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Injury type-dependent differentiation of NG2 glia into heterogeneous astrocytes

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Abstract

The glial scar is comprised of a heterogeneous population of reactive astrocytes. NG2 glial cells (also known as oligodendrocyte progenitor cells or polydendrocytes) may contribute to this heterogeneity by differentiating into astrocytes in the injured CNS, but there have been conflicting reports about whether astrocytes comprise a significant portion of the NG2 cell lineage. By using genetic fate mapping after spinal cord injury (SCI) and experimental autoimmune encephalomyelitis (EAE) in mice, the goal of this study was to confirm and extend upon previous findings, which have shown that NG2 cell plasticity varies across CNS injuries. We generated mice that express tdTomato in NG2 lineage cells and express GFP under the *Aldh1l1* or *Glt1* promoter so that NG2 glia-derived astrocytes can be detected by their expression of GFAP and/or GFP. We found that astrocytes comprise approximately 25% of the total NG2 cell lineage in the glial scar by 4 weeks after mid-thoracic contusive SCI, but only 9% by the peak of functional deficit after EAE. Interestingly, a subpopulation of astrocytes expressed only GFP without co-expression of GFAP, uncovering their heterogeneity and the possibility of an underestimation of NG2 glia-derived astrocytes in previous studies. Additionally, we used high performance liquid chromatography to measure the level of tamoxifen and its metabolites in the spinal cord and show that genetic labeling of NG2 glia-derived astrocytes is not an artifact of residual tamoxifen. Overall, our data demonstrate that a heterogeneous population of astrocytes are derived from NG2 glia in an injury type-dependent manner.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.expneurol.2018.07.001>.

Keywords

Oligodendrocyte progenitor cells; Astrocytes; Spinal cord injury; Experimental autoimmune encephalomyelitis; Glial scar; Tamoxifen; Astrocyte heterogeneity

1. Introduction

NG2 progenitor cells were first discovered when Raff et al. isolated cells from rat optic nerve using an antibody against A2B5 (Raff et al., 1983). They showed that these progenitors could differentiate into either oligodendrocytes or astrocytes depending on the culture conditions. Due to their plasticity *in vitro*, they were originally called oligodendrocyte-type 2 astrocyte (O2A) progenitor cells (Raff et al., 1983), and were later discovered to also express NG2 (Stallcup and Beasley, 1987) and PDGFR α (Nishiyama et al., 1996) in the CNS. The possibility of NG2 cells serving as multipotent neural stem cells in the CNS caused wide-spread excitement until careful genetic lineage tracing studies demonstrated that while NG2 cells can differentiate into astrocytes in specific regions during development (Zhu et al., 2008a; Zhu et al., 2008b), they differentiate only into oligodendrocytes in the adult CNS (Kang et al., 2010).

The concept of NG2 cell lineage plasticity has recently been revived with the discovery that, in addition to oligodendrocytes, they may differentiate into astrocytes as well as Schwann cells after CNS injury. One of the first attempts to perform genetic lineage tracing of NG2 cells in the injured CNS used a retrovirus expressing a fluorescent marker driven by the NG2 promoter (Sellers et al., 2009). This study reported that between 35 and 50% of reporter-labeled NG2 cells became GFAP⁺ astrocytes after dorsal hemisection spinal cord injury. Labeling NG2 glia using a retrovirus potentially overestimated the amount of NG2 glia-derived astrocytes in this study since any virally transduced cell that upregulated NG2 expression after the injury, including astrocytes, would also be fluorescently labeled. The use of transgenic reporter mice to investigate cell fate *in vivo* can overcome the technical issue of off-target labeling that can occur with retroviral lineage tracing. However, studies using tamoxifen-inducible cre transgenic mice (*e.g.* NG2-CreER, Pdgfra-CreER, Olig2-CreER) have shown NG2 cells to have varying degrees of plasticity after CNS injury. We have previously shown that a substantial percentage of NG2 cells differentiate into astrocytes after contusive SCI (25%, ((Hackett et al., 2016), while others have shown that fewer NG2 cells, if any, become astrocytes after dorsal hemisection (5%, (Barnabe-Heider et al., 2010)), cortical stab (5%, (Dimou et al., 2008); 8% (Komitova et al., 2011)), focal demyelination (3%, (Zawadzka et al., 2010)), EAE (1%, ((Tripathi et al., 2010)), or in a model of ALS (0%, (Kang et al., 2010)). It has therefore been difficult to draw conclusions about the capacity of NG2 cells to differentiate into astrocytes after CNS injury (Dimou and Gallo, 2015; Levine, 2015; Richardson et al., 2011).

