993; Levine and Nishiyama, 1996; Nishiyama et al., 1996; Reynolds and Hardy, 1997; Diers-Fenger et al., 2001; Liu et al., 2002; Mallon et al., 2002). Following spinal cord injury (SCI) in adult rodents, NG2⁺ cells respond by increased proliferation (McTigue et al., 2001; Zai and Wrathall, 2005; Lytle and

Wrathall, 2007) and may serve as an endogenous source of glial replacement chronically (Zai and Wrathall, 2005; Horky et al., 2006; Lytle and Wrathall, 2007; Rabchevsky et al., 2007; Tripathi and McTigue, 2007).

However, the NG2⁺ cell population in the adult central nervous system is heterogeneous (Butt et al., 2002; Horner et al., 2002; Nishiyama et al., 2002; Stallcup, 2002; Chittajallu et al., 2004). In addition to NG2⁺ OPCs, and potentially other types of NG2⁺ progenitor cells, NG2⁺ synantocytes have been described. Although this finding has made it difficult to define the adult OPC response to injury, this may be important in devising therapeutic approaches to mitigate abnormal myelination (Gledhill et al., 1973; Blakemore, 1974; Blight, 1983b, 1985; Wrathall et al., 1998) and resultant abnormalities of conduction (Blight, 1983a; Dimitrijevic et al., 1984; Bunge, 1994; Dimitrijevic et al., 1997) of spared white matter chronically after SCI.

There is indirect evidence suggesting that more than one type of NG2⁺ cells responds to SCI. In a recent study of cells dissociated from the rat spinal cord at 3 days after a standardized contusion SCI, we found that not all NG2⁺ cells could be accounted for by oligodendrocyte lineage markers A2B5, O4 and O1 (Lytle et al., 2006). *In vitro* clonal analysis showed that NG2⁺ cells from the injured spinal cord produce oligodendrocytes, and to a lesser extent astrocytes, or mature into morphologically complex NG2 cells (Yoo and Wrathall, 2007). Furthermore, in examining tissue from C57Bl6 mice subjected to a murine version of the same SCI (Kuhn and Wrathall, 1998), we found two populations of NG2⁺ cells, a nestin⁺ (neural progenitor marker) and nestin^{neg} population of NG2-expressing cells (Lytle and Wrathall, 2007). Taken together, these results suggest that more than one type of NG2⁺ cells may proliferate in response to SCI. Importantly, previous studies have been unable to distinguish between the response of OPCs and non-OPC NG2⁺ cells in the spinal cord after traumatic injury, and to what extent the adult NG2⁺ cell response might recapitulate developmental events in this progenitor population.

We examined the NG2 cell response in a transgenic mouse expressing enhanced green fluorescent protein (EGFP) driven by the 2–3-cyclic nucleotide 3-phosphodiesterase (CNP) promoter (Yuan et al., 2002). In these mice, all cells in the oligodendrocyte lineage express EGFP (Yuan et al., 2002), allowing us to study the response of NG2⁺ OPCs to SCI and that of other non-OPC lineage NG2⁺ cells. We analyzed spinal cords of both normal CNP-EGFP mice and of mice after standardized contusive SCI, using BrdU-labeling of proliferating cells, together with immunohistochemistry for cellular antigens and transcription factors, and patch clamp electrophysiology of cells in spinal cord slices. Our data demonstrate that two different populations of NG2⁺ cells - EGFP⁺ and EGFP^{neg}, respectively - proliferate in response to SCI. We show not only that EGFP⁺NG2⁺ cells generate oligodendrocytes after SCI, but also that these progenitors undergo cellular and physiological changes in response to SCI that are similar to those that occur in early postnatal NG2⁺ cells during developmental oligodendrogenesis.

METHODS

Spinal Cord Injury

Surgery was performed on 5–7 week old CNP-EGFP mice. Mice were anesthetized with 0.4–0.6mg/g of Avertin (2,2,2-tribromoethanol, Sigma), and a laminectomy was performed at thoracic level 8/9. The spinal column was stabilized via the lateral processes at T7 and T10. Contusion was produced by dropping a 2g weight from a height of 2.5cm (mild injury) onto a teflon impounder 1.5mm in diameter, as previously described (Kuhn and Wrathall, 1998). The muscle was sutured and the skin closed with wound clips. Animals were injected subcutaneously with 2ml saline at the hip area and recovery was allowed on a heating pad until the animals awoke. Bladder expression was performed manually until a reflex bladder was

established, usually 3–6 days post-injury. All procedures were approved by the Children's National Medical Center Animal Care and Use Committee.

Behavioral Assessment

Mice were tested for hindlimb functional deficits at 24h after SCI, and again at 3 and 7 days post-injury, with further behavioral testing at 1 week intervals for chronic animals. For acute studies, 8 animals were used for each terminal timepoint and control groups. For chronic studies, 6 animals were used, as well as 5 controls. Hindlimb locomotor recovery was assessed using the Basso Mouse Scale (BMS) for locomotion, an open-field locomotor score (Engesser-Cesar et al., 2005; Basso et al., 2006). The BMS scale ranges from 0 to 9, with 0 indicating complete paralysis of the hindlimbs.

Bromodeoxyuridine Incorporation Experimental Design

Study I: Acute Proliferation—Mice (N = 8 per group) were injected i.p. with BrdU (17mg/kg) at 2, 4, and 6 hours prior to perfusion 1, 3, 4 and 7 days post-injury. Uninjured controls (N = 5) were also injected under the same conditions.

Study II: Chronic Replacement—Mice (N = 6) were injected with BrdU (17mg/kg) 2 times at 2 hour intervals on days 2, 3 and 4 post-injury. Mice were sacrificed at 42 days post-injury.

Histology

At 1, 3, 7 and 42 days post injury, mice were anesthetized with Avertin and perfused with 50 ml each of 0.9% saline and 4% buffered paraformaldehyde (PFA). Spinal cords were post-fixed for 1 hour in PFA, and cryoprotected in a sucrose gradient. Cords were quickly embedded and frozen in OCT (Fisher, Fairlawn, NJ) in blocks of 4, with each block containing injured and control tissue, and stored at -20°C . Ten μm coronal sections were cut from a 1cm length of spinal cord centered on the injury epicenter. Representative slides were stained with eriochrome cyanine (ECRC), hemotoxylin, and eosin and phyloxine to assess tissue morphology and determine the location of the injury epicenter, as previously described (Grossman et al., 2001).

Immunohistochemistry

Immunohistochemistry was performed on cross sections of tissue at specified distances rostral and caudal to the injury epicenter, at the specified terminal timepoints. Primary antibodies against Cd11b (1:50, Calbiochem, San Diego, CA), nestin (1:500, DSHB), BrdU (1:50, BD Biosciences), Nkx2.2 (1:200, Developmental Studies Hybridoma Bank), Mash1 (1:100, BD Biosciences), and APC/CC1 (1:1000, Calbiochem) were monoclonals raised in Mouse. Anti-AN2 (1:50), a monoclonal raised in mouse, was a kind gift from Dr. Jacqueline Trotter at the University of Mainz. Antibodies against Olig1 (1:500) and Olig2 (1:500) were a kind gift from the Dana Farber Cancer Institute. Antibodies to NG2 (1:200) and glial fibrillary acid protein (1:100, GFAP, Chemicon, Temecula CA), neurofilament (1:600) (Sigma, St. Louis MO), Sox10 (1:200, Abcam) and Ki67 (1:50, Novo Castra) were polyclonals raised in rabbit. The anti-PDGFR- α antibody (1:50, Santa Cruz, Caramillo, CA) and the anti-human Sox17 (1:100, R&D Systems, Minneapolis, MN) were raised in goat. Fluorescent immunohistochemistry was performed using Cy5-conjugated or Rhodamine-conjugated secondary antibodies against goat, mouse and rabbit (MP Biomedicals, Solon, OH, and Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). All tissue blocks contained both injured and uninjured tissue. No-primary controls were performed alongside all immunostaining procedures. All procedures were optimized for cell-specific staining and to reduce background and non-specific staining.

BrdU labeling

Slides were treated with 10% formalin, for 10 minutes and washed with phosphate buffered saline. DNA was denatured using 2M HCl for 45 minutes at room temperature. Tissue was then neutralized using 0.1M borate buffer pH 8.5 for 10 minutes, and blocked for 1 hour in 20% serum. Tissue was incubated with primary antibody against BrdU (BD Biosciences mouse monoclonal, 1:50) at 4°C for 24 hours. Tissue was washed, and incubated with secondary antibody (TRITC- α -mouse) for 1 hour at room temperature. Slides were washed and mounted with Vectashield (Vector) containing DAPI (4',6-diamidino-2-phenylindole, Vector) for visualization of nuclei. For double labeling, antigen labeling was performed first, as described above, followed by BrdU labeling.

Cell Counts: Sampling and Statistics

Cells were counted within a reticule of specified area ($200\mu\text{m}^2$) positioned in the ventral-lateral portion of spared white matter, between the tips of the ventral horns (at distal rostral and caudal locations) or the border of the spared white matter and the lesion (at the epicenter), and at the perimeter of the tissue, as previously described (Lytle and Wrathall, 2007). This area was counted because it is free of overt lesion at all distances examined. Labeling was assessed every 0.5 mm distal to the epicenter, up to 2mm. At all locations, three randomly selected sections separated by 10 μm were examined from each slide. For each distance (e.g. epicenter), 3 sections were counted per animal, on both left and right sides, for a total of 6 fields of view per distance, per animal, and 5 animals in each group. On average there are about 50 DAPI⁺ nuclei per field of view, with minor differences depending on time after injury and distance from epicenter. Thus the data point for each animal at each location and time represents the average from the analysis of approximately 300 cells. The N number used for statistical analysis was the number of mice per group. Significance was determined using Repeated Measures 2-Way ANOVA or One-Way ANOVA with Tukey's post-hoc analysis. Significance was set at $p < 0.05$.

