Age-Dependent Netrin-1 Signaling Regulates NG2⁺ Glial Cell Spatial Homeostasis in Normal Adult Gray Matter

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Neuron–glial antigen 2-positive (NG2⁺) glial cells are the most proliferative glia type in the adult CNS, and their tile-like arrangement in adult gray matter is under tight regulation. However, little is known about the cues that govern this unique distribution. To this end, using a NG2⁺ glial cell ablation model in mice, we examined the repopulation dynamics of NG2⁺ glial cells in the mature and aged mice gray matter. We found that some resident NG2⁺ glial cells that escaped depletion rapidly enter the cell cycle to repopulate the cortex with altered spatial distribution. We reveal that netrin-1 signaling is involved in the NG2⁺ glial cell early proliferative, late repopulation, and distribution response after ablation in the gray matter. However, ablation of NG2⁺ glial cell in older animals failed to stimulate a similar repopulation response, possibly because of a decrease in the sensitivity to netrin-1. Our findings indicate that endogenous netrin-1 plays a role in NG2⁺ glial cell homeostasis that is distinct from its role in myelination.

Key words: NG2⁺ glia; homeostasis; Netrin-1

Introduction

NG2⁺ glial cells are unique among glial cells of the adult CNS in that they are both uniformly distributed and actively cycling (Nishiyama et al., 1999; Hill and Nishiyama, 2014). Their best recognized role is as precursors to myelinating oligodendrocytes during development and remyelination after demyelinating insults (Richardson et al., 2011). Recent investigation demonstrated that NG2⁺ glial cells respond to the loss and regeneration of the neighboring NG2⁺ cells by rapid dividing and through active repulsion between cells to regulate their density (Nishiyama et al., 2009; Hughes et al., 2013). Furthermore, recent sensory deprivation studies in mice have uncovered a functional coupling between the NG2⁺ glial cell landscape and alterations in neurotransmission in response to sensory experiences (Mangin et al., 2012). However, the signals in the naive adult neural microenvironment that instruct NG2⁺ glial cell density and organization are essentially unknown.

Netrin-1 (NT-1) is a chemotropic axon guidance cue that has been previously shown to influence the behavior of NG2⁺ glial cells. NT-1 serves as a dispersal factor for early NG2⁺ glial cells in the embryonic (Jarjour et al., 2003) and developing (Tsai et al., 2003) spinal cord and optic nerve (Sugimoto et al., 2001), acting through the NT-1 receptors deleted in colorectal cancer (DCC)

and uncoordinated family member 5 (UNC-5), which are both expressed by NG2⁺ glial cells (Spassky et al., 2002). In addition to having promigratory roles in the developing brain, NT-1 in the adult CNS has been shown to promote myelination by driving differentiation of NG2⁺ progenitors toward mature oligodendrocytes in various demyelinating injuries (He et al., 2013; Tepavčević et al., 2014). However, such studies on the adult CNS often use exogenous sources of NT-1, masking endogenous NT-1 contributions to NG2⁺ glial cell mobility and proliferation. Overall, it remains unknown whether NT-1 plays an active role in the steady-state regulation of NG2⁺ glial cell number and distribution in the unperturbed, adult gray matter.

In the present study, using a NG2⁺ glial cell ablation model, we survey the spatial dynamics of NG2⁺ cells in the adult gray matter after their mass ablation, tracking their regeneration, proliferation, migration, and distribution. We observed that NG2⁺ cells that escaped depletion rapidly enter the cell cycle, expand their pool, and efficiently repopulate the adult cortex [postnatal day 90 (P90); P90–P120], albeit with altered spatial distribution. We observed that the NT-1 signaling pathway is required for the development of a NG2⁺ glial cell tile-like distribution during repopulation. However, NG2⁺ glial ablation in older animals (P270–P350) failed to stimulate a repopulation response, possibly due to a decrease in DCC levels. Our findings implicate NT-1 as a signal responsible for the maintenance of NG2⁺ glial cell distribution in adult gray matter.

