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Transient *Cnp* Expression by Early Progenitors Causes Cre-Lox-based Reporter Lines to Map Profoundly Different Fates

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Abstract

NG2 expressing oligodendroglial precursor cells are ubiquitous in the central nervous system and the only cell type cycling throughout life. Previous fate mapping studies have remained inconsistent regarding the question whether NG2 cells are capable of generating certain types of neurons. Here, we use CNP-Cre mice to map the fate of a sub-population of NG2 cells assumed to be close to differentiation. When crossing these mice with the ROSA26/YFP Cre-reporter line we discovered large numbers of reporter-expressing pyramidal neurons in the piriform and dorsal cortex. In contrast, when using Z/EG reporter mice to track the fate of *Cnp*-expressing NG2 cells only oligodendroglial cells were found reporter positive. Using BrdU-based birth dating protocols and inducible NG2CreER:ROSA26/YFP mice we show that YFP positive neurons are generated from radial glial cells and that these radial glial cells display temporary and low level activity of certain oligodendroglial genes sufficient to recombine the Cre-inducible reporter gene in ROSA26/YFP but not in Z/EG mice. Taken together, we did not obtain evidence for generation of neurons from NG2 cells. Our results suggest that with an appropriate reporter system *Cnp* activity can be used to define a proliferative subpopulation of NG2 cells committed to generate oligodendrocytes. However, the strikingly different results obtained from ROSA26/YFP versus Z/EG mice demonstrate that the choice of Cre-reporter line can be of crucial importance for fate mapping studies and other applications of the Cre-lox technology.

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Keywords

NG2 cells; fate mapping; transient Cre expression; *Cnp*, radial glial cells

Introduction

NG2 glial cells (NG2 cells), used synonymously for oligodendroglial precursor cells (OPCs) in this text, are ubiquitous throughout the central nervous system including white and gray matter and represent ~5% of total brain cell count (Dawson et al., 2003). NG2 cells have received much attention in the past as they not only generate all oligodendrocytes but are also the major cycling population of cells in the healthy postnatal and adult CNS (Dawson et al., 2003; Robins et al., 2013). In addition, NG2 cells show a high degree of lineage plasticity and can be instructed to generate astrocytes and neurons under appropriate conditions *in vitro* (Kondo and Raff, 2000; Raff et al., 1983; Rao and Mayer-Proschel, 1997).

From previous studies we know that the lineage plasticity of NG2 cells *in vivo* is much more restricted compared to cultured OPCs. Genetic fate mapping studies have consistently shown that the vast majority of cells generated by postnatal NG2 cells in the forebrain are oligodendrocytes and NG2 cells (Dimou et al., 2008; Huang et al., 2014; Kang et al., 2010; Rivers et al., 2008; Zhu et al., 2011). In addition, embryonic NG2 cells seem to be capable of generating larger numbers of astrocytes in parts of the ventral forebrain (Huang et al., 2014; Zhu et al., 2011). It is further established that NG2 cells do not generate a substantial number of neurons but there are several reports that small but distinct subpopulations of neurons may be derived from postnatal and/or adult OPCs (Guo et al., 2010; Rivers et al., 2008; Robins et al., 2013). Even if the number of potentially NG2 cell-derived neurons may be small compared to the total population of neurons it is still important to identify the neurogenic potential of NG2 cells *in vivo* as they could potentially be used as a ubiquitous pool of resident precursors for replacement and regeneration if it were known how to activate and recruit these cells into a neuronal lineage. Unfortunately, the fate mapping studies published so far provide conflicting evidence regarding regional production of neurons from postnatal or embryonic NG2 cells (also expressing PDGFalphaR) (Clarke et al., 2012; Dimou et al., 2008; Guo et al., 2009, 2010; Huang et al., 2014; Kang et al., 2010; Rivers et al., 2008; Robins et al., 2013; Tsoa et al., 2014; Zhu et al., 2008, 2011). It is difficult to reconcile all of these studies as they differ in many aspects: for example, the promoters used to drive expression of Cre in NG2 cells, the usage of different Cre-reporter mice, the time and protocol of induction of Cre-expression, the success rate of birth-dating newly generated neurons and the degree of specificity of the initial reporter expression in OPCs.