Electrophysiology

Mice (5–7 weeks of age) were anaesthetized with 4% isoflurane, decapitated, and spinal cords quickly removed. The dura was removed. Longitudinal $200\mu\text{m}$ thick slices were cut. Following a 1 hour recovery period, slices were transferred to a recording chamber and perfused with extracellular solution of the following composition (in mM): NaCl 124, KCl 3, CaCl_2 2.5, MgSO_4 1.3, NaHCO_3 26; NaHPO_4 1.25; glucose 15; tetrodotoxin 1mM; saturated with 95% O_2 /5% CO_2 at room temperature. CNP-EGFP⁺ cells were identified in the ventral lateral white matter of the spinal cord. One cell was analyzed per slice. Patch electrodes had resistances between 6 and 8 M Ω when filled with intracellular solution of the following composition (in mM): K-gluconate 130; NaCl 10; Mg-ATP 2; Na-GTP 0.3, HEPES 10; EGTA 0.6, 0.3% biocytin, adjusted to pH 7.2 and 275 mOsm. Prior to whole-cell recording of currents, series resistances values ranged between 4 to 12 M Ω and were compensated by at least 75%. Delayed rectifying (K_{DR}) and transient (K_A) potassium currents were isolated as previously described (Chittajallu et al., 2004; Chittajallu et al., 2005). Biocytin/NG2 and biocytin/O4 staining was performed as previously described (Chittajallu et al., 2005).

Glial Growth Factor 2 (GGF2) and Fibroblast Growth Factor 2 (FGF2) Treatment

Standardized contusive injury was performed, as described above. Mice (N = 3) were injected with 0.8mg/kg recombinant human GGF2 (Acorda Pharmaceutical, Hawthorne NY) and 0.02mg/kg FGF2 (Peprotech, Rocky Hill NJ) in 500 μl of sterile 0.9% saline once daily, subcutaneously, for 8 days following injury, beginning 1 day post injury. Control mice (N = 3) were injected with 500 μl of saline once daily, subcutaneously. Mice were perfused and spinal cords post-fixed and sectioned, as described above, 9 days post-injury.

RESULTS

Histopathology and functional deficit after SCI in the CNP-EGFP mouse

In the CNP-EGFP mouse, EGFP is expressed by oligodendrocyte lineage cells, neural progenitor cells that express the CNP gene and Schwann cells (Yuan et al., 2002; Aguirre et al., 2004; Chittajallu et al., 2005). In the normal spinal cord, EGFP⁺ cells were easily visualized in both grey and white matter. As previously reported, in rats and wild-type mice, spinal cord contusion in the CNP-EGFP mouse (Supplemental Figure 1) produced a dorso-central lesion at the T8/T9 vertebral level, surrounded by residual white matter (Kuhn and Wrathall, 1998; Seki et al., 2002; Ghasemlou et al., 2005; Basso et al., 2006; Lytle and Wrathall, 2007). Within the overt lesion, normal spinal cord tissue including EGFP-expressing cells, was lost. In addition, the total amount of myelinated white matter in cross-sections through the epicenter was significantly reduced after SCI.

There also appeared to be a loss of glial cells in the residual white matter surrounding and adjacent to the overt lesion. To examine cell loss quantitatively we compared overall DAPI⁺ cells, oligodendrocytes (EGFP⁺CC1⁺) and astrocytes (EGFP^{neg}GFAP⁺) in the spared ventrolateral white matter (Supplemental Figure 1), and found a similar pattern as previously reported in C57Bl/6 wild-type mice (Lytle and Wrathall, 2007). The density of mature EGFP⁺CC1⁺ oligodendrocytes and EGFP^{neg}GFAP⁺ astrocytes was significantly reduced by 1 day post injury (DPI) at the injury epicenter, with decreasing loss distally. Loss of mature glia within the first week predominantly occurred by 1 DPI, when $47 \pm 4\%$ of EGFP⁺CC1⁺ cells were lost in spared ventrolateral white matter at the injury epicenter: little additional loss occurred though day 7. At 1 and 2 mm rostral and caudal the epicenter, loss of EGFP⁺CC1⁺ cells in residual white matter was significant at 3 and 7 DPI ($p < 0.05$). These results are similar to those previously reported for astrocyte and oligodendrocyte loss after SCI in C57Bl/6 wild-type mice (Lytle and Wrathall, 2007).

To verify that SCI in the CNP-EGFP mouse also produced expected functional deficits, we assessed locomotor activity using the BMS scale for murine hindlimb function that rates open-field performance from 0 (complete loss of hindlimb function) to 9 (normal) (Engesser-Cesar et al., 2005; Basso et al., 2006). Animals were severely impaired at 1 DPI (BMS score 1.6 ± 0.1), exhibiting only slight to extensive ankle movement and no hind limb weight support. Significant improvements in hindlimb function occurred at days 3 and 7 DPI, before leveling off. At 35 and 42 DPI, hindlimb function was significantly increased over 7 days. By 42 days, mice had a BMS score of 6.8 ± 0.5 , indicating frequent/consistent plantar stepping, some coordination, paws parallel at initial contact, and severe trunk instability. This pattern is consistent with what we have previously reported for wild-type C57Bl/6 mice (Lytle and Wrathall, 2007), and was not statistically different from CNP-EGFP^{neg} littermates.

Proliferative response of EGFP⁺NG2⁺ cells following spinal cord injury

In addition to loss of mature glial cells in the residual white matter, we found evidence for the proliferative response of different cell types that may contribute to glial replacement after SCI. To address the issue of whether proliferative NG2⁺ cells migrated to their positions or proliferated locally, a short timeframe of BrdU incorporation was employed, and BrdU specificity was confirmed with Ki67 double-labeling and nuclear colocalization (Figure 1A). Greater than 99% of BrdU⁺ cells were Ki67⁺. The extremely small percentage of BrdU⁺Ki67^{neg} cells likely represent BrdU uptake by cells undergoing DNA repair. EGFP⁺NG2⁺ cells were analyzed for proliferation patterns in response to injury, and showed a peak of BrdU incorporation in the rostral spinal cord at 3 DPI (Figure 1B).

During development, NG2⁺ cells do not generate oligodendrocytes *in vivo* in the absence of appropriate axon-derived signals, and axon loss prevents oligodendrocyte development (Greenwood and Butt, 2003). We therefore investigated axon preservation after SCI, comparing the relative amount of neurofilament immunoreactivity in sections at different locations with respect to the injury epicenter (Figure 1C–D). These data indicate that while there are likely to be many contributing factors to NG2 cell proliferation and differentiation after SCI, the relatively greater axon preservation rostral to the injury site may contribute to relative higher EGFP⁺NG2⁺ cell proliferation. There was a significant increase in total EGFP⁺NG2⁺ cells (Figure 1E–F) in the rostral cord beginning 3 DPI followed by an increase in EGFP⁺O4⁺ pre-oligodendrocytes in the rostral spinal cord by 7 DPI (Figure 1G–H), suggesting differentiation of EGFP⁺NG2⁺ OPCs.

EGFP⁺NG2⁺ cells in the adult spinal cord display electrophysiological characteristics of early postnatal OPCs in the developing CNS

One distinct advantage offered by the CNP-EGFP mouse is the visual identification of oligodendrocyte lineage cells for patch clamp analysis. Passive membrane properties and voltage-gated potassium channel profiles have been well characterized in NG2⁺ cells and later stage O4⁺ cells during development (Chittajallu et al., 2004; Chittajallu et al., 2005). In order to determine whether adult EGFP⁺NG2⁺ cells physiologically resemble early postnatal OPCs in the developing animal, we performed *in situ* patch clamp analysis. To initially identify EGFP⁺NG2⁺ cells, 200µm-thick longitudinal sections of the normal and injured spinal cords at 3 DPI were immunostained for NG2. As NG2⁺ cells express PDGFαR in the developing CNS, EGFP⁺NG2⁺ cells were also immunolabeled with anti-PDGFαR antibodies for identification (Figure 2A, top). Out of approximately 200 EGFP⁺NG2⁺ cells analyzed (N = 3 mice), 95 +/- 2% were PDGFαR⁺ in uninjured controls, and 93 +/- 6% at 7 DPI. PDGFαR labeling, however, was weak and observed only on cell bodies. EGFP⁺NG2⁺PDGFαR⁺ cells expressed low levels of EGFP and had cell bodies 5–10µm in diameter that were generally elongated. Recorded cells were filled with biocytin for post-hoc immunocytochemical analysis to confirm their phenotype (Figure 2A, bottom).

We first established the distinct passive membrane properties associated with EGFP⁺NG2⁺ cells or later stage EGFP⁺O4⁺ cells (Table 1). All cells were analyzed for resting membrane potential (RMP), membrane resistance (R_m) and capacitance. Consistent with previous reports during development in the CNS (Yuan et al., 2002; Chittajallu et al., 2005), EGFP⁺O4⁺ cells in the uninjured animal had a more hyperpolarized RMP (-52 ± 5 mV) than EGFP⁺NG2⁺ cells (-33 ± 3 mV), and lower R_m (183 ± 59 MΩ) than NG2⁺ cells (306 ± 77 MΩ). EGFP⁺O4⁺ cells also displayed higher capacitance (75 ± 16 pF) than EGFP⁺NG2⁺ cells (8 ± 1 pF in control), indicative of the increase in cell size associated with acquisition of a more mature phenotype.

Following SCI (3 DPI), EGFP⁺NG2⁺ cells exhibited no change in RMP (-33 ± 3 mV) or capacitance (6.9 ± 0.9 pF). The R_m was significantly increased (1267 ± 450 MΩ), which may be indicative of a more immature stage of progenitor cell development. Also EGFP⁺O4⁺ cells exhibited no change in RMP (-45 ± 4 mV), R_m (122.2 ± 22.5 MΩ), or capacitance (81.7 ± 25.7 pF) following SCI.