Materials and Methods

Animals. All animal procedures were performed according to the Institutional Animal Care and Use Committee of Division of Laboratory Animal Resources, State University of New York Stony Brook School of Medicine and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The transgenic mouse strains *Tg(Cspg4-cre)1Akik/J* (NG2CreBAC) and *Gt(Rosa)26Sor^{tm1(HBEGF)Awai/J}* were purchased from The Jackson Laboratory. NG2CreBAC mice were back-

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crossed to generate inducible Diphtheria Toxin Receptor iDTR mice. In the iDTR mouse line, the gene encoding diphtheria toxin receptor [DTR simian heparin-binding epidermal growth factor-like growth factor (HBEGF)] is under the control of the constitutive Rosa26 locus promoter, and its expression is blocked by an upstream loxP-flanked STOP sequence. The DTR is expressed after Cre recombinase removes the STOP cassette, rendering only NG2-expressing cells susceptible to DT. Wild-type littermates were also injected with DT and used as control animals for the experiments with systemic DT administration. No specific adverse side effects of DT were observed when administered to the control and iDTR mice.

DT administration. Adult mice (P90–P120) received an intraperitoneal injection of DT (100 ng) for 7 consecutive days (designated a 1DT through 7DT). Mice were analyzed at 7 d after the first injection (acute depletion phase; 7DT) and 3 d and 1, 2, and 3 weeks after 7DT administration (7DT+d). These time points were chosen to include the onset of NG2⁺ glia death and acute depletion (3–7 d) and recovery (1–3 weeks).

Immunohistochemistry. Immunostaining was performed using free-floating coronal brain slices included the following antibodies: anti-bromodeoxyuridine (BrdU; Accurate Chemical and Scientific Corporation), anti-NG2 (Millipore Bioscience Research Reagents, R&D Systems), anti-PDGFR α (BD Biosciences, Santa Cruz Biotechnologies), anti-Ki67 (Novocastra), anti-proliferating cell nuclear antigen (PCNA; Millipore Bioscience Research Reagents), anti-DCC (Santa Cruz Biotechnologies), and anti-NT-1 (Abcam). For newly generated cells, BrdU was dissolved in drinking water (1 mg/ml), and mice were given access to the water *ad libitum* for 3 weeks after 7DT. Mouse anti-BrdU (Abcam) and rat anti-BrdU (Abcam) was used for 5-chloro-2'-deoxyuridine (CldU) and 5-iodo-2'-deoxyuridine (IdU) detection, respectively. The CldU/IdU staining was done as described previously (Tuttle et al., 2010). For BrdU staining, sections were incubated with 2N hydrochloric acid (HCl) for 20 min at 37°C and then washed with PBS for 30 min. For Ki67 or PCNA staining *in situ*, sections were boiled in 0.1 M sodium citrate, pH 4.5, in a water-steamer bath apparatus.

Western blots and immunoprecipitation. For Western blots, somatosensory cortices were dissected out at the end of the experiments and used for protein extraction using lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1% 2-mercaptoethanol, 1% Triton X-100, plus proteases inhibitor cocktail; Sigma). Protein samples (10 mg) were separated on GENE Mate express gels and transferred to PVDF membranes (Millipore). The membranes were incubated with primary antibodies overnight at 4°C.

CldU/IdU chase. CldU (8.4 mg/ml) and IdU (11.3 mg/ml) injections were done as described previously (Bauer and Patterson, 2006).

Neutralizing antibody injection. NT-1 neutralizing antibody (10 ng/ml) was infused into the somatosensory cortices of control and iDTR animals via cannula implantation. Stereological coordinates used were as follows: ventral, 2.0 mm; lateral, 1.3 mm; and bregma, 0.54 mm.

Sholl analysis. Sholl analysis to measure branch complexity was done as described previously (Eyer mann et al., 2012). The following parameters were used for all Sholl analyses: 10 μ m starting radius, 40 μ m ending radius, 10 μ m step size, and 0.07 μ m span.

NG2 glia area coverage analysis. The distance between the closest neighbors of each NG2 glia in a $384 \times 384 \times 20 \mu\text{m}^3$ area was measured. Each neighboring couple was counted once. An average of 100–150 cells and 10–20 fields were counted per brain.