The discrepancy of NG2 glial cell fate between CNS injury studies may be explained by inherent differences between injury types as well as the method of astrocyte identification, or technical issues arising from tamoxifen metabolism. NG2 glia-derived astrocytes are most commonly identified by the use of GFAP immunohistochemistry, but not all astrocytes

express GFAP (Kimelberg, 2004). Thus, the detection of GFAP expression alone may lead to an underestimation of NG2 cell-derived astrocytes after injury. Furthermore, a potential confound with tamoxifen-inducible mouse studies is the effect of residual tamoxifen in the CNS at the time of injury. Most studies typically wait 3–7 days after the last tamoxifen injection assuming this is sufficient time for tamoxifen to be fully metabolized and expelled from the mice. A recent study has shown that it takes 6–8 days for tamoxifen and its metabolites to be degraded to ineffective concentrations (Valny et al., 2016), but to our knowledge, similar measurements have not been performed in the spinal cord, which could display different degradation kinetics due to the higher density of myelin in the spinal cord compared to the brain. Presence of residual tamoxifen metabolites presents a potential problem because if astrocytes increase expression of NG2 after injury, they can undergo recombination and express the reporter that was meant to be specific for NG2 cells. This off-target effect can lead to the false impression that these reporter-labeled astrocytes were derived from NG2 cells.

Therefore, the goal of this study was to perform a more detailed investigation of the extent of NG2 cell differentiation into astrocytes after CNS injury. We generated transgenic mice in which NG2 cells are labeled with tdTomato and astrocytes express GFP under either the *Aldh111* or *Glt1* promoter. These mice allowed us to address the limitations of GFAP immunohistochemistry by identifying NG2 glia-derived astrocytes by GFP expression in addition to GFAP. To address the possibility that NG2 glia lineage plasticity is injury-type dependent, we compared NG2 cell fate between distinct types of CNS injury: SCI and EAE. In addition, we used HPLC (high performance liquid chromatography) to demonstrate that the levels of tamoxifen and its metabolites are significantly reduced by the time of injury, and that NG2 glia give rise to astrocytes even when levels of tamoxifen and its metabolites are below the limit of detection by HPLC. Taken together, we show that the proportion of NG2 glia that differentiate into astrocytes differs between CNS injury models, and that NG2 glia-derived astrocytes are a heterogeneous population.

2. Materials and methods

2.1. Animals

NG2-CreER mice (Jackson Laboratory stock 008538 (Zhu et al., 2011)) were bred to Rosa26-tdTomato reporter mice (kindly donated by Dr. Fan Wang, Duke University, Durham, NC. (Arenkiel et al., 2011)) to produce NG2^{tdTom} mice in which Cre is hemizygous and Rosa26-tdTomato is either heterozygous or homozygous. NG2^{tdTom} mice were bred to Aldh111^{GFP} transgenic mice (Yang et al., 2011) to generate NG2^{tdTom}/Aldh111^{GFP} offspring. NG2^{tdTom} mice were also bred to Glt1^{GFP} transgenic mice (Regan et al., 2007) to generate NG2^{tdTom}/Glt1^{GFP} offspring. Aldh111^{GFP} and Glt1^{GFP} mice were kindly donated by Dr. Jeffrey Rothstein at Johns Hopkins University, Baltimore, MD. Plp1-CreER mice (Jackson Laboratory stock 005975 (Doerflinger et al., 2003)) were bred to Rosa26-tdTomato mice to generate Plp1^{tdTom} offspring in which Rosa26-tdTomato is heterozygous. All CreER and GFP transgenics were used as hemizygous. Pdgfra-CreER mice (Jackson Laboratory stock 018280 (Kang et al., 2010)) were bred to Rosa26-tdTomato mice to generate Pdgfra^{tdTom} offspring. All mice were in a C57BL/6 genetic background

(backcrossed at least 10 times). All procedures involving animals were approved by the University of Miami Institutional Animal Care and Use Committee and followed NIH guidelines.