As voltage-gated potassium channels are involved in proliferation, differentiation and maturation of NG2⁺ oligodendrocyte progenitors during development (Borges et al., 1994; Gallo et al., 1996; Knutson et al., 1997; Chittajallu et al., 2002; Neusch et al., 2003; Vautier et al., 2004; Chittajallu et al., 2005), analysis of potassium currents (K_A, K_{DR}, and K_{IR}) was performed in both EGFP⁺NG2⁺ and EGFP⁺O4⁺ cells. In control spinal cords, both EGFP⁺NG2⁺ and EGFP⁺O4⁺ cells expressed K_A and K_{DR} (Figure 2B). No EGFP⁺NG2⁺ cells expressed K_{IR}. However, consistent with previous developmental analysis (Borges et al.,

1994; Gallo et al., 1996; Knutson et al., 1997; Chittajallu et al., 2002; Neusch et al., 2003), K_{IR} was expressed by $EGFP^{+}O4^{+}$ cells (data not shown).

As increased K_{DR} expression is correlated with a proliferative phenotype during development, we also analyzed $EGFP^{+}NG2^{+}$ cells from 3-day injured spinal cords rostrally, i.e. when and where $EGFP^{+}NG2^{+}$ proliferation peaks (Figure 1B). There was an increase in the K_{DR} in $EGFP^{+}NG2^{+}$ cells after injury at all test pulses greater than 10mV (Figure 2B), consistent with the observed increase in proliferation. There was also an increase in the percentage of $EGFP^{+}NG2^{+}$ cells expressing K_{DR} (Table 2). While K_A current density did not significantly increase at 3 DPI, the percentage of $EGFP^{+}NG2^{+}$ cells expressing K_A did (Table 2). Single Boltzmann curve fits for K_A and K_{DR} were constructed (data not shown), and half-activation values ($V_{1/2}$) for $EGFP^{+}NG2^{+}$ cells were calculated (data not shown). These were significantly different for both K_A (-2.3 mV in control animals vs. 11.1 mV in injured) and K_{DR} (4.4 mV in control animals vs. 17.1 mV in injured), and showed a right shift in the voltage response curve after injury, which may be indicative of different potassium channel subunit compositions (Figure 2C). Conversely, cells at the $EGFP^{+}O4^{+}$ stage displayed no differences in K_A , K_{DR} , or K_{IR} current amplitude between the uninjured and injured populations (data not shown).

Expression of cell-specific markers in $EGFP^{+}NG2^{+}$ cells

As $NG2^{+}$ cells are known to give rise to astrocytes in culture (Raff et al., 1983; Belachew et al., 2003), and $Cd11b^{+}$ cells express $NG2$ transiently after injury (Bu et al., 2001), $EGFP^{+}NG2^{+}$ cells were examined for co-expression of the astrocyte marker $GFAP$ and the macrophage/microglial marker $Cd11b$. All cells were negative for $GFAP$ and $Cd11b$ (data not shown). Additionally, no $NeuN^{+}$ cells were $EGFP^{+}$ (data not shown).

As nestin is upregulated in OPCs that are actively in the cell cycle during development (Gallo and Armstrong, 1995), $EGFP^{+}NG2^{+}$ cells were also examined for nestin expression (Supplemental Figure 2A–B). In control animals, only 7% of $EGFP^{+}NG2^{+}$ cells expressed nestin (2 ± 0.4 cells/mm²). This percentage increased at all locations and time points after injury. Consistent with the observed rostral increase in proliferation of $EGFP^{+}NG2^{+}$ cells, total nestin⁺ $EGFP^{+}NG2^{+}$ cells were greatest in the rostral cord at 3 DPI, where approximately 30% of $EGFP^{+}NG2^{+}$ were nestin⁺ (6 ± 0.7 cells/mm², Supplemental Figure 2B).

$S100\beta$ is a known astrocyte precursor cell marker (Langley et al., 1984) and is also expressed in oligodendrocyte lineage cells (Deloulme et al., 2004; Hachem et al., 2005). Approximately 65% of $EGFP^{+}NG2^{+}$ cells in the uninjured animal expressed $S100\beta$ (16 ± 3 cells/mm², Supplemental Figure 2C–D). However, this was significantly decreased at 1 DPI (Supplemental Figure 2D). At 3 DPI, numbers remained low caudally, but returned to normal levels rostrally and at the epicenter (Supplemental Figure 2D). By 7 DPI, rostral and caudal levels were normal, with a significant increase at the epicenter to 137% of control (44 ± 5 cells/mm²).

The transcription factor expression pattern is altered in $EGFP^{+}NG2^{+}$ cells after spinal cord injury

The transcription factors $Olig1$, $Olig2$, $Nkx2.2$, $Sox10$, $Sox17$ and $Mash1$ play essential roles in oligodendrocyte progenitor cell specification and differentiation in the developing embryonic spinal cord and brain (Woodruff et al., 2001; Ligon et al., 2006a; Sohn et al., 2006; Sugimori et al., 2007). Immunopositive cells were quantified to investigate possible roles that these transcription factors play in the injured adult spinal cord. In normal tissue, $Olig1$, $Olig2$ and $Nkx2.2$ expression was observed in both grey and white matter, and was not limited

to EGFP-expressing cells (data not shown). Sox10, Sox17 and Mash1 were also expressed in grey and white matter, although in only less than 1% of cells (data not shown).

In control animals, 58% of EGFP⁺NG2⁺ cells were Olig2⁺, averaging 50 ± 5 cells/mm². At 1 DPI, 80–98% of EGFP⁺NG2⁺ cells were Olig2⁺, with highest coexpression (98%, or 45 ± 2 cells/mm²) in the rostral spinal cord. Furthermore, there was an overall increase in the total number of Olig2⁺NG2⁺ cells by 1 DPI in the rostral cord (Supplemental Figure 3A–B). At 3 and 7 DPI, Olig2 expression in EGFP⁺NG2⁺ cells was still greatest in the rostral spinal cord. While the percentage of EGFP⁺NG2⁺ cells that express Olig2 decreased with time, the density of EGFP⁺NG2⁺ cells that express Olig2 remained at or above control levels (Supplemental Figure 3B), suggesting consistent replacement of this population. By 6 weeks, the density of Olig2-expressing EGFP⁺NG2⁺ cells was generally decreased (Supplemental Figure 3C). These data support the observation that Olig2 plays an important role in the acute injury phase and may induce programs of gene activity acutely after injury similar to those necessary for oligodendrocyte formation during development.

After injury, there was considerable loss of overall Nkx2.2 and Olig1 expression in the spared white matter (data not shown). While Nkx2.2 was expressed in uninjured mice at a density of approximately 35 ± 4 EGFP⁺ cells/mm², only $6 \pm 0.9\%$ of EGFP⁺Nkx2.2⁺ cells were NG2⁺, indicating that most Nkx2.2 expression occurred in more mature oligodendrocyte lineage cells. After injury, Nkx2.2 expression in EGFP⁺NG2⁺ increased from a density of 2 cells/mm² in control animal white matter to an average of 5 ± 0.4 cells/mm² (approximately 15% of total EGFP⁺NG2⁺ cells, depending on location vis-à-vis the injury epicenter and time after injury) at rostral and caudal locations in the spared white matter at all acute time points. However, Nkx2.2 expression in EGFP⁺NG2⁺ cells decreased to control levels at 6 weeks, indicating a role for Nkx2.2 in the acute injury phase. Olig1 was similarly expressed in a small population of EGFP⁺NG2⁺ cells in control animals. There was an overall decrease from control levels (approximately 7 ± 1 cells/mm²) in EGFP⁺NG2⁺Olig1⁺ cells after injury, both acutely (approximately 4 ± 0.6 cells/mm²) and chronically (approximately 2 ± 1 cells/mm²).

Sox10 and Sox17, transcription factors that promote differentiation of oligodendrocytes (Sohn et al., 2006; Liu et al., 2007), were expressed in 21% (5 ± 0.13 cells/mm², Supplemental Figure 3D–E) and 20% (5.2 ± 0.08 cells/mm², Supplemental Figure 3D, F) of EGFP⁺NG2⁺ cells in uninjured controls, respectively. By 3 DPI, there was a significant decrease in EGFP⁺NG2⁺Sox10⁺ cells at all locations. By 7 DPI, EGFP⁺NG2⁺Sox10⁺ cells returned to near-normal levels at the injury epicenter and rostrally, with a significant increase in EGFP⁺NG2⁺Sox10⁺ cells caudally. EGFP⁺NG2⁺Sox17⁺ cells significantly decreased rostrally and at the injury epicenter by 3 DPI. While expression was still down at the epicenter at 7 DPI, rostral expression returned to control levels, and a significant increase in caudal expression was observed.

Finally, Mash1, which promotes differentiation of oligodendrocytes and neurons in the embryonic spinal cord (Sugimori et al., 2007), was rarely observed in uninjured or injured spinal cord tissue, with fewer than 1 immunopositive cell per field of view (data not shown). This is consistent with previous reports (Yamamoto et al., 2001; Ohori et al., 2006).

These results show that, during the first week after injury, expression of the transcription factor Olig2, which is necessary for oligodendrocyte specification during development, was upregulated while transcription factors necessary for lineage progression to a differentiated stage (Nkx2.2, Sox10, Sox17) and (re)myelination (Olig1) were generally downregulated during the first week.

A subpopulation of NG2⁺ cells of the spinal cord does not express EGFP before or after injury

If all NG2⁺ cells were strictly oligodendrocyte lineage cells, then we would expect expression of EGFP in all NG2⁺ cells, as CNP mRNA is expressed earlier than NG2 in the developing rodent spinal cord (Yu et al., 1994; Nishiyama et al., 1996). In other regions of the postnatal and adult brain of the CNP-EGFP mouse, all NG2⁺ cells are also EGFP⁺ (Yuan et al., 2002; Aguirre and Gallo, 2004; Aguirre et al., 2004; Chittajallu et al., 2004). Immunohistochemical analysis revealed that in adult spinal cord, while CC1⁺ oligodendrocytes and O4⁺ pre-oligodendrocytes expressed EGFP at greater than 99% frequency, NG2 was expressed in both EGFP⁺ (Figure 1E) and EGFP^{neg} cells (Figure 3A). Furthermore, EGFP⁺NG2⁺ and EGFP^{neg}NG2⁺ cells were morphologically distinct. EGFP⁺NG2⁺ cells were generally bipolar in appearance (Figure 1E), typical of OPCs, whereas EGFP^{neg}NG2⁺ cells ranged from multipolar cells with secondary branching to a simple, bipolar morphology, with the former being the predominant phenotype (Figure 3A). We then immunolabeled EGFP^{neg}NG2⁺ cells with anti-GFP to ensure that EGFP was not being expressed at levels undetectable by fluorescence microscopy. EGFP^{neg}NG2⁺ cells were not stained with anti-GFP antibodies (Figure 3B). Lastly, we labeled EGFP^{neg}NG2⁺ cells for PDGF α R and observed weak expression on nearly all EGFP^{neg}NG2⁺ cell bodies (Figure 3C). In approximately 200 EGFP^{neg}NG2⁺ cells (N = 3 mice), 91 \pm 4% were PDGF α R⁺ in uninjured controls, and 93 \pm 3% rostrally at 7 DPI. These data are consistent with studies published by Berry et al. (2002), suggesting that morphologically complex NG2⁺ cells express PDGF α R, and can be distinguished from OPCs by a lack of CD9 antigenicity.