Most of the previous studies applied tamoxifen-inducible expression of Cre, which elegantly allows following up the fate of cells after a specific time point defined by tamoxifen administration. In an attempt to ideally map the fate of the entire population of OPCs following tamoxifen injection, Cre-driving promoters, which were known to be expressed early in the life of an OPC, in “young” OPCs, and very sensitive reporter mouse lines were

chosen. Potential pitfalls of this approach are that such early promoters may also be transiently or weakly expressed by different lineages and thereby may readily be picked up by very sensitive reporter mouse lines. Further, because the development of oligodendroglial cells proceeds asynchronously through the brain, activation of an early promoter will unavoidably label NG2 cells in quite distinct stages of their cell cycle and development in different regions of the brain.

Taken together, present studies on neurogenesis by NG2 cells are still inconclusive regarding the question whether there may be a certain stage in the life of an OPC after which the cell is committed to an oligodendroglial lineage and how such a stage could be identified. Furthermore, it is unclear, how the differences in recombination efficiency between Cre reporter strains affect the outcome of fate mapping studies and what the confounding effect of low-level oligodendroglial promoter activity in non-oligodendroglial cells is.

To shed light on these questions we crossbred a mouse line (CNP-Cre) in which Cre is knocked-into the 2',3' -cyclic nucleotide 3' -phosphodiesterase (CNPase) gene, *Cnp* (Lappe-Siefke et al., 2003), with two Cre-reporter strains with assumed different recombination efficiencies. As CNPase protein in the CNS is only found in mature myelinating oligodendrocytes (Trapp et al., 1988) we reasoned that only a subpopulation of OPCs being close to differentiation into oligodendrocytes would express *Cnp*.

We employ these CNP-Cre mice and mice allowing for conditional and tamoxifen-inducible expression of Cre-recombinase under the NG2 promoter (NG2creERTMBAC) (Zhu et al., 2011) to substantiate our conclusions in combination with efficiently and weakly recombining Cre-reporter mice and BrdU-based birth dating protocols. Our results show that the *Cnp* promoter can identify a “late” subpopulation of OPCs which are exclusively committed to oligodendrogenesis and self-renewal. We further demonstrate that radial glial cells, and possibly other progenitors, transiently activate oligodendroglial genes during the embryonic period and that the outcome of fate mapping studies can be dramatically affected by the efficiency of the Cre-reporter mouse line employed.

Materials and Methods

Mice

CNP-Cre mice expressing Cre recombinase under control of the endogenous *Cnp* regulatory sequences (Lappe-Siefke et al., 2003) were obtained from the Max Planck Institute of Experimental Medicine (Göttingen) and maintained on a C57BL/6 background. Z/EG (Novak et al., 2000) (JAX stock 3920, Tg(CAG-Bgeo/GFP)21Lbe/J), and ROSA26/YFP (Srinivas et al., 2001) (JAX stock 6148, B6.129X1-*Gt(ROSA)26Sor^{tm1(EYFP)Cos}/J*) Cre reporter mice were obtained from The Jackson Laboratory. CNP-Cre^{+/+} and CNP-Cre^{+/-} mice were interbred with Z/EG and ROSA26/YFP reporter lines (both homozygous and/or heterozygous), generating CNP-Cre:Z/EG and CNP-Cre:ROSA26/YFP, respectively. To verify Cre dependence of reporter protein expression, P10 CNP-Cre^{wt/wt}:ROSA26/YFP mice were examined by GFP immuno-labelling. No YFP⁺ cells were seen throughout gray and white matter regions in frontal forebrain sections.