2.2. SCI surgery and EAE induction

For SCI, 6 to 8 week old female mice were injected daily with tamoxifen (MP Biomedicals, 0.124 mg/g body weight, i.p) for 5 consecutive days. One or eight weeks after the last injection, mice were anesthetized (ketamine/xylazine, 100 mg/15 mg/kg i.p.) and received a T8 laminectomy. The spinal column was stabilized using spinal clamps, and then mice received a moderate (75 kDyne) SCI using an Infinite Horizons impactor device (Precision Systems and Instrumentation, LLC). Mice received post-operative treatment of antibiotics (Baytril, 10 mg/kg), and analgesics (buprenorphine, 0.05 mg/kg) diluted into 1 mL of Lactated Ringer's solution injected subcutaneously twice per day for the first week following surgery. Mice received manual bladder expression twice daily until the end of the experiment.

For EAE induction, 2 to 4 month old mice were injected daily with tamoxifen (MP Biomedicals, 0.124 mg/g body weight, i.p) for 5 consecutive days. One week after the last tamoxifen injection, mice received an injection of pertussis toxin (500 ng/mouse; day 0 and 2, i.p.) and an injection of MOG₃₅₋₅₅ peptide (300 ng/mouse; day 1, i.p.) emulsified in Complete Freund Adjuvant. Clinical symptoms were recorded daily starting on day 7 until the end of the experiment. The scoring criteria was based on a commonly used 6 point scale: 0-no apparent symptoms, 1-loss of tail tone, 2-flaccid tail, 3-complete hindlimb paralysis, 4-complete forelimb paralysis, 5-moribund, 6-dead (Brambilla et al., 2011). We also included half-points to more precisely assess the disease progression: 2.5-hindlimb weakness, 3.5-forelimb weakness. Mice were sacrificed 8 days after the onset of symptoms during the acute disease phase. Disease onset was considered the second consecutive day of scoring at least a 2. All mice had reached a clinical score of at least a 3 by the time of sacrifice.

2.3. Histology

Mice were anesthetized and then transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Spinal cords were dissected and postfixed in 4% paraformaldehyde in PBS for 2 h, and cryoprotected in 30% sucrose in PBS overnight. For spinal cord injury tissue, 8 mm segments of the spinal cord centered at the injury site were embedded in OCT compound (Tissue-Tek) and serial sagittal sections (16 μ m) were cut using a cryostat and thaw mounted onto slides. For EAE tissue, 3 mm segments of cervical, thoracic, and lumbar spinal cord were embedded in OCT compound and serial cross sections (16 μ m) were cut using a cryostat and thaw mounted onto slides. Sections were immunostained with primary antibodies in 5% Normal Goat Serum in PBS with 0.3% Triton-X overnight at 4 °C. Primary antibodies used were: RFP (Rockland 600-401-379S, 1:1000), GFAP (Invitrogen 130300, 1:1000; Abcam ab4674, 1:1000), APC/CC1 (Millipore OP80 Ab-7, 1:500), GFP (Abcam ab13970, 1:2000), PDGFR β (Abcam ab32570, 1:500), and p75 (Neuromics GT15057, 1:500). Following primary antibody incubation, sections were washed and incubated in species-appropriate Alexa Fluor IgG (H + L) secondary

antibodies (Invitrogen, 1:500) at room temperature for 1 h. Slides were mounted using Vectashield with DAPI (Vector Laboratories H-1200).

2.4. Histological quantification

For histological quantifications in Figs. 1, 2, and 3, images were first acquired using a 60× objective on an Olympus FluoView 1000 confocal microscope. For spinal cord injury tissue, 6 fields (3 rostral and 3 caudal to the injury site) were taken within the first 500 μm segment of the glial scar borders. For EAE tissue, a single field was taken per lesion, which was identified by increased cell density (DAPI nuclei) and astrogliosis (GFP and GFAP), and at least 7 lesion areas were imaged per animal. All tdTomato⁺/DAPI⁺ cells within the images were counted, excluding those with a tubular pericyte morphology. The results reported for Figs. 1, 2, and 4 are the averaged cell counts of two independent observers. The NG2-CreER mice used in this study did not label macrophages or fibroblasts at the injury site. Since Pdgfra-CreER mice also label fibroblasts at the injury site, PDGFRβ⁺/tdTomato⁺ fibroblasts were excluded from our quantifications. For histological quantifications in Fig. 5, spinal cords from uninjured Plp1^{tdTom} mice were sectioned coronally and images of whole spinal cord were taken using a Nikon Eclipse Ti fluorescent microscope. Subsequently, 225 by 225 μm² boxes were drawn in the dorsal column, the gray matter, and the lateral white matter, and then all tdTomato⁺ cells were quantified within these regions. Virtually all tdTomato⁺ cells were CC1⁺, indicating that they were mature oligodendrocytes (data not shown).