EGFP^{neg}NG2⁺ cells incorporated BrdU in a distinct spatio-temporal pattern compared to EGFP⁺NG2⁺ cells, with a peak at 7 DPI at the injury epicenter (Figure 3D). It is important to note that EGFP^{neg}NG2⁺ cells displayed the highest proliferation rate where the lesion is largest, i.e. at the epicenter.

Expression of cell-specific markers in EGFP^{neg}NG2⁺ cells

EGFP^{neg}NG2⁺ cells were also examined for co-expression of the astrocyte marker GFAP, the neuronal marker NeuN and the macrophage/microglial marker Cd11b. The EGFP^{neg}NG2⁺ population was negative for GFAP and NeuN (not shown). A small subpopulation of EGFP^{neg}NG2⁺ cells transiently expressed Cd11b at 3 DPI (not shown). However, these cells morphologically resembled activated macrophages/microglia and were therefore not included in the analysis.

EGFP^{neg}NG2⁺ cell populations were also examined for nestin and S100 β (Figure 4A–D). About 9% of EGFP^{neg}NG2⁺ cells express nestin in the uninjured animal (4 ± 0.5 cells/mm²), and these were generally cells that did not display a complex morphology (Figure 4A). However, after SCI, up to 35% of EGFP^{neg}NG2⁺ cells expressed nestin (12 ± 1.5 cells/mm²), depending upon time after injury and location (Figure 4B). The peak of nestin expression was at 7 DPI in EGFP^{neg}NG2⁺ cells at the injury epicenter. The observed increase in nestin expression may be indicative of a progenitor cell subtype. Alternatively, as nestin is expressed by reactive astrocytes, and as expression is greatest at the injury epicenter, EGFP^{neg}NG2⁺nestin⁺ cells may represent a “reactive” NG2⁺ cell subtype.

In the EGFP^{neg}NG2⁺ population, 17% of cells from the uninjured spinal cord, generally bipolar cells, expressed S100 β (7 ± 1 cells/mm², Figure 4C–D). This decreased by 1 DPI, and rebounded by 3 DPI. By 7DPI, S100 β levels were at or significantly greater than normal levels (Figure 4D).

We also examined the expression of oligodendrocyte lineage transcription factors Olig1, Olig2, Nkx2.2, Sox10, Sox17 and Mash1 in EGFP^{neg}NG2⁺ cells. Sox10, Sox17 and Mash1 were only

rarely observed in these cells. In addition, EGFP^{ne}NG2⁺ cells rarely expressed Olig2 in control or acutely injured animals through 7 days after injury (Figure 4E). However, Olig2 was expressed in approximately 15% of EGFP^{ne}NG2⁺ cells in the chronically injured animals at 6 weeks (9 ± 2 cells/mm², Figure 4F).

Nkx2.2 was expressed in approximately 8% of EGFP^{ne}NG2⁺ cells in the normal spinal cord (3 ± 0.5 cells/mm²). While the total number of EGFP^{ne}NG2⁺Nkx2.2⁺ cells showed a slight increase in the acute injury phase, the percentage of EGFP^{ne}NG2⁺ cells that expressed Nkx2.2 significantly decreased to approximately 5% (2 ± 0.3 cells/mm²), with no obvious trend vis-à-vis the injury epicenter and time after injury. Only 6% of EGFP^{ne}NG2⁺ cells expressed Nkx2.2 at 6 weeks post-injury (2 ± 0.4 cells/mm²). Olig1 was expressed by approximately 12% of EGFP^{ne}NG2⁺ cells in control animals (5 ± 1 cells/mm²), and expression of this transcription factor decreased by 50% in this cell population during the acute injury phase at most locations and time points. At 6 weeks, while expression was still down significantly at the injury epicenter, levels returned to normal at rostral and caudal locations.

Chronic Replacement of Mature Glial Cells

Mature glial cells were lost from residual white matter by 24 hours after injury. EGFP⁺NG2⁺ cells, among other cells, proliferated within the first week with a peak at 3 DPI in the rostral spinal cord (Figure 1B). By 7 DPI, there was an increase in total EGFP⁺NG2⁺ cells and EGFP⁺O4⁺ cells in the rostral spinal cord (Figure 2A–D). We wanted to examine whether cells that proliferate in the first week survive chronically and whether they produce mature glia, particularly oligodendrocytes. Therefore, mice were injected with BrdU on days 2, 3 and 4 post-injury, and allowed to survive for 6 weeks.

In uninjured tissue, BrdU⁺ cells were occasionally observed, and were generally EGFP⁺CC1⁺, EGFP^{ne}GFAP⁺, EGFP^{ne}NG2⁺ or EGFP⁺NG2⁺. Cells that incorporated BrdU in the first week following injury survived to 6 weeks (Figure 5A), and included EGFP⁺CC1⁺ oligodendrocytes, EGFP^{ne}GFAP⁺ astrocytes, EGFP^{ne}NG2⁺ cells or EGFP⁺NG2⁺ cells (Figure 5B–D), indicative of replacement of mature glia that were lost in the acute injury phase. Approximately 13–28% of CC1⁺ oligodendrocytes were BrdU⁺ (15 ± 2 to 28 ± 3 cells/mm²) and 12–36% of GFAP⁺ astrocytes were BrdU⁺ (4 ± 1 to 18 ± 1 cells/mm²). In total, the EGFP⁺CC1⁺ cell density returned to normal levels and the EGFP^{ne}GFAP⁺ astrocyte density was increased as much as 2–3 fold in the spared ventral-lateral white matter (Figure 5D). It is important to note that all BrdU⁺CC1⁺ oligodendrocytes expressed EGFP. These data indicate that proliferative populations in the acutely injured animal contribute to the replacement of mature glial cells chronically.

Up to 50% of EGFP⁺NG2⁺ cells were BrdU⁺ (6 ± 1 cells/mm²), and up to 50% of EGFP^{ne}NG2⁺ cells were also BrdU⁺ (24 ± 4 cells/mm²), depending upon location with respect to the injury epicenter (Figure 5E). Notably, there were approximately four times as many EGFP^{ne}NG2⁺ cells as compared to EGFP⁺NG2⁺ cells chronically, depending upon location vis-à-vis the injury epicenter. If EGFP⁺NG2⁺ cells were indeed OPCs, then a significant number of cells that divided in the first week would be expected to have matured by 6 weeks. If the EGFP^{ne}NG2⁺ cell population is comprised largely of non-progenitor cells which do not contribute to glial replacement after SCI, then this population should increase by the chronic time point. Figure 5E demonstrates that indeed a significant increase in the density of EGFP^{ne}NG2⁺ cells was observed at 6 weeks after SCI. NG2, CC1, and GFAP accounted for approximately half of all BrdU positive cells (Figure 5F), indicating a contribution from other cell types that respond to acute injury, and survive chronically following SCI.

Treatment with Glial Growth Factor 2 and basic Fibroblast Growth Factor *in vivo* results in increased numbers of EGFP⁺CC1⁺ oligodendrocytes and EGFP^{neg}NG2⁺ cells

Basic fibroblast growth factor (FGF2) and glial growth factor 2 (GGF-2) have been shown to increase the number of NG2⁺ cells and/or oligodendrocytes after SCI (Zai et al., 2005; Ohori et al., 2006). Furthermore, increases in endogenous FGF2 coincide with NG2⁺ cell upregulation after SCI (Zai et al., 2005). However, it is still undefined whether the responsive cells are OPCs. Therefore, we treated CNP-EGFP mice with FGF2 and GGF-2 after contusive SCI. As previously reported, we found an increase in the total number of NG2⁺ cells *in vivo* (Figure 6A–B, D). However, this increase was only observed in EGFP^{neg}NG2⁺ cells, with the greatest increase at the injury epicenter, consistent with the correlation of endogenous levels of these growth factors with EGFP^{neg}NG2⁺ cell density (Figure 6C). There was no significant difference in the EGFP⁺NG2⁺ cell population between treated and un-treated mice (Figure 6D). We did, however, observe a significant increase in the number of EGFP⁺CC1⁺ oligodendrocytes (Figure 6C, E). These data suggest that EGFP⁺NG2⁺ cells maintain a consistent population even when differentiation is enhanced. Moreover, these data show that EGFP⁺NG2⁺ cells and EGFP^{neg}NG2⁺ cells show a differential response to treatment with FGF2 and GGF2 following SCI.

DISCUSSION

We utilized the CNP-EGFP mouse to investigate the response of different subpopulations of NG2⁺ cells to SCI. We show that at least two types of NG2⁺ cells exist in the normal spinal cord and proliferate in response to SCI with spatio-temporal differences, which suggest that they may respond to distinct signals. In the white matter of the normal adult spinal cord, approximately one-third of NG2⁺ cells expressed EGFP. These EGFP⁺NG2⁺ cells were generally bipolar, which is typical of OPCs. Conversely, approximately two-thirds were EGFP^{neg}NG2⁺ cells, whose morphology ranged from bipolar to more complex, but was generally multipolar.