NG2creERTMBAC transgenic mice (Zhu et al., 2011) were obtained from University of Connecticut and maintained as homozygotes against a C57BL/6 background. To generate NG2creERTM-BAC:ROSA26/YFP mice they were inter-bred with the ROSA26/YFP Cre reporter line. To check for any “leaky Cre expression,” P9 NG2creERTMBAC:ROSA26/YFP mice were administered 50 μ L corn oil or NaCl (2 intraperitoneal injections per day for 3 days). No YFP⁺ cells were observed in these control animals. In this study both double heterozygotes and double homozygotes were used. Because no differences were observed between male and female mice, data from both sexes has been pooled.

Induction of Cre-mediated Recombination with Tamoxifen

Cre activity was induced in embryonic NG2creERTMBAC:ROSA26/YFP transgenic mice by administering 6 mg of tamoxifen (TMX) to pregnant dams at E16.5 via oral gavage. TMX (Sigma) was dissolved in corn oil preheated to 35°C, at a concentration of 20 mg mL⁻¹, mixed with 5 mg mL⁻¹ Progesterone by repetitive sonications at the same temperature. In postnatal NG2creERTMBAC:ROSA26/YFP mice, 0.5 mg TMX was administered at P6 at a concentration of 10 mg mL⁻¹ (single intraperitoneal injection).

BrdU Labelling

P10 CNP-Cre:ROSA26/YFP mice semi daily received intraperitoneal BrdU injections for 3 consecutive days (100 mg kg⁻¹ BrdU, Sigma, dissolved at 16.2 mg mL⁻¹ in 0.9% NaCl). These mice were sacrificed and analyzed at P30. Pregnant dams of the CNP-Cre:ROSA26/YFP line at E12.5 received two applications of BrdU via oral gavage at a dosage of 50 mg kg⁻¹ (Sigma, dissolved at 16.2 mg mL⁻¹ in 0.9% NaCl, 4 h apart). Litters were analyzed at P32.

Tissue Processing

Brains of postnatal and embryonic animals were dissected in ice-cold saline of the following composition (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 7 MgSO₄, 0.5 CaCl₂, 25 NaHCO₃, 25 glucose, 75 sucrose, gassed with 95% O₂ and 5% CO₂ mixture. 400 μ m frontal sections were prepared on a vibratome (Leica VT1200 S). Subsequently, the 400 μ m slices were kept at 35°C for 25 min. The slices were allowed to cool in the same solution to room temperature before being fixed overnight in 8% paraformaldehyde (PFA) (in PBS; pH 7.44). Following this the tissue was re-sectioned to 50 μ m. Slices were pre-embedded in agar to facilitate re-sectioning.

Immunohistochemistry

50 μ m free-floating sections were incubated with primary antibodies in TBS containing 0.2% Triton X-100 overnight at 4°C. Secondary antibodies and detection were each applied for 3 h at 35°C to sections incubated in TBS containing 0.2% Triton X-100. Secondary detection was carried out using biotinylated secondary antibodies, followed by incubation with streptavidin-conjugates or by dyeconjugated secondary antibodies. HCl pretreatment (2N HCl for 30 min) was applied for BrdU-GFP double immunohistochemistry, and the GFP and BrdU labeling steps were performed sequentially. In this protocol only 0.06%

Triton X-100 was used at each step. For the CC1-GFP double immunolabelling, 2% normal horse serum was applied in the primary antibody reaction.

Dilutions of primary antibodies and their sources are as follows: rabbit anti-NG2 (1:500, Chemicon), rabbit anti-GPR17 (1:1000, kindly provided by Dr. Patrizia Rosa, CNR, Milano, Italy (Vigano et al. 2016), rabbit anti-GFAP (1:500, DakoCytomation), mouse anti-NeuN (1:250, Chemicon), rabbit anti-BLBP (1:250, Abcam), mouse anti-APC (CC1 clone) (1:400, Chemicon), rat anti-BrdU (1:71.4, AbD Serotec), rabbit and chicken anti-GFP (1:200, Invitrogen).