2.5. High Performance Liquid Chromatography (HPLC)

HPLC was performed as previously described with some modifications (Johnson et al., 2013). To generate a standard curve, 6 to 8 week old female mice were anesthetized (ketamine/xylazine, 100 mg/15 mg/kg i.p.), transcardially perfused with cold PBS, the spinal cords were dissected, and 10–15 mg piece was homogenized in 150 μL of normal saline with 25 μL of internal standard (0.1 μg/mL propranolol in methanol) and 25 μL of tamoxifen (MP Biomedicals), or 4-hydroxy-tamoxifen (Sigma, H6278–10MG) or *N*-desmethyl-tamoxifen (Sigma, D9069–5 mg). Samples were moved to a 10 mL glass tube and 7 mL of 2.5% isopropanol in n-hexane was added and then mixed for 5 min at 2000 rpm on a cyclomixer. Samples were then centrifuged for 5 min at 2000g. The organic layer was separated and evaporated to dryness at 40 °F using TurboVap LV (Caliper). The residue was reconstituted in 200 μL of the mobile phase (720 μL Acetonitrile + 480 μL HPLC water with 0.1% formic acid (6:4 acetonitrile:HPLC water with 0.1% formic acid)) and 2 μL was injected into an analytical column. A gradient HPLC method on a reversed phase column (Atlantis dc18 Column, 100 Å, 3 μm, 2.1mm × 150 mm; Atlantis Columns; 186001299) was used on an API 4000 Q-Trap mass spectrometer (Applied Biosystems; Carlsbad, CA, USA) equipped with 2 HPLC pumps and a column oven (Perkin Elmer; Waltham, MA, USA) set at 60 °C. Flow rate was set at 0.9 mL/min. For experimental tissue, 6 to 8 week old female mice (n = 4–5 per time point) were injected with tamoxifen for 5 consecutive days as described above. At 1, 7, or 28 days after the last injection, mice were anesthetized (ketamine/xylazine, 100 mg/15 mg/kg i.p.), transcardially perfused with cold PBS, spinal cords were dissected, and processed for HPLC as described above.

3. Results

3.1. Identification of heterogeneous NG2 glia-derived astrocytes after SCI

Since GFAP immunohistochemistry does not label all astrocytes, we generated NG2^{tdTom}/Aldh111^{GFP} mice as well as NG2^{tdTom}/Glt1^{GFP} mice for a more complete assessment of NG2 glia-derived astrocytes after SCI. NG2^{tdTom} mice label approximately 30% of all NG2 glia (Zhu et al., 2011), whereas virtually all astrocytes are labeled in Aldh111^{GFP} and Glt1^{GFP} mice (Yang et al., 2011). In the uninjured spinal cord of NG2^{tdTom}/Aldh111^{GFP} or NG2^{tdTom}/Glt1^{GFP} mice, we observed no co-localization of tdTomato with GFP (Fig. 1Y–AB; Fig. 2Y–AB), consistent with previous reports that NG2 cells do not give rise to astrocytes in the normal adult CNS (Kang et al., 2010). However, around 18% and 28% of NG2 glia (tdTomato⁺ cells) at 1 and 4 weeks after SCI respectively gave rise to astrocytes that expressed GFAP and/or Aldh111^{GFP} (Fig. 1). The relative proportion of the three types of astrocytes (GFAP⁺, or GFAP⁺/Aldh111^{GFP}+, or Aldh111^{GFP}+) were similar between the two time points. Although most NG2 glia-derived astrocytes expressed GFAP, around 2–3% expressed only Aldh111^{GFP} at both time points. Using NG2^{tdTom}/Glt1^{GFP} mice, we found that only about 5% of NG2 glia (tdTomato⁺ cells) were Glt1^{GFP} astrocytes and 9% were GFAP⁺/Glt1^{GFP} astrocytes at 1 week after SCI (Fig. 2A–L, AC). The percentage of NG2 glia (tdTomato⁺) that were Glt1^{GFP} astrocytes significantly increased to about 20% by 4 weeks after SCI, and the majority of GFAP⁺/tdTomato⁺ astrocytes were also Glt1^{GFP} (Fig. 2M–X, AC). Taken together, our data demonstrate that chronically after SCI, around 25% of NG2 glia differentiate into a heterogeneous population of astrocytes that express GFAP and/or Aldh111 or Glt1.