We provide further support for a growing body of evidence that a *subset* of adult NG2-expressing cells, and specifically EGFP⁺NG2⁺ cells, recapitulate developmental programs of early postnatal OPCs to give rise to replacement oligodendrocytes observed chronically after SCI. We extend these findings with novel electrophysiological analysis of proliferative NG2⁺ cells after SCI. Specifically, using an *in situ* preparation of adult spinal cord tissue, we found that the passive membrane properties and voltage gated potassium channel profile of EGFP⁺NG2⁺ cells at 3 DPI were consistent with those reported for proliferative OPCs *in vitro* and *in situ* (Bevan et al., 1987; Sontheimer et al., 1989; Barres et al., 1990; Kettenmann et al., 1991; Berger et al., 1995; Borges and Kettenmann, 1995; Attali et al., 1997; Knutson et al., 1997; Chittajallu et al., 2002). We identified an increase in the amplitude of the delayed rectifier K⁺ current coincident with the peak in BrdU incorporation after injury, consistent with a proliferative OPC population during development. In particular, increased R_m and upregulation of and K_{DR} were observed at 3 DPI, when proliferation of EGFP⁺NG2⁺ cells, as measured by BrdU incorporation, was greatest. These data indicate that reactivation of EGFP⁺NG2⁺ cells after SCI involves specific changes in their membrane ionic channels, and suggest that EGFP⁺NG2⁺ cells in the adult spinal cord may rely on similar mechanisms of proliferation as observed during embryonic and early postnatal development.

Similar to OPCs, EGFP⁺NG2⁺ cells after SCI expressed cellular proteins and transcription factors associated with OPCs during developmental oligodendrogenesis. In particular, we observed an increase in the expression of cellular markers associated with OPC proliferation and early lineage progression (nestin, Olig2 and Nkx2.2), and a decrease in transcription factors associated with oligodendrocyte maturation (Olig1, Sox17 and Sox10). As previously reported, Mash1 was rarely observed (Yamamoto et al., 2001; Ohori et al., 2006), and only a small

fraction of EGFP⁺NG2⁺ cells expressed Olig1, Nkx2.2, Sox10 or Sox17 at the acute time points after injury investigated. These transcription factors play a key role in oligodendrocyte maturation and myelination, and it is likely that expression increases later than 7 DPI. Olig2, which is required early in lineage progression and for acquisition of the O4⁺ pre-myelinating stage (Yue et al., 2006), was expressed by more EGFP⁺NG2⁺ cells than any other transcription factor examined. An increase in the number of Olig2-expressing cells has been demonstrated in various injury paradigms in different regions of the CNS (Fancy et al., 2004; Han et al., 2004; Talbott et al., 2005). We observed up-regulation of Olig2 rostral to the injury epicenter, prior to the observed increase in O4⁺ cells rostrally, as it would be expected if Olig2 expressing EGFP⁺NG2⁺ are OPCs.

We show here, for the first time, that in the adult spinal cord, a subset of NG2-expressing cells, EGFP^{neg}NG2⁺ cells, are potentially not OPCs and that they respond to SCI in a robust manner *in vivo*. The peak of proliferation of EGFP^{neg}NG2⁺ cells was observed at the injury epicenter, where scar formation is greatest. EGFP^{neg}NG2⁺ cells did not express transcription factors consistent with OPC development and oligodendrogenesis. Considering that the increase in NG2⁺ cell density persists as long as 9 weeks post-injury (McTigue et al., 2001), after replacement of lost oligodendrocytes in spared white matter had been accomplished (Lytle and Wrathall, 2007; Zai and Wrathall, 2005), it is possible that a subpopulation of NG2-expressing cells in the adult CNS may be a differentiated glial phenotype that does not serve as a macroglial reservoir (Levine and Nishiyama, 1996; Horner et al., 2002). One study concluded that BrdU⁺NG2⁺ cells were destined to participate in scar formation in a cortical stab wound study (Alonso, 2005), consistent with the recently hypothesized role of NG2⁺ synantocytes in homeostatic function (Berry et al., 2002; Butt et al., 2002; Horner et al., 2002; Lin and Bergles, 2002; Nishiyama et al., 2002). Our results are also consistent with results from clonal analysis of rat NG2⁺ cells after SCI studied *in vitro* (Yoo and Wrathall, 2007), where some clones of purified NG2⁺ cells produced glia when permitted to differentiate, while others remained NG2⁺ cells. An alternative possibility is that the EGFP^{neg}NG2⁺ cell population may itself be heterogeneous, as demonstrated by the finding that S100 β was expressed in a subset of bipolar EGFP^{neg}NG2⁺ cells, which may represent an immature astrocyte population that does not yet express GFAP.

It is, of course, a possibility that EGFP^{neg}NG2⁺ cells and EGFP⁺NG2⁺ cells share a common lineage. Even though oligodendrocytes and ventral motor neurons arise from a common progenitor during embryonic spinal cord development (Miller, 2002), it is well established that the adult spinal cord is not conducive to neurogenesis after injury, even in the presence of exogenous growth factors (Ohuri et al., 2006). Olig2 is down-regulated to allow progression along a neuronal lineage during development (Lu et al., 2001; Fancy et al., 2004; Hack et al., 2005; Lee et al., 2005) and may be a repressor of neurogenesis after injury (Buffo et al., 2005). Therefore, Olig2⁺EGFP^{neg}NG2⁺ cells in the chronic animal may be progenitors that failed to differentiate along the oligodendrocyte lineage acutely and also did not receive the proper signals to become neurons. It has also been demonstrated that ablation of Olig2 function during early development leads to a complete lack of OPCs and synantocytes (Ligon et al., 2006b). Therefore, Olig2⁺EGFP^{neg}NG2⁺ cells after chronic SCI may be synantocytes derived from Olig2⁺EGFP⁺NG2⁺ cells stimulated during the acute injury phase.

GGF2 and FGF2 have been shown to increase the number of NG2⁺ cells and/or oligodendrogenesis (Zai et al., 2005; Ohori et al., 2006). We therefore treated SCI animals with GGF2 and FGF2 to determine the specific response of EGFP⁺NG2⁺ cells and EGFP^{neg}NG2⁺ cells. While the number of EGFP⁺NG2⁺ cells was not affected, the observed increase in EGFP⁺CC1⁺ oligodendrocytes suggests that we stimulated differentiation of the EGFP⁺NG2⁺ cell population, which might have strong implications for therapeutic intervention. However, the observed increase in survival and/or proliferation of

EGFP^{neg}NG2⁺ cells in response to growth factor treatment has therapeutic implications as well. It is also likely that treatment with GGF2 and FGF2 has led to more axonal preservation/regeneration, providing more available axons onto which OPCs can differentiate. It has, however, been shown GGF2 infusion alone into areas of primary demyelination does not lead to remyelination enhancement (Penderis J, et al., 2003b).

Survival, proliferation and/or differentiation of OPCs and other NG2-expressing cells may be only part of a complicated path to remyelination in the injured adult spinal cord. And while we did not study remyelination per se, our studies may indicate a need for combinatorial treatment. The role of these cells is not yet clear, and whether they are beneficial or detrimental to recovery remains unknown. Optimizing therapeutic agents and time windows for increased response of specific subsets of NG2-expressing cells should be considered in the future design of regeneration and repair therapies.

In conclusion, the data presented here provide strong evidence that the EGFP⁺NG2⁺ cell population is morphologically and electrophysiologically similar to perinatal OPCs that give rise to mature oligodendrocytes. We also provide support for the assertion that NG2⁺ cells in the adult and after SCI may include immature astrocytes (S100 β ⁺), other progenitor cell subtypes (nestin⁺, Olig1/2⁺, Nkx2.2⁺) and possibly synantocytes. The complexity of the cellular response after SCI indicates that, in the future, specific treatment paradigms targeted to enhance the response of certain subtypes of NG2⁺ cells may improve the potential for regeneration and remyelination after SCI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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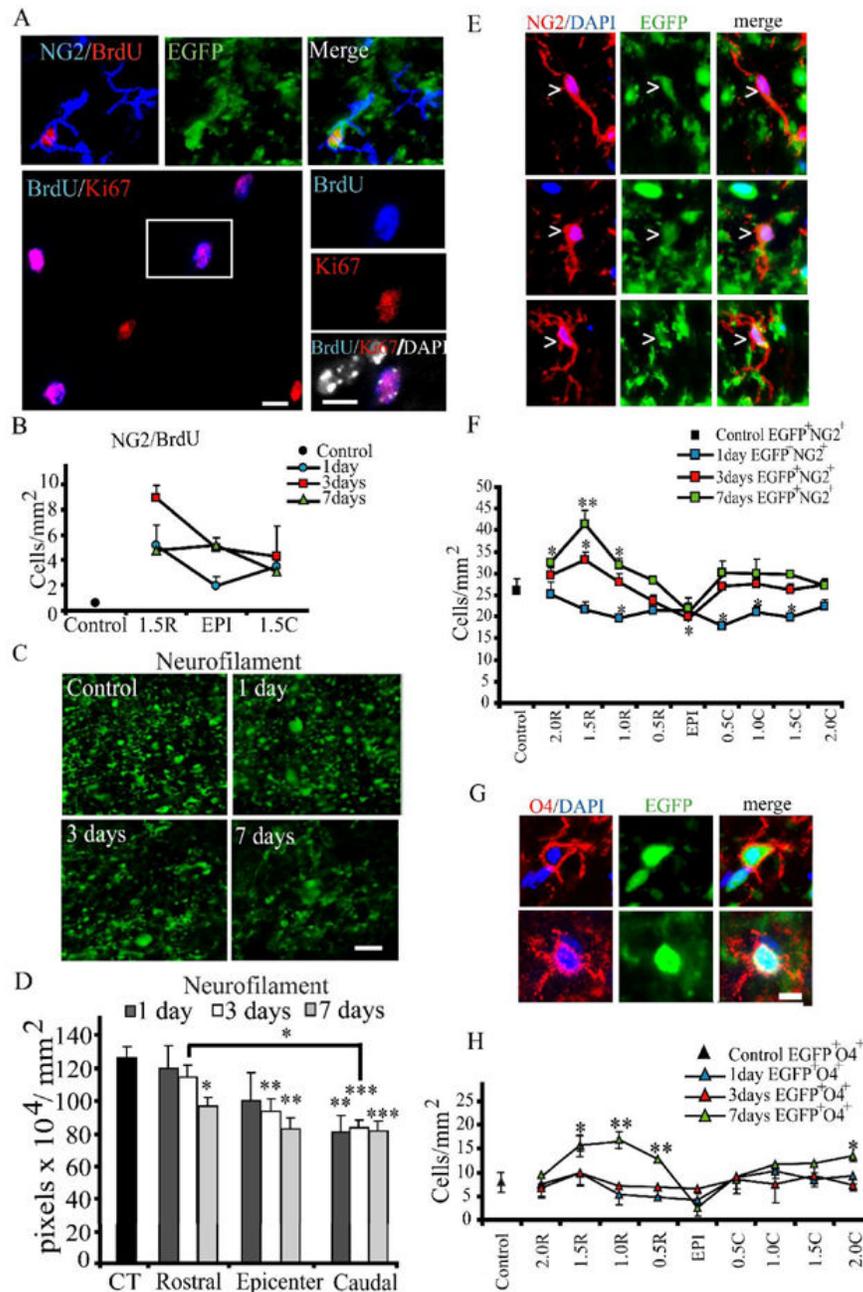