Dilutions of secondary antibodies and their sources are as follows: 1:200 for Streptavidin-cy2, -cy3, -cy5, -Rhodamine Red-X, - DyLight 488 (Jackson ImmunoResearch). Dye conjugated and biotinylated secondary antibodies were used in a concentration of 1:200: goat anti-mouse Alexa 555 and goat anti-rabbit Alexa 488 (Invitrogen), donkey anti-chicken biotin and highly cross-adsorbed anti-rat biotin (Jackson ImmunoResearch), sheep anti-mouse biotin (Serotec) and goat anti-rabbit biotin (Vector). 1:100 of Nissl deep red counterstain (Invitrogen).

All sections were mounted with Vectashield (Vector Laboratories).

Image Acquisition

Sections were scanned with a confocal laser scanning microscope (Leica TCS NT, equipped with an Argon-Krypton laser) and scans of different dyes (e.g., DyLight 488, RRX and fluorescent Nissl Cy5) were acquired sequentially. For DyLight 488 or Cy2, the following laser lines and filters were used: excitation 488 nm and emission band pass 530/30 nm. For RRX or Cy3 we used excitation 568 nm and band pass 600/30 nm. For Cy5 we used: excitation 647 nm and emission long pass 665 nm. Scans were acquired with a 63× objective (water; NA 1.2, correction ring, Leica) and the pinhole was set to 1 Airy unit. Laser power, detector gain and offset were adjusted such that in the final scan (4–10 averages) only a few pixels displayed zero or maximal (255) digital units. Typically, detector gain and offset were set to ~60–70% and ~6 digital units, respectively. Images were post-processed in Photoshop: maximum brightness was rescaled to ~80% and a gamma correction factor of ~1–1.2 was introduced to increase visibility of details at the small scale used for figures.

Identification of YFP+ Neurons

In BrdU labeling experiments, reporter positive neurons were identified on the basis of morphology, as having a large round to oval cell body and a prominent, apical main dendrite. NeuN staining could not be used to ascertain the neuronal phenotype, since NeuN immunohistochemistry did in our hands not work with the HCl pretreatment applied for BrdU labelling. To substantiate that neurons were identified correctly based on their morphology, analogous scans were performed on NeuN-GFP labelled sections. Almost all cells identified only by morphology as neurons proved to be NeuN+: 0.99 ± 0.004 (total of 208 YFP+ neurons, $n = 3$ mice), 0.97 ± 0.004 (total of 112 YFP+ neurons, $n = 3$ mice) and 0.98 ± 0.01 (total of 77 YFP+ neurons, $n = 3$ mice), in the dorsal, piriform and ventral cortices.

Cell Count and Quantification

To assess the phenotype of reporter positive cells, frontal brain sections at postnatal ages (−1.64 mm from Bregma) and embryonic ages (0–1 mm from Bregma) were examined. Cell count analysis were performed randomly within the indicated gray and white matter regions (Fig. 1A), and all analysis included three to four animals for each time point, with two to three brain sections per animal. Each confocal scan volume under analysis was $9.763 \times 10^5 \mu\text{m}^3$. Image analysis processing software LSM 5 Image browser (Carl Zeiss) was used for cell identification and counts. The “Fractions of GFP or YFP cells that were positive for a cell specific antigen” were calculated as follows: for each region, this fraction was calculated separately for each confocal stack, the fractions from different stacks were then averaged to obtain a value which represents the mean fraction for that region for each animal. Final fractions for each region were calculated by averaging values across the number of animals.

Results

To study whether *in vivo* there is a certain differentiation stage of NG2+ oligodendroglial precursor cells during which these cells are committed to an oligodendroglial fate and have lost lineage plasticity we followed the fate of cells expressing recombinase in CNP-Cre knock-in mice (Lappe-Siefke et al., 2003). This mouse line was chosen since in the CNS the CNPase protein is found only in mature myelinating oligodendrocytes (Trapp et al., 1988) and we therefore expected that only those NG2+ OPCs would express Cre which are just prior to transforming into pre-myelinating oligodendrocytes and have already started to transcribe some oligodendrocyte-specific genes. To assess Cre expression we generated two mouse lines by crossing CNP-Cre mice with two different Cre-reporter mice, Z/EG (GFP) and ROSA26/YFP, yielding CNP-Cre:Z/EG and CNP-Cre:ROSA26/YFP mouse lines, respectively (see Methods for details). For analysis we chose hippocampal CA1 stratum radiatum, dorsal cortex, ventral cortex, and piriform cortex as representative gray matter regions, and corpus callosum and fimbria as partially and strongly myelinated white matter tracts, respectively (Fig. 1A).