Confocal microscopy and cell counting. A confocal laser-scanning microscope TCS-SP5 (DM16000 B instrument; Leica) was used for image localization of FITC (488 nm laser line excitation; 522/35 emission filter), Cy3 (570 nm excitation; 605/32 emission filter), and Cy5 (647 nm excitation; 680/32 emission filter). At least four different brains for each strain and each experimental condition were analyzed and counted. Cell counting was performed blindly, and tissue sections were matched across samples. An average of 15–20 sections were quantified using unbiased stereological morphometric analysis to obtain an estimate of the total number of positive cells.

Statistical analysis and experimental design. All statistical analysis was performed using the Student's *t* test. In all cases, replicates refer to biological rather than technical replicates. NG2Cre/iDTR breeding crosses

were set so that ablation controls were of the same litter. Equal numbers of adult (P70–P120) males and females were used for ablation studies. Statistical analyses were performed using SigmaPlot software.

Results

NG2⁺ glial cell proliferation rate is increased after their massive ablation in the adult gray matter

To investigate the signaling mechanisms involved in controlling NG2⁺ glial cell density and distribution in the adult CNS, we generated a mouse line that enabled us to massively ablate NG2⁺ glial cells and track their regeneration *in vivo* specifically in the somatosensory cortex, a region in which NG2⁺ glial cell distribution is shown to be self-regulated (Hughes et al., 2013). In this mouse line, DTR is expressed selectively by NG2⁺ glial cells, rendering them susceptible to DT (NG2Cre/iDTR; iDTR mouse). Our DT injection paradigm reduced NG2⁺ glial cell density in the somatosensory cortex of these mice by ~65–75% (7DT; Fig. 1A), as determined by immunostaining for NG2 and PDGFR α (a second marker for NG2 glia; Fig. 1B). 3 days after the last DT injection (7DT+3d), our analysis showed that NG2⁺ glial cell ablation stimulates survivor NG2⁺ glial cells to rapidly reenter the cell cycle, since $82 \pm 7.3\%$ of the remaining NG2⁺ glia coexpressed PCNA; only $21.7 \pm 2.1\%$ coexpressed PCNA in control mice (Fig. 1C). We next labeled slowly dividing (CldU⁺) and rapidly dividing (IdU⁺) cells in the adult cortex by sequential administration of thymidine analogs before the DT administration and examined the behaviors of NG2⁺ cells after ablation (Fig. 1D). We found two population of NG2⁺ cells: (1) one population of cells that retained CldU⁺ for up to 3 weeks after its administration, indicating that they were dividing slowly; and (2) another population lacking the CldU signal but retaining the more recently administrated IdU, indicating more rapid division and dilution of CldU. Both CldU⁺ and IdU⁺ NG2⁺ glial cells were independently present in the control mice, indicating a mitotically heterogeneous population in the adult cortex. In the cortex of iDTR mouse, during the early stages of repopulation, we observed a CldU⁺ IdU⁺ cell population, suggesting that a slowly dividing pool survives ablation and reenters the cell cycle to contribute to the repopulation (Fig. 1E). Unexpectedly, even at 3 weeks (7DT+23d), when NG2⁺ glial cell repopulation was close to completion (Fig. 1F), NG2⁺ glial cells still exhibited a high proliferation rate in the iDTR mice relative to the control mice (Fig. 1G). Our data indicate that a heterogeneous NG2⁺ glial cell population, based on proliferation dynamics, is present in the adult cortex and that, during ablation, the slowly dividing NG2⁺ glial cells undergo to a rapid mitotic phase to maintain their normal density.