Figure 1. Evidence for proliferation of EGFP⁺NG2⁺ cells after spinal cord injury

A. BrdU incorporation (red) in EGFP⁺ (green) NG2⁺ (blue) cells. BrdU incorporation (blue, bottom panels) always occurs in Ki67-expressing (red) cells and is always nuclear, as indicated by labeling with DAPI (white). Tissue shown is from the rostral spinal cord, 3DPI. Scale bar = 10 μ m.

B. Quantitative analysis of EGFP⁺NG2⁺BrdU⁺ cells (bottom panel) in control animals and at 1, 3 and 7 DPI at 1.5mm rostral, injury epicenter, and 1.5mm caudal. Cell counts were performed in the ventral-lateral white matter. Immunoreactive cells were quantified from 5 animals at the injury epicenter, and at 1.5mm rostral and caudal in the spared ventral-lateral white matter. For each distance at each timepoint, three 10 μ m-thick sections were counted,

left and right hemispheres, for a total of six fields per animal. Therefore, thirty 200 μm^2 fields of view were quantified for each. One-Way ANOVA with Tukey's post-hoc analysis for variance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control.

C–D. The proliferative response of EGFP⁺NG2⁺ cells is correlated to neurofilament (NF) immunoreactivity (IR). Representative NF staining in the spared ventral lateral white matter at the injury epicenter in control and 1, 3 and 7 days post-injury and quantification of NF immunoreactivity (pixels/mm²) at 1.5mm rostral and caudal and at the injury epicenter. There is degradation of NF structure by 1 DPI, with significant loss at 3 and 7 days. IR was quantified for 5 animals at the injury epicenter and at 1.5mm rostral and caudal in the spared ventral-lateral white matter. For each distance at each timepoint, three 10 μm -thick sections were counted, left and right hemispheres, for a total of six fields per animal. Therefore, thirty 200 μm^2 fields of view were quantified for each. There is significant loss in the caudal cord at all timepoints examined. Loss at the epicenter is significant at 3 and 7 days, and in the rostral cord at 7 days. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control. There is a significant difference between the rostral and caudal cords at 3 days ($p < 0.05$). Bars represent mean \pm SEM. Two-way ANOVA with Bonferroni's post-hoc analysis. Scale bar = 5 μm .

E. EGFP-expressing (green) NG2 (red) cells (arrowheads) are typically bipolar (top panels, uninjured tissue shown) or have very few branches (middle panels, injured tissue shown 3 DPI). Occasionally, cells have more complex branching (bottom panels, uninjured tissue shown). DAPI (blue) labels cell nuclei. Scale bar = 10 μm .

F. Cell counts in control and injured animals for EGFP⁺NG2⁺ cells every 0.5 mm from the injury epicenter, up to 2 mm rostral and caudal. There is a significant increase in EGFP⁺NG2⁺ cells in the rostral spinal cord at 3 and 7 dpi. One-Way ANOVA with Tukey's post-hoc analysis for variance. * $p < 0.05$; ** $p < 0.01$ vs. control.

G. Preoligodendrocytes labeled with O4 (top panels) express EGFP. Scale bar = 10 μm .

H. There is a significant increase in EGFP⁺O4⁺ cells in the rostral spinal cord at 7 dpi. One-Way ANOVA with Tukey's post-hoc analysis for variance. * $p < 0.05$; ** $p < 0.01$ vs. control.

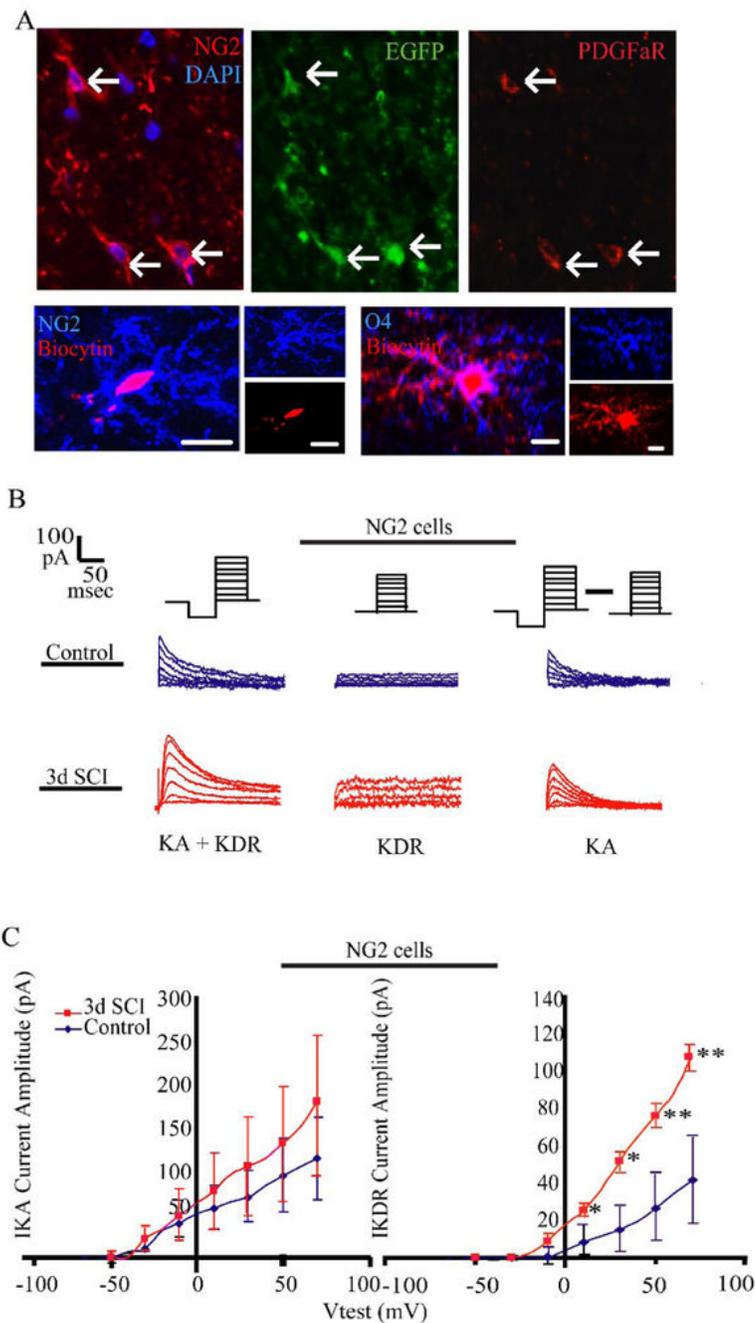


Figure 2. Electrophysiological analysis of EGFP⁺NG2⁺ cells after spinal cord injury indicates an OPC-like phenotype

A. Immunostaining for NG2 and PDGF α R identifies EGFP⁺ OPC-like cells. Expression of PDGF α R was observed on NG2 cells, but was weak and limited to the cell body. After electrophysiological analysis, cells were filled with biocytin (red) for post-hoc immunocytochemical analysis to distinguish between EGFP⁺NG2⁺ (blue, top panels) and EGFP⁺O4⁺ (blue, bottom panels) stage cells, and to confirm identification of EGFP⁺NG2⁺ cells.

B. Proliferative electrophysiological phenotype of EGFP⁺NG2⁺ cells in the rostral spinal cord of uninjured and injured animals at 3 DPI. Representative traces from EGFP⁺NG2⁺ cells

depicting total outward current ($K_A + K_{DR}$, top panel), isolated K_{DR} (middle panel) and isolated K_A (bottom panels) for control (left panels) and 3-day injured (right panels) cells.

C. Current-voltage curves constructed from a series of depolarizing voltage steps from -70mV to 70mV ; 10mV increments. Holding potential was -60mV . For isolation of K_A , K_{DR} amplitude was digitally subtracted from the total outward current. For K_{DR} , this followed a pre-pulse to -40mV to inactivate K_A . All values are after junction potential correction. $N = 32$ cells for control, and 32 cells for injured. Prior to recording, series resistance compensation to at least 75% was performed. Symbols represent mean \pm SEM.