A Fraction of NG2+ OPCs in CNP-Cre:Z/EG and CNP-Cre:ROSA26/YFP Mice Was Reporter Positive

In CNP-Cre:Z/EG mice at postnatal day (P6), the proportion of NG2 cells that were GFP+ in the gray matter regions ranged between 0.2 and 0.35 (Fig. 1B,C, dorsal cortex: 0.22 ± 0.02 , $n = 4$ mice; CA1: 0.2 ± 0.03 , $n = 4$ mice; ventral cortex: 0.35 ± 0.03 , $n = 3$ mice). In the white matter regions only a fraction of ~0.13 of NG2 cells expressed GFP (Fig. 1B,E, corpus callosum: 0.15 ± 0.06 ; fimbria: 0.11 ± 0.06 , $n = 4$ mice for both regions).

In CNP-Cre:ROSA26/YFP mice at the same age (P6), at least two-fold higher fraction of NG2 cells expressed the fluorescent reporter protein YFP throughout all analyzed regions (Fig. 1B,D, dorsal cortex: 0.64 ± 0.09 ; CA1: 0.48 ± 0.06 ; ventral cortex: 0.79 ± 0.03 , $n = 4$ mice) with the difference to CNP-Cre:Z/EG mice being most pronounced in white matter (Fig. 1B,F, corpus callosum: 0.72 ± 0.07 ; fimbria: 0.74 ± 0.05 , $n = 4$ mice).

As expected, in both mouse lines and in all regions studied there is a considerable fraction of NG2 cells, presumably “younger” OPCs, that did not display any fluorescent reporter protein (between 0.26 and 0.89 of NG2 cells, Fig. 1B). To test whether reporter-expressing NG2 cells are ready to differentiate, we co-stained slices with GPR17 a marker of this transitional stage which has lost proliferative activity (Fumagalli et al., 2011). Indeed, we found that GFP positive NG2 cells significantly more frequently co-expressed GPR17 when compared to GFP negative NG2 cells (0.22 ± 0.08 vs. 0.06 ± 0.01 , $n = 4$ mice, KS-test, Fig. 1G,H), consistent with our view that reporter positive NG2 cells represent more mature or “late” OPCs.

In CNP-Cre:Z/EG but Not in CNP-Cre:ROSA26/YFP Mice, Reporter Positive Cells Are Identified by Oligodendroglial Markers

As oligodendrocytes express CNPase, it can be expected that they produce the reporter protein themselves thereby constituting the fraction of reporter positive cells which is not (anymore) NG2 positive. We identified mature oligodendrocytes with CC1 antibodies directed against the adenomatosis polyposis coli antigen (APC) (Bhat et al., 1996). By the end of the 4th postnatal week (P30) myelination in most areas of the forebrain has progressed very far and numerous oligodendrocytes have been generated from NG2 cells (Kang et al., 2010; Sturrock, 1980). Indeed, in the dorsal cortex of CNP-Cre:Z/EG mice the fraction of NG2+ cells decreases from P10 to P30 from 0.74 ± 0.09 ($n = 4$ mice) to 0.3 ± 0.1 ($n = 4$ mice, Fig. 2A,B), and the fraction of CC1+ cells among GFP+ cells reaches 0.73 ± 0.01 in the dorsal cortex at P30 ($n = 4$ mice, Fig. 2A). As myelination starts much earlier in white matter than in the above lying grey matter, in corpus callosum already at P10 only a small fraction of GFP+ cells is still labelled with NG2 antibodies and at P30 almost all GFP+ cells are also CC1+ (Fig. 2A,C). Nevertheless, at P30 in both regions, dorsal cortex and corpus callosum the sum of the fractions of NG2+ cells and CC1+ cells is close to unity implying that in CNP-Cre:Z/EG mice the vast majority of reporter positive cells belongs to the oligodendroglial lineage and represents OPCs and oligodendrocytes (Fig. 2A).