To test whether the presence of NG2 glia-derived astrocytes after SCI is specific to NG2^{tdTom} mice, we also assessed their presence in Pdgfra^{tdTom} mice. Since perivascular fibroblasts are also genetically labeled in Pdgfra^{tdTom} mice, we excluded tdTomato⁺ cells that were also PDGFRβ⁺ from our quantification. Interestingly, we found that approximately 40% of the NG2 cells (tdTomato⁺ cells) in the glial scar close to the lesion border were GFAP⁺ at 1 week after SCI in Pdgfra^{tdTom} mice (Fig. 3). This percentage is greater than that observed in NG2^{tdTom} mice (15%) at 1 week after injury, demonstrating that the percentage of genetically traced NG2 cells that express astrocyte markers after injury varies between transgenic mouse lines.

3.2. Identification of heterogeneous NG2 glia-derived astrocytes after EAE

To determine potential differences in NG2 glia-derived astrocytes after different CNS injuries, we induced EAE in the NG2^{tdTom}/Glt1^{GFP} mice. During the acute phase of EAE in which animals display the greatest severity of physical symptoms, approximately 9% of the NG2 glia (tdTomato⁺ cells) in white matter lesions differentiated into either GFAP⁺ (4%) or Glt1^{GFP} (5%) astrocytes (Fig. 4). Interestingly, we did not observe any NG2 glia-derived astrocytes that expressed both GFAP and Glt1^{GFP}, unlike what we observed after SCI. Taken together, our data suggest that the amount and type of NG2 glia-derived astrocytes differ between SCI and EAE.

3.3. Consideration of residual tamoxifen in off-target labeling of NG2 glia-derived astrocytes

Reactive astrocytes may upregulate NG2 after SCI which, in the presence of residual tamoxifen, could lead to recombination and expression of tdTomato in astrocytes. To address this potential technical confound, we used HPLC to measure the amount of tamoxifen and its metabolites, 4-hydroxy-tamoxifen (4-OH-Tam) and *N*-Desmethyl-tamoxifen (N-Des-Tam), in the mouse spinal cord at 1, 7, or 28 days after the last tamoxifen injection (Fig. 5A). We observed that the level of tamoxifen was over 350-fold lower at 1 week (17.18 ± 4.52 ng/mL) after the last tamoxifen injection compared to just 1 day (6342 ± 654 ng/mL), and that levels were too low to detect using HPLC by 4 weeks (Fig. 5B).

To determine whether the residual amounts of tamoxifen and its metabolites (4-OH-Tam, N-Des-Tam) present at 1 week after the last tamoxifen injection were sufficient to induce additional recombination, we used Plp1^{tdTom} mice, which express tdTomato in mature oligodendrocytes. We reasoned that since oligodendrocytes are post-mitotic, if we see an increased density of genetically labeled oligodendrocytes (tdTomato⁺ cells) at 2 weeks compared to 1 week after the last tamoxifen injection, then this suggests that the small amount of residual tamoxifen and its metabolites is sufficient to induce recombination (Fig. 5C). We quantified the number of tdTomato⁺ oligodendrocytes in the dorsal column (DC), lateral white matter (LWM) and the gray matter (GM) of spinal cord cross sections (Fig. 5D) and found that there was no significant difference between the density of oligodendrocytes between the two time points (Fig. 5E). Our data suggest that the small amount of tamoxifen metabolites present one week after tamoxifen administration is insufficient to promote significant recombination.

To demonstrate that NG2 glia-derived astrocytes can be observed after SCI even when tamoxifen and its metabolites before injury are below the HPLC lower limit of detection, we performed SCI on NG2^{tdTom}/Aldh111^{GFP} and NG2^{tdTom}/Glt1^{GFP} mice 4–8 weeks after the last tamoxifen injection. We again observed colocalization of tdTomato with GFAP and GFP using this paradigm (images shown in Fig. 1A–L and Fig. 2A–L) thus confirming that the expression of astrocyte markers in NG2-lineage cells is not due to off-target recombination in the presence of residual tamoxifen.