Altered branch complexity of newly generated NG2⁺ glial cells after repopulation

We next analyzed NG2⁺ glial cell morphology during regeneration. Although newly born NG2⁺ glia (NG2⁺ BrdU⁺ cells) at 7DT+3d and 7DT+7d display a few short, thick processes, a more complex morphology with longer, more numerous, and more branched processes was observed at later stages (7DT+15d and 7DT+23d; Fig. 2A). We next examined the morphology of repopulating NG2⁺ glial cells in iDTR mice midway through repopulation (7DT+15d) and compared actively dividing cells (NG2⁺ BRDU⁺ Ki67⁺) with cells that had divided and exited the cell cycle (NG2⁺ BRDU⁺ Ki67⁻; Fig. 2B). Sholl analysis showed that actively dividing NG2⁺ glial cells had less complex branch morphologies, further indicating that NG2⁺ glial cells go through proliferation-dependent developmental stages during

Figure 1. Quiescent, resident NG2⁺ glial cells that escape ablation rapidly enter the cell cycle and regenerate in the adult cortex. **A**, Representative images and cell number quantification for NG2⁺ and PDGFRα⁺ cells at 7DT. **B**, Time points of repopulation analysis. **C**, Representative images and cell number quantification for PCNA⁺ PDGFRα⁺ cells at 7DT+3d. **D**, CldU/IdU administration paradigm. **E**, Representative images of CldU, IdU, and PDGFRα⁺ IdU⁺ CldU⁺ cells at 7DT+7d. **F**, Representative images and cell number quantification for NG2⁺ glial cells at 7DT+23d. **G**, Representative images and cell number quantification for BrdU⁺ PCNA⁺ PDGFRα⁺ cells at 7DT+23d. BrdU (100 mg/ml) was injected intraperitoneally at 7DT+14d. Error bars indicate mean ± SEM (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). *n* = 3–5 per group. Scale bars, 40 μm. CTRL, Control.

their repopulation. When examined at the final post-ablation stage (7DT+30d), NG2⁺ glial cell morphology in the iDTR cortices had more complex branching patterns (Fig. 2C). This observation suggests that post-ablation NG2⁺ glial cells are in a constant state of development and reorganization to maintain optimal branch coverage, even after the restoration of normal density.

NT-1 plays a role in regulating NG2⁺ glial cell proliferation and unique tile-like pattern after their ablation

We next asked whether, along with the intrinsic mechanism of density self-regulation observed during regeneration, an extrinsic signaling cue could also participate in determining the spatial organization and morphologies of NG2⁺ glial cells during repopulation. We focused on NT-1 and its receptor DCC, based on their involvement in promoting oligodendrocyte progenitor physiology (Spassky et al., 2002). NT-1 levels were upregulated during the early phase of regeneration (7DT+3d) and later stabilized (7DT+15d and 7DT+23d; Fig. 3A), suggesting that the NT-1 pathway is involved in facilitating early NG2⁺ glial cell dispersal in the adult brain, akin to previously reported NT-1 migratory events during developmental spinal cord myelination (Tsai et al., 2006). Conversely, the receptor DCC was at low and high levels at early and late stages of NG2⁺ glial cell regeneration, respectively, implicating a DCC-mediated late phase of self-repulsion in the establishment of NG2⁺ glial cell domains once

optimal density is reached. Our *in situ* analysis confirmed this idea, because NG2⁺ glia had high expression of DCC in the tips of cell processes at 7DT+23d but not at 7DT+3d (Fig. 3B). We next blocked NT-1 in the somatosensory cortex at 7DT+3d, a time when we find a major increase in NT-1 expression, via cannula-assisted focal infusion of an NT-1 neutralizing antibody. We then assessed whether blocking the early NT-1 response affects subsequent repopulation (Fig. 3C). The NT-1 neutralizing antibody significantly reduced NG2⁺ glial cell density focally around the site of infusion, almost completely abolishing the regeneration response. This was attributable to the reduced total numbers of proliferating NG2⁺ glial cells, because the numbers of actively dividing NG2⁺ glial cells (PCNA⁺ PDGFRα⁺ cells) of the total pool (total PDGFRα⁺ cells) were significantly reduced (Fig. 3D). NT-1 blocking further reduced NG2⁺ glial cell branch length (Fig. 3E) and impeded their normal spatial distribution (Fig. 3F), further indicating that, in addition to compromised density attributable to reduced proliferation, blocking NT-1 signaling affects morphology, density, and normal distribution. Our analysis indicates that NT-1 is involved in determining NG2⁺ glial cell landscape organization and density in the adult CNS.