When compared to CNP-Cre:Z/EG mice, CNP-Cre:ROSA26/YFP mice had similar and slightly higher fractions of YFP+ cells colabeled with NG2 antibodies in gray (dorsal cortex: 0.66 ± 0.13 at P10, 0.31 ± 0.03 at P30, Fig. 2D,E, $n = 4$ mice for each) and white matter (corpus callosum: 0.28 ± 0.05 at P10, 0.07 ± 0.02 at P30, $n = 4$ mice for each), respectively, but these fractions likewise underwent a developmental decrease in favor of the fraction of CC1+ YFP+ oligodendrocytes (dorsal cortex: 0.24 ± 0.02 , corpus callosum: 0.87 ± 0.01 , both at P30, Fig. 2D,F, $n = 4$ mice). However, in striking contrast to the CNP-Cre:Z/EG line, the fractions of NG2+ and CC1+ cells did not add up to near unity in the dorsal cortex of CNP-Cre:ROSA26/YFP mice (Fig. 2D,F). At P30 a fraction of ~0.45 of YFP+ cells in the dorsal cortex did neither label with NG2 nor CC1 antibodies (Fig. 2D).

Reporter Positive Cells in Ventral Cortex of CNP-Cre:ROSA26/YFP Mice Express Astroglial Marker

OPCs in culture and *in vivo* have been shown to be at least bipotential and able to also generate astrocytes (Huang et al., 2014; Raff et al., 1983; Rao and Mayer-Proschel, 1997; Zhu et al., 2011). To test whether the fraction of reporter positive cells being NG2- and CC1-

in CNP-Cre:ROSA26/YFP mice represent astrocytes, possibly derived from OPCs, we stained P30 brains for glial fibrillary acidic protein (GFAP) which is selectively expressed by mature astrocytes (Bignami et al., 1972; Kalman and Hajos, 1989). We did not observe any YFP+ or GFP+ astrocytes in dorsal cortex or corpus callosum in either CNP-Cre:ROSA26/YFP or CNP-Cre:Z/EG mice (Fig. 3A,B,E). However, we discovered a notable population of YFP expressing cells showing the highly ramified bushy arbor of processes characteristic of protoplasmic astrocytes (Bushong et al., 2004) specifically in the ventral cortex. These cells very frequently expressed GFAP (Fig. 3D) and GFAP+ cells represented 0.12 ± 0.02 of all YFP+ cells in the ventral cortex (Fig. 3E, $n = 4$ mice). Moreover, these cells even made up a significant fraction of all GFAP+ astrocytes in ventral cortex (0.13 ± 0.01 , total of 155 GFAP+ astrocytes, $n = 4$ mice). In contrast, the ventral cortex of CNP-Cre:Z/EG mice did not show GFAP+ GFP+ cells or GFP1 cells exhibiting an astrocyte-like morphology (except for 1 individual GFP+ GFAP+ cell, $n = 4$ mice). Similarly, we did not find GFP+ astrocytes in corpus callosum and dorsal cortex of CNP-Cre:Z/EG mice (total of 688 GFP+ cells analyzed, $n = 4$ mice, Fig. 3E). Taken together, these results indicate that astrocytes do not account for the substantial fraction of non-oligodendroglial cells (YFP+, NG2-, CC1-) in the dorsal cortex of CNP-Cre:ROSA26/YFP mice (cf. Fig. 2D) but they rather point at a qualitative difference in lineage tracking between the two Cre-reporter mouse lines.