4. Discussion

The primary goal of our study was to address whether NG2 glia indeed become astrocytes after CNS injury. Using NG2 reporter mice (NG2^{tdTom}) crossed to two different astrocyte reporter mice (Aldh111^{GFP} or Glt1^{GFP}), as well as Pdgfra reporter mice (Pdgfra^{tdTom}), we showed that about 25–40% of the NG2 cells in the glial scar express one of the GFP astrocyte markers and/or GFAP by 4 weeks after SCI. This confirms that a substantial proportion of NG2 cells differentiate into astrocytes after contusive SCI, and interestingly, that there is heterogeneity in the NG2 glia-derived astrocyte population after SCI. In contrast, we found that only about 9% of the NG2 glia in EAE lesions become astrocytes by the peak of the disease. Whereas NG2 glia-derived astrocytes after SCI expressed GFAP and/or GFP, astrocytes in EAE lesions expressed only one of the two markers, and we did not detect any cells that expressed both GFAP and GFP. This difference between our SCI

and EAE results suggests that the number and types of NG2 glia-derived astrocytes differ between injury types. Therefore, besides inter-lab variability in quantification methods, the discrepancy between previous findings may be due to the type of CNS injury under investigation.

Most of the astrocytes markers in previous studies were either intermediate filaments or cell surface proteins, which can make cell counting difficult, especially in the complicated interweaving network of reactive astrocytes in the glial scar. We took advantage of the sparse labeling in NG2^{tdTom} mice (around 30% recombination efficiency) combined with cytoplasm-filling properties of GFP in Aldh111^{GFP} and Glt1^{GFP} mice to facilitate cell counting for this study. *Aldh111* encodes the protein aldehyde dehydrogenase 1 family member 11 which is important for folate metabolism and cell division (Krupenko, 2009). The Aldh111^{GFP} mouse line has been shown to specifically label astrocytes in the CNS, and *Aldh111* expression is increased in astrocytes after injury perhaps as a requirement for proliferation (Yang et al., 2011). *Glt1* encodes glutamate transporter-1 which plays a prominent role in extracellular glutamate homeostasis, and Glt1^{GFP} mice are fairly specific in labeling astrocytes (Regan et al., 2007). After SCI, we found that over half of the GFAP⁺/tdTomato⁺ cells were also Aldh111^{GFP+} at both 1 week and 4 weeks. Using the Glt1-GFP mouse however, we observed that approximately 30% of the GFAP⁺/tdTomato⁺ cells colocalized with Glt1^{GFP} at 1 week after SCI. This percentage increased by 4 weeks when the majority (72%) of GFAP⁺/tdTomato⁺ cells were Glt1^{GFP+}. Previous studies have reported that *Glt1* expression is decreased in the majority of astrocytes after contusive SCI in the Glt1^{GFP} mice (Lepore et al., 2011), suggesting that astrocytes downregulate *Glt1* acutely after SCI. To detect even low levels of expression after injury, we used an antibody against GFP. It is possible that the population of GFAP⁺/Glt1^{GFP-} astrocytes observed by Lepore et al. included newly derived astrocytes from NG2 cells that did not highly express *Glt1* until further maturation, thus undetected without antibody labeling as we used in our studies.

The generation of NG2 glia-derived astrocytes in EAE did not mirror our observations after acute or chronic SCI. Fewer NG2 glia-derived astrocytes were observed in EAE lesions compared to SCI, and they were either GFP⁺ or GFAP⁺, not both, pointing to differences in heterogeneity between the two injury types. We showed that approximately 4% of tdTomato⁺ cells expressed GFAP, which is slightly greater than what has been previously reported. Tripathi et al. showed that only 1% of NG2 cells express GFAP after EAE, but this was likely due to the fact that they did not restrict their cell counts to lesion sites (Tripathi et al., 2010). It should be noted that not all EAE lesions contained NG2^{tdTom+} cells likely owing to the low recombination efficiency in this mouse line. The surprising presence of a GFAP⁻/Glt1^{GFP+} population of NG2 cells in EAE suggests that previous studies may have underestimated the lineage plasticity of NG2 glia based on GFAP expression alone. The disparity between SCI and EAE suggests that the alteration of NG2 cell fate depends on the type of injury. It is possible that NG2 cell fate is altered to a greater extent in injuries like contusive SCI where inflammation, secondary damage, and astrogliosis contribute to large chronic injury areas. For example, since contusive SCI leads to greater hemorrhage than EAE, it is possible that factors in blood, such as fibrinogen, may contribute to differences in NG2 glia proliferation and differentiation between these types of injuries (Petersen et al., 2017).