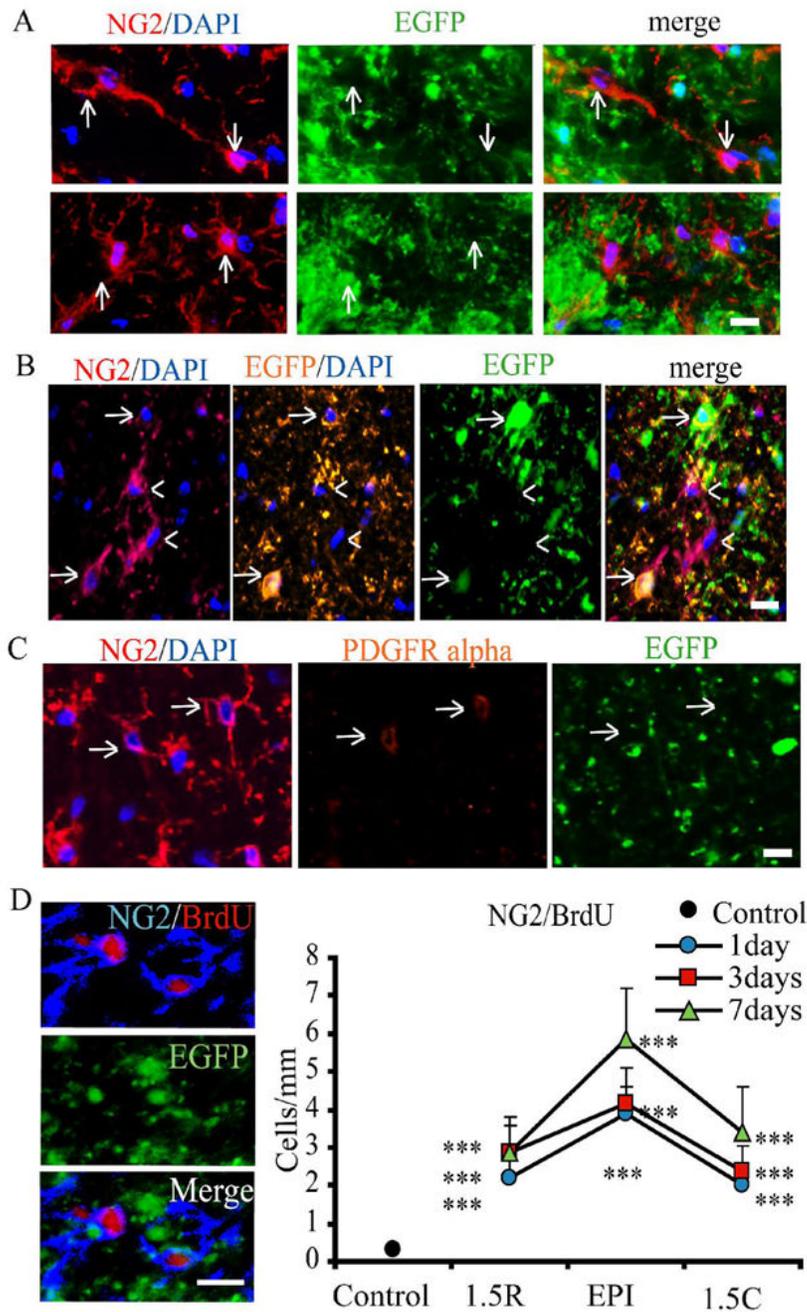


Figure 3. EGFP^{neg}NG2⁺ cells also proliferate after spinal cord injury and do not resemble OPCs

A. Non-EGFP-expressing NG2 (red) cells (arrows) range in morphology from simple, bipolar (top panels, injured tissue shown 3 DPI) to branched, multipolar (bottom panels, uninjured tissue shown). DAPI (blue) labels cell nuclei. Scale bar = 10 μ m.

B. Non-EGFP-expressing NG2⁺ cells (arrow heads) are negative for anti-EGFP (orange). EGFP⁺NG2⁺ cells can also be seen (arrows) and are immuno-positive for GFP. Scalebar = 10 μ m.

C. Non-EGFP-expressing NG2⁺ cells express PDGFR α . However, expression is weak and was limited to the cell body.

D. BrdU (red) is incorporated into EGFP^{neg}NG2⁺ cells (blue) in the first week after injury. Scale bar = 10µm. Quantitative analysis of EGFP^{neg}NG2⁺BrdU⁺ cell counts in control animals and after injury. Cell counts were performed in the ventral-lateral white matter. Immunoreactive cells were quantified for 5 animals every at the injury epicenter and at 1.5mm rostral and caudal in the spared ventral-lateral white matter. For each distance at each timepoint, three 10 µm-thick sections were counted, left and right hemispheres, for a total of six fields per animal. Therefore, thirty 200 µm² fields of view were quantified for each. One-Way ANOVA with Tukey's post-hoc analysis for variance. * p < 0.05; ** p < 0.01; *** p < 0.001 vs. control.

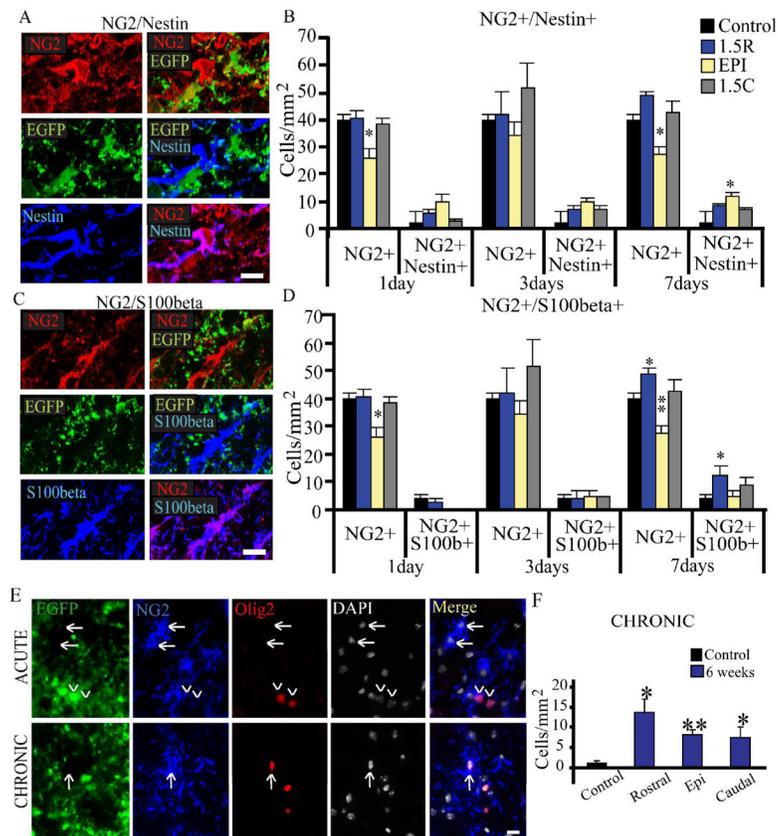


Figure 4. The EGFP^{neg}Olig2⁺ cell population displays different patterns of expression of S100 β , nestin, and transcription factors associated with OPCs

A. Example of a nestin⁺ (blue) EGFP^{neg} (green) NG2⁺ (red) cell in the ventrolateral white matter in injured tissue at 3 DPI. Scale bar = 10 μ m.

B. Quantitative analysis for EGFP^{neg}NG2⁺nestin⁺ cells (top) in the control animal and in the injured animal at 1, 3 and 7 DPI, at 1.5mm rostral, epicenter, and 1.5mm caudal. Cell counts were performed in the ventral-lateral white matter. A side-by-side comparison for EGFP^{neg}NG2⁺ and EGFP^{neg}NG2⁺nestin⁺ cells is shown for each timepoint. Immunoreactive cells were quantified for 5 animals every 0.5 mm from the injury epicenter, and at the injury epicenter in the spared ventral-lateral white matter. For each distance at each timepoint, three 10 μ m-thick sections were counted, left and right hemispheres, for a total of six fields per animal. Therefore, thirty 200 μ m² fields of view were quantified each. One-Way ANOVA with Tukey's post-hoc analysis for variance. * p<0.05, ** p<0.01 vs. control.

C. Example of S100 β ⁺ (blue) EGFP^{neg} (green) NG2⁺ (red) cells in the ventrolateral white matter in injured tissue at 3 DPI. Scale bar = 10 μ m.

D. Quantitative analysis for EGFP^{neg}NG2⁺S100 β ⁺ cells in the control animal and in the injured animal at 1, 3 and 7 DPI, at 1.5mm rostral, epicenter, and 1.5mm caudal. Cell counts were performed in the ventral-lateral white matter. A side-by-side comparison for EGFP^{neg}NG2⁺ and EGFP^{neg}NG2⁺S100 β ⁺ cells and is shown for each timepoint. A subset of EGFP^{neg}NG2⁺ cells express S100 β . Immunoreactive cells were quantified for 5 animals every 0.5 mm from the injury epicenter, and at the injury epicenter in the spared ventral-lateral white matter. For each distance at each timepoint, three 10 μ m-thick sections were counted, left and right hemispheres, for a total of six fields per animal. Therefore, thirty 200 μ m² fields of view were quantified each. One-Way ANOVA with Tukey's post-hoc analysis for variance. * p<0.05, ** p<0.01 vs. control.

E. Representative images of Olig2 staining in EGFP^{neg}NG2⁺ cells, acutely and chronically. Olig2 was not observed in the acute injury phase, but was observed chronically.

F. Quantification of EGFP^{neg}Olig2⁺ cells in the chronic animal. Immunoreactive cells were quantified for 5 animals every 0.5 mm from the injury epicenter, and at the injury epicenter in the spared ventral-lateral white matter. For each distance at each timepoint, three 10 μ m-thick sections were counted, left and right hemispheres, for a total of six fields per animal. Therefore, thirty 200 μ m² fields of view were quantified for each. Represented as mean \pm SEM. One-Way ANOVA with Tukey's Post-hoc test Symbols represent mean \pm SEM. One-way ANOVA with Tukey's post-hoc analysis. * = $p < 0.05$, ** = $p < 0.01$ vs. Control.