NG2⁺ glial cells in the aged CNS lack proliferative response and repopulation after its massive ablation

The surprising observation that NG2⁺ glial cells in the mature CNS exhibited higher proliferation capacities than reported pre-

Figure 2. NG2⁺ glial cells undergo morphological development in preparation for their tile-like distribution and density in the gray matter. **A**, Representative images of newly generated NG2⁺ glial cells (NG2⁺ BrdU⁺ cells) throughout the repopulation stages. **B**, Representative images of NG2⁺ BrdU⁺ Ki67⁺ cells at 7DT + 15d and quantification of branch complexity by Sholl analysis (for details, see Materials and Methods). **C**, Sholl analysis at the 7DT + 30d. Error bars indicate mean $\pm$ SEM (* p < 0.05, ** p < 0.01, *** p < 0.001). n = 4–6 per group. Scale bars: **A** and inset 10 μ m; **B**, 40 μ m. CTRL, Control.

viously when large-scale ablation of their neighbors led us to consider whether similar capacities exist in the aged CNS. We analyzed the regeneration potential of NG2⁺ glial cells in older mice (P270–P350) using BrdU administration as above. In contrast to NG2⁺ glial cells of adult mice, the NG2⁺ glial cells in the aged cortex that escape ablation failed to repopulate the areas of depletion in the cerebral cortex, even as late as 7DT + 23d (Fig. 4A). Consistent with the idea that NG2⁺ glial cell morphology is functionally linked to cell-intrinsic regulation of their density and distribution, the branch complexity (Fig. 4B) and overall cell number (Fig. 4C) of the NG2⁺ glia in the aged iDTR cortex were reduced. Based on our data above, we next asked whether compromised regeneration and morphology could be linked to alter NT-1 signaling. Protein expression analysis assessing NT-1 and DCC levels from P15 to P300 in mice indicated that NT-1 levels increase progressively as DCC levels decrease throughout the aging process, further indicating that alterations in the NT-1 signaling pathway regulate NG2⁺ glial cell development and density to maintain their domains in the CNS (Fig. 4D).

Discussion

NG2⁺ glial cells represent an actively cycling glial subtype that is found throughout the adult CNS. NG2⁺ glial cells have been shown to dramatically increase their proliferation rate and pop-

ulate the site of insult after acute brain injury (Hampton et al., 2004) and demyelinating injury (Keirstead et al., 1998) and as a result of neurodegeneration (Magnus et al., 2008). It has been further established that the changes in the local CNS environment may enable NG2⁺ glial cells to exhibit greater lineage plasticity and participate in cell replacement (Magnus et al., 2008; Sellers et al., 2009). A recent study has corroborated this idea by showing tight homeostatic self-regulation of unique territories maintained by NG2⁺ glial cells through self-avoidance (Hughes et al., 2013). In the present study, we sought to identify underlying processes behind this autoregulation to unravel potential cell-intrinsic and -extrinsic mechanisms involved in maintaining the NG2⁺ glial cell landscape in normal adult gray matter. We focused on uninjured somatosensory cortex in an effort to identify signals that are independent of the need for heavy myelination and injury response.

Using the NG2Cre/iDTR mouse model of NG2⁺ glial cell ablation, we were able to emulate a highly regenerative and proliferative state in the repopulating NG2⁺ glial cells, which is usually a response to a pathological condition, such as demyelination or trauma, in the healthy mature CNS. We showed that the response of surviving NG2⁺ glial cells to the mass ablation of neighbors was prompt, because we observed that virtually all

Figure 3. NT-1 regulates NG2⁺ glial cell development and repopulation dynamics in the adult gray matter. **A**, Protein levels of NT-1 and DCC throughout the repopulation stages. **B**, Representative images of NG2⁺ DCC⁺ cells at 7DT+23d. **C**, Administration protocol for NT-1 neutralizing antibody infusion. **D**, Representative images and cell number quantification for PDGFRα⁺ and PCNA⁺ PDGFRα⁺ cells at 7DT+15d, 1 week after blocking antibody infusion. **E**, Sholl analysis of iDTR cortices injected with NaCl or NT-1 blocking antibody at 7DT+15d. **F**, Quantification of area coverage by close-neighbor analysis (distance between the closest NG2⁺ glial cells). iDTR cortices injected with NaCl or NT-1 blocking antibody at 7DT+15d. Error bars indicate mean $\pm$ SEM (* p < 0.05, ** p < 0.01, *** p < 0.001). n = 5–6 per group. Scale bars, 40 μ m. CTRL, Control.