To ensure that the NG2 glia-derived astrocytes we observed after SCI and EAE were not an artifact of residual tamoxifen, we implemented a series of experiments. First, we performed HPLC to determine how long tamoxifen and its metabolites remained in the spinal cord after injection. We found that tamoxifen levels were reduced over 350-fold by 1 week (17.18 ± 4.52 ng/mL) after injection compared to 1 day (6342 ± 654 ng/mL) after injection and levels were undetectable at 4 weeks after injection. The small amount of residual tamoxifen 1 week after injection did not induce further recombination when tested in the Plp1-CreER mouse line suggesting that a 1 week waiting period before injury allows for sufficient reduction of tamoxifen and its metabolites to prevent off-target labeling. To our knowledge, this is the first study to measure tamoxifen metabolite concentrations in the mouse spinal cord, and largely corroborate recent similar study on mouse brain (Valny et al., 2016). In addition, we observed NG2 glia-derived astrocytes even when SCI was performed 4–8 weeks after tamoxifen injection when tamoxifen and its metabolites have reaches undetectable levels.

Since non-myelinating Schwann cells also express GFAP (Jessen et al., 1990; Yang and Wang, 2015) and are observed in the spinal cord after a contusive injury, we wanted to ensure that the NG2 glia-derived astrocytes we observed after injury were not in fact invading Schwann cells labeled by the transgenic reporter mice. There were virtually no $\text{Pdgfra}^{\text{tdTom}+}/\text{GFAP}^+$ cells in the lesion border that were also positive for the Schwann cell marker p75 (Supplementary Fig. 1). The majority of p75^+ cells were observed in the dorsal column and within the GFAP^- fibrotic scar, while only a few were observed in the glial scar borders where we observed the majority of NG2 glia-derived astrocytes. These data indicate that Schwann cells were not included in our quantifications.

To determine if NG2 glia-derived astrocytes are specific to the NG2-CreER mouse line, we used Pdgfra-CreER mice, which also label NG2 glia, and found an even greater percentage of NG2 glia-derived astrocytes after injury. This may in part be explained by a difference in the recombination efficiency between the two mouse lines (30% for the NG2-CreER mouse (Zhu et al., 2008a) and 90% for the Pdgfra-CreER mouse (Kang et al., 2010)) potentially resulting in a dissimilar sampling of a heterogeneous population of NG2 cells. Additionally, pericytes and fibroblasts labeled with tdTomato were excluded by $\text{PDGFR}\beta$ staining in the Pdgfra-CreER mice (but only by morphology in the NG2-CreER mice) which may have resulted in a more accurate quantification of the total number of NG2 glia. Together, the data from both mouse lines demonstrate that indeed, NG2 glia differentiate into astrocytes after contusive SCI. It is not yet clear whether NG2 cell-derived astrocytes are beneficial or detrimental to proper wound healing and regeneration in CNS injury and disease. While astrogliosis has been largely regarded as inhibitory to regeneration in SCI (Silver and Miller, 2004), the astroglial scar may in fact perform some beneficial functions after SCI (Anderson et al., 2016), and astrocytes could promote myelination in EAE (Ishibashi et al., 2006; Ishibashi et al., 2009). The contribution of NG2 glia to the heterogeneity of astrocytes after injury may help explain some of the dichotomous functions attributed to the astroglial scar. For example, an attractive possibility is that the newly generated NG2 glia-derived astrocytes are permissive for axon regeneration, while pre-existing astrocytes that contribute to the glial scar are inhibitory. This possibility remains to be tested in the future.

Overall, we have confirmed previous studies by us and others that NG2 glia do indeed give rise to astrocytes after SCI and EAE. We extend upon these findings by demonstrating that NG2 lineage plasticity is dependent on injury types in terms of both total percentage and heterogeneity of NG2-glia derived astrocytes. Future studies will need to address the biological basis for these differences, including functional significance of the NG2 glia-derived astrocyte heterogeneity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALDH1L1	Aldehyde Dehydrogenase 1 Family Member L1
DAPI	4',6-diamidino-2-phenylindole
EAE	Experimental Autoimmune Encephalomyelitis
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GLT1	Glutamate Transporter 1
HPLC	High Performance Liquid Chromatography
NG2	Neural Glial Antigen 2
OPCs	Oligodendrocyte Progenitor Cells
PDGFRα	Platelet Derived Growth Factor Receptor α
PLP1	proteolipid protein 1
RFP	Red Fluorescent Protein
SCI	Spinal Cord Injury

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Fig. 1.