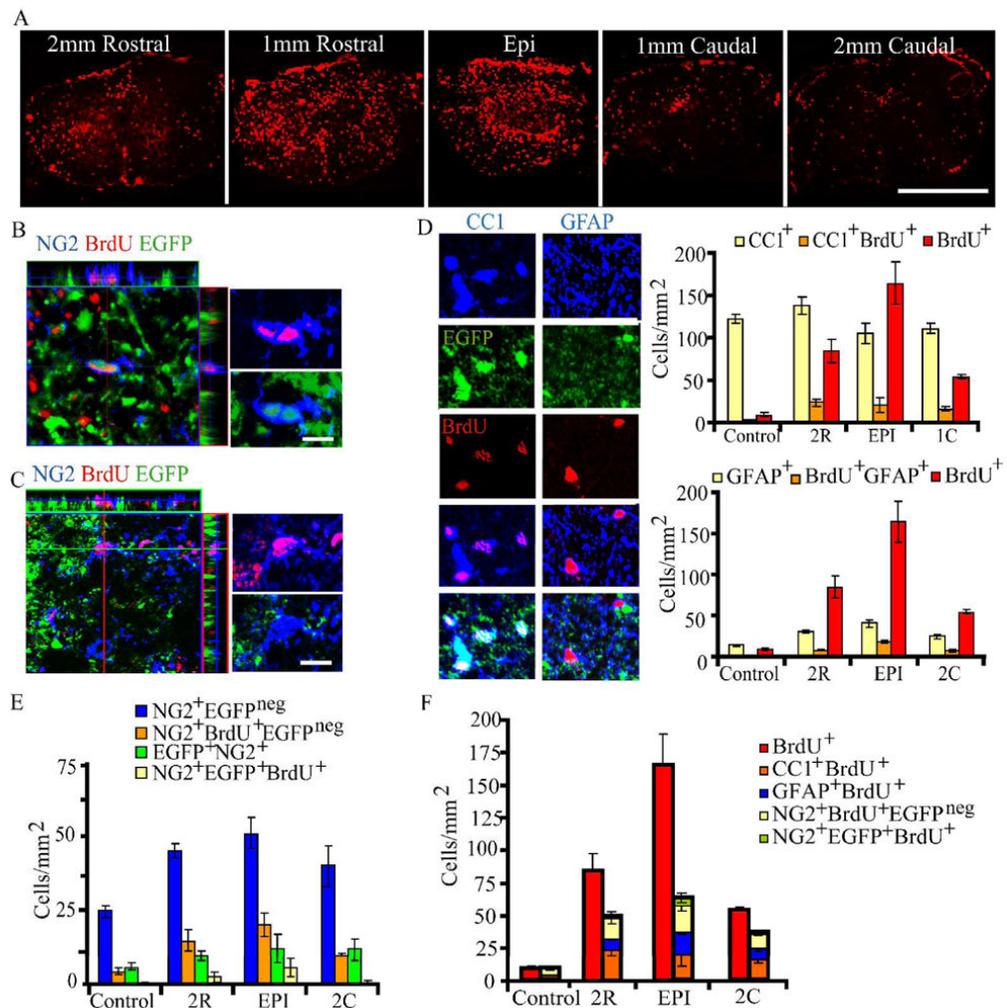


Figure 5. Fate of cells that incorporate BrdU in the first week following spinal cord injury
Six mice were injected with bromodeoxyuridine (BrdU) at 2–4 dpi, and were sacrificed at 6 weeks. Five control animals were also injected. Cell counts were performed in the spared ventral-lateral white matter. Six fields of view were counted per distance per animal, for a total of 36 fields of view per distance in injured animals, 30 in control.

A. Low-power view of BrdU incorporated in cells at the injury epicenter, and 1mm and 2mm rostral and caudal to it. Scalebar = 500 μ m

B – C. Representative confocal microscopy images with orthogonal views. Both EGFP⁺ and EGFP⁻NG2⁺ cells display BrdU incorporation. Scalebars = 5 μ m.

D. Representative confocal microscopy images of EGFP⁺CC1⁺ oligodendrocytes and EGFP⁻GFAP⁺ astrocytes that have incorporated BrdU. Scalebar = 10 μ m.

Top right panel: Quantification of average EGFP⁺CC1⁺ oligodendrocytes, BrdU⁺ nuclei, and EGFP⁺CC1⁺ cells that have incorporated BrdU. Bars represent mean \pm SEM. One-way ANOVA with Tukey's post-hoc analysis was performed. BrdU incorporation is significantly greater at all distances ($p < 0.001$).

Bottom right panel: Quantification of average EGFP⁻GFAP⁺ astrocytes, BrdU⁺ nuclei, and EGFP⁻GFAP⁺ cells that have incorporated BrdU. Bars represent mean \pm SEM. One-way ANOVA with Tukey's post-hoc analysis. GFAP⁺ cell density in the chronic spinal cord is

significantly greater than control levels at all distances ($p < 0.05$). BrdU incorporation is significantly greater at all distances ($p < 0.01$).

E. Quantification of EGFP⁺NG2⁺ cells and EGFP^{neg}NG2⁺ cells. Bars represent mean \pm SEM. One-way ANOVA with Tukey's post-hoc analysis. EGFP^{neg}NG2⁺ cells are significantly greater than control at all distances ($p < 0.05$). EGFP^{neg}NG2⁺BrdU⁺ cells are significantly greater than control at all distances ($p < 0.05$). EGFP⁺NG2⁺ cells are significantly greater than normal at rostral locations, and at 1mm caudal ($p < 0.01$). EGFP⁺NG2⁺BrdU⁺ cells are significantly greater than normal at the injury epicenter and at 1mm rostral and caudal ($p < 0.05$).

F. BrdU⁺ cell population breakdown. EGFP⁺CC1⁺ oligodendrocytes, EGFP^{neg}GFAP⁺ astrocytes and NG2⁺ cells comprise about half of all BrdU⁺ cells in the chronic animal.

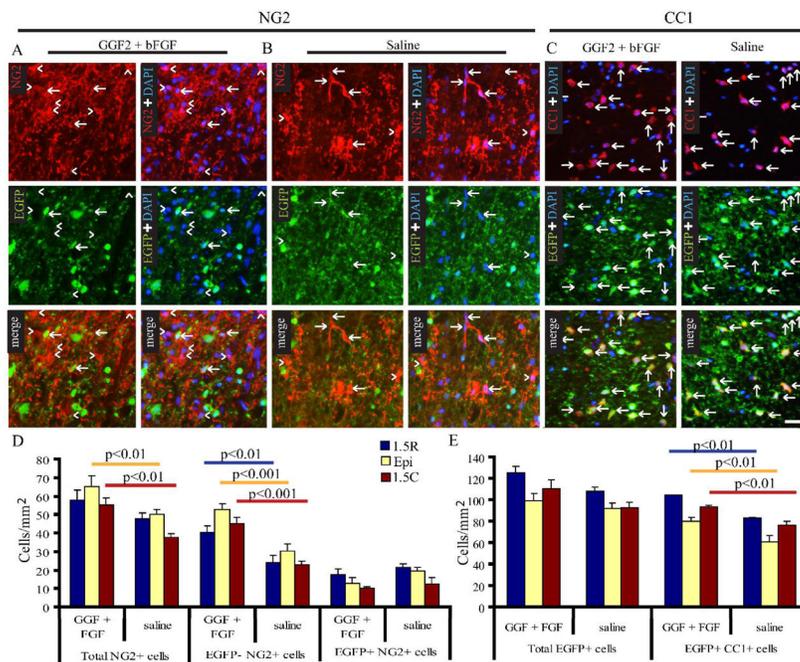


Figure 6. *In vivo* treatment with glial growth factor 2 and basic fibroblast growth factor spinal cord injury results in increased numbers of EGFP⁺CC1⁺ oligodendrocytes

SCI Mice were injected subcutaneously with 0.02mg/kg FGF2 and 0.8mg/kg GGF2 for 8 days, beginning at 1 DPI, and sacrificed on day 9. NG2 immunoreactivity was increased in GGF2 + FGF2 mice (A), compared to saline-injected controls (B). EGFP⁺CC1⁺ oligodendrocytes were also increased in GGF2 + FGF2 treated mice (C). D. Quantification of NG2⁺ cells indicating a significant increase in EGFP^{neg}NG2⁺ cells. E. Quantification of CC1⁺ cells indicating a significant increase in EGFP⁺CC1⁺ oligodendrocytes; bars represent mean \pm SEM. Two-way ANOVA was performed with Bonferroni's post-hoc analysis.

Table 1

Passive membrane properties of NG2 and O4 cells

		RMP (mV)	Rm (MOhm)	Cm (pF)
		Mean \pm SEM; Range	Mean \pm SEM; Range	Mean \pm SEM Range
NG2	Control	-33.2 ± 3.2 ; -15 to -40	306.2 ± 77 ; 200 to 1200	8 ± 0.9 ; 4 to 20
	3d SCI	-30.2 ± 3.9 ; -13 to -40	1267.9 ± 450.7 ; 150 to 5000	6.9 ± 0.9 ; 4 to 15
O4	Control	-52.2 ± 5.3 ; -32 to -77	183.6 ± 59.4 ; 35 to 150	75.7 ± 16.2 ; 12 to 150
	3d SCI	-45.6 ± 3.7 ; -30 to -73	122.2 ± 22.5 ; 47 to 300	81.7 ± 25.7 ; 13 to 265

Passive membrane properties of NG2 and O4 cells were determined. Resting membrane potential (RMP), membrane resistance (Rm) and capacitance (Cm) reveal distinct properties associated with NG2 or O4 cells in both control and injured preparations. Confirmation of cell types was performed with biocytin filling in combination with post-hoc immunocytochemical analysis. Eighty-eight cells were analyzed: N = 32 NG2 cells (control), N = 32 NG2 cells (injured), N = 8 O4 cells (control), N = 16 O4 cells (injured).

Table 2
Percentage of Cells Expressing Voltage-Gated Potassium Currents

	K_A	K_{DR}	K_{IR}		K_A	K_{DR}	K_{IR}
NG2	25% (8/32)	75% (24/32)	0% (0/32)	O4	Control	100% (8/8)	75% (6/8)
	59% (19/32)	100% (32/32)	0% (0/32)		3d SCI	83% (12/14)	100% (14/14)

Not all cells displayed all voltage-gated potassium currents. There was an increase in the percentage of NG2 cells expressing K_A and K_{DR} after injury. No NG2 cells expressed K_{IR}. There was a decrease in the percentage of O4 cells expressing K_A after injury, and an increase in K_{IR}. Eighty-eight cells were analyzed: N = 32 NG2 cells (control), N = 32 NG2 cells (injured), N = 8 O4 cells (control), N = 16 O4 cells (injured).