Figure 4. Failed repopulation of NG2⁺ glial cells and altered NT-1/DCC expression levels in aged gray matter. **A**, Representative images of NG2⁺ BRDU⁺ cells throughout the repopulation stages in aged cortices. **B**, Representative images of NG2⁺ glial cell morphology between aged control and iDTR cortices. **C**, Representative images and cell number quantification of PDGFRα⁺ cells in adult and aged cortices. **D**, Protein expression levels and associated quantification of DCC and NT-1 in the cortices of mice at different developmental ages. Error bars indicate mean $\pm$ SEM (* p < 0.05). n = 3 per group. Scale bars, 40 μ m; Fig 4B, 10 μ m. CTRL, Control.

PDGFRα⁺ cells were actively cycling (PCNA⁺) at 3 d after the last DT administration. We subsequently demonstrated two mitotically distinct populations of these cells: (1) previously described rapidly cycling NG2⁺ glial cells that are mostly depleted by DT administration (IdU⁺); and (2) slowly dividing NG2⁺ glial cells

that are resistant to DT administration and mostly constitute the source of the repopulating pool. Importantly, we show that, although the density recovers within 3 weeks, the newly generated NG2⁺ glial cells still exhibit enhanced proliferation capacities, indicating a cell-intrinsic, density-independent cue

mediating NG2⁺ cell proliferation and density in the adult gray matter.

NT-1 signaling has been shown previously to control attraction/repulsion in migrating oligodendrocyte precursor cells (Sugimoto et al., 2001; Jarjour et al., 2003; Petit et al., 2007). However, these studies often involved analyses either during developmental stages of the NG2⁺ glial cell population or using demyelinating injury models, in which exogenous and transient NT-1 overexpression is often used to mobilize NG2⁺ glial cells. It remains unknown whether endogenous NT-1 is actively involved in the steady-state NG2⁺ glial cell architecture in the unperturbed adult gray matter. We found that NT-1 signaling is involved intimately in repopulation dynamics. Blocking NT-1 hindered the proper distribution and density of repopulating NG2⁺ glial cells by regulating their proliferative status and morphological development.

The proliferative abilities of NG2⁺ glial cells have been shown to diminish with age (Zhu et al., 2011). In line with this, the surviving NG2⁺ glial cells after ablation in aged mice (P300⁺) was not able to repopulate, even after a 3 week recovery period. Attenuation of NT-1 signaling might be partially responsible for the diminishing proliferative state and density of NG2⁺ cells in the aged brain, because we detected that the NT-1 receptor DCC levels decrease with age. It is important to note that there was no significant difference in DCC levels between adult and aged cortices, indicative of the possible contributions of other NT-1 receptors, such as Unc-5 (Jarjour et al., 2003), to NT-1-mediated NG2 glial cell dynamics in the aged brain. The source of NT-1 during the early stages of repopulation remains unclear, although neurons have been shown previously to be a major cell type that produces NT-1 (Petit et al., 2007).

We additionally correlate the NG2⁺ glial cell morphology with proliferation capacities, in both aged and adult mice. We show that the branching of NG2⁺ glial cells develops as repopulation progresses from early to late phases. Aged NG2⁺ glial cells, like adult NG2⁺ glial cells after NT-1 blocking, had similarly reduced branch complexities. We propose functional links in the response of NG2⁺ glial cell development to NT-1 among proliferation, spatial distribution, and branch morphology, as processes that mediate NG2⁺ cell dynamics in the normal adult brain.

The participation of NG2⁺ glial cells in cell-replacement in the adult brain has been an attractive therapeutic avenue for neurodegenerative and autoimmune diseases. It will be important to determine how to manipulate spatial signaling elements, such as NT-1 signaling pathway, towards functionally diverting the proliferative and regenerative potentials of NG2⁺ glial cells.

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