NG2 cells differentiate into Aldh111^{GFP+} astrocytes after contusive SCI. NG2 lineage cells do not express Aldh111^{GFP} in the uninjured condition (Y-AB). However, a population of NG2^{tdTom+} cells in the glial scar express GFAP and/or Aldh111^{GFP} by 1 week (18%) (A-L, AC) and 4 weeks (28%) (M-X, AC) after SCI. All images are from sagittal spinal cord sections. (E-H) are magnified views of boxed regions in (A-D). (I-L) are magnified views of boxed regions in (E-H). (Q-T) are magnified views of boxed regions in (M-P). (U-X) are magnified views of boxed regions in (Q-T). Scale bars = 500 μ m for (A-D, M-P), 50 μ m for

(E-H, Q-T, Y-AB), and 10 μm for (I-L, U-X). $n = 4\text{--}5$ animals per group. Percentages of each category (GFP⁻/GFAP⁺, GFP⁺/GFAP⁺, GFP⁺/GFAP⁻) were compared between 1 week and 4 weeks using a Student's *t*-test. Error bars = SEM.

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Fig. 2.

NG2 cells differentiate into GLT1^{GFP+} astrocytes after contusive SCI. NG2 lineage cells do not express GLT1^{GFP} in the uninjured condition (Y-AB). A population of NG2^{tdTom+} cells in the glial scar express GFAP and/or GLT1^{GFP} by 1 week (14%) (A-L, AC) and 4 weeks (25%) (M-X, AC) after SCI. All images are from sagittal spinal cord sections. (E-H) are magnified views of boxed regions in (A-D). (I-L) are magnified views of boxed regions in (E-H). (Q-T) are magnified views of boxed regions in (M-P). (U-X) are magnified views of boxed regions in (Q-T). Scale bars = 500 μ m for (A-D, M-P), 50 μ m for (E-H, Q-T, Y-AB), and 10 μ m for (I-L, U-X). n = 4–5 animals per group. Percentages of each category (GFP⁻/GFAP⁺-, GFP⁺/

GFAP⁺, GFP⁺/GFAP⁻) were compared between 1 week and 4 weeks using a Student's t-test. Error bars = SEM. * $p < 0.05$.

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Fig. 3.

NG2 glia-derived astrocytes are also present in $Pdgfra^{tdTom}$ mice after SCI. A large population (approximately 40%) of $Pdgfra^{tdTom+}$ cells (B, E, H) in the glial scar close to the lesion border express GFAP (C, F, I) at 1 week after SCI. Colocalization of tdTomato (red) with GFAP (white) is indicated by blue arrows. Scale bars = 500 μ m (A-C), 50 μ m (D-F), and 10 μ m (G-I). $n = 3$ animals. Error bar = SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

NG2 cells differentiate into Glt1^{GFP+} astrocytes after EAE. A cross section of cervical spinal cord shows a lesion in the dorsal white matter indicated by increased cell density and astrogliosis. A population of NG2^{tdTom+} cells in EAE white matter lesions express Glt1^{GFP}. Approximately 4% of NG2^{tdTom+} cells were GFAP⁺, and 5% of NG2^{tdTom+} cells were Glt1^{GFP+} but GFAP⁻ (M). (E-H) are magnified views of boxed regions in (A-D). (I-L) are magnified views of boxed regions in (E-H). Scale bars = 250 μ m (A-D), 50 μ m (E-H), and 10 μ m (I-L). n = 4 animals. Error bars = SEM.

Fig. 5. NG2 cell-derived astrocytes are not an artifact of residual tamoxifen. (A) Experimental timeline for HPLC experiments. (B) Concentrations (ng/mL) of tamoxifen (Tam) and its metabolites: 4-hydroxyl-tamoxifen (4-OH-Tam), and *N*-Desmethyl-tamoxifen (N-Des-Tam), detected in the spinal cords of mice by HPLC at 1, 7, and 28 days after the last tamoxifen injection. LLD: lower limit of detection. *n* = 4–5 mice per time point. ± SEM (C) Experimental timeline for testing for further recombination beyond 1 week after last tamoxifen injection (D-E). (D) Diagram showing Plp1^{tdTom} labeled oligodendrocytes and the areas used for quantification: dorsal column (DC), gray matter (GM) and lateral white matter (LWM). (E) Graph showing the number of plp1^{tdTom} oligodendrocytes at 1 week or 2 weeks after the last tamoxifen injection. *n* = 8 per time point. Two-way ANOVA with Tukey's multiple comparisons *post hoc* test. Error bars = SEM. Scale bar = 500 μm in (D).