Many Cortical Reporter Positive Cells in CNP-Cre:ROSA26/YFP Mice Represent Neurons

Because there is conflicting evidence regarding whether postnatal NG2 cells may generate cortical neurons we tested if a subpopulation of YFP expressing cells in the dorsal cortex of P30 CNP-Cre:ROSA26/YFP mice stained with NeuN, a nuclear marker of mature neurons. Indeed, we found numerous YFP+ NeuN+ double positive cells in particular in layer 2 of the dorsal cortex (Fig. 4A). A fraction of 0.55 ± 0.07 of YFP+ cells also displayed NeuN immunoreactivity (Fig. 4, total of 607 YFP1 cells, $n = 4$ mice) suggesting that the fraction of YFP+ cells, which neither expressed NG2 nor CC1 (cf., Fig. 2D, DCtx, P30) consists of neurons. Furthermore, these cells also represented a substantial fraction of the neuronal population: 0.23 ± 0.05 of layer II dorsal cortex neurons (NeuN+ cells, Fig. 4B) expressed the oligodendroglial promoter driven reporter protein YFP (total of 1462 neurons, $n = 4$ mice). Moreover, further populations of YFP+ NeuN+ cells were found in ventral and piriform cortex (Fig. 4C,D,F). In striking contrast, we did not observe GFP expressing neurons in P30 CNP-Cre:Z/EG mice (dorsal cortex, ventral cortex and piriform cortex, $n = 4$ mice, Fig. 4E). Taken together, the fraction of YFP+ cells in dorsal cortex which did not label with NG2 or CC1 and which was exclusively observed in CNP-Cre:ROSA26/YFP mice (cf., Fig. 2) can be classified as neurons triggering the question about the origin of these cells.

Reporter Positive Cortical Neurons in CNP-Cre:ROSA26/YFP are Generated during the Embryonic Period

To explore whether YFP+ neurons are conventionally generated from embryonic precursors or may be derived from postnatal YFP-expressing NG2 cells in CNP-Cre:ROSA26/YFP mice we birth-dated YFP1 neurons with BrdU application experiments. In dorsal cortex the clear and typical arrangement of YFP1 neurons in layer II (cf., Fig. 4A) suggests that these

cells were generated during the classical embryonic wave of neurogenesis, around E16 (Bayer and Altman, 1990). We therefore first designed the birth-dating experiments for YFP + neurons in the ventral cortex, as their spatial arrangement in this region does not provide a clue for their generation (cf., Fig 4C). Anatomically, most YFP+ neurons we found in the ventral cortex belong to the anterior amygdaloid area and the cortical amygdaloid nuclei. Neurons in these regions as well as neurons in the anterior piriform cortex are generated around E12–14 (Bayer, 1980, 1986; Sarma et al., 2011). As expected, if YFP+ neurons in these regions were generated embryonically, application of BrdU at E12.5 (Fig. 5A) resulted in a substantial fraction of these large pyramidal-shaped neurons to be labelled by anti-BrdU antibodies later at P30 (Fig. 5B,C). The percentages of YFP+ neurons which have accumulated BrdU is high (~ 0.6 – 0.8 , $n = 6$ mice, Fig. 5G) and given the fact that our single BrdU application cannot label all cells generated throughout the relevant period, these fractions are consistent with the view that the YFP labelled neurons are born embryonically around E12–14.

We performed an additional birth-dating experiment to directly test whether postnatal NG2 cells may contribute to the population of YFP+ cells observed at P30. BrdU was applied for 3 days around P10, a time point at which in CNS largely NG2 cells are known to actively proliferate (Bu et al., 2004; Clarke et al., 2012; Kukley et al., 2008). As previously reported by other investigators (Clarke et al., 2012), we found that at P11, after the 3-day BrdU injection a large fraction of NG2 cells had taken up BrdU (0.758 ± 0.036 , 0.757 ± 0.037 , and 0.67 ± 0.1 in the dorsal, ventral and piriform cortex, respectively, total of 149, 130, and 102 NG2 cells, respectively, $n = 2$ mice, not shown). If these cells generated some of the YFP+ neurons one would expect to see some YFP+ BrdU+ neurons. However, after administration of BrdU from P10 to P12 to (Fig. 5D) CNP-Cre:ROSA26/YFP mice revealed no YFP+ BrdU+ neurons when assessed with an immunohistochemical analysis at P32 (Fig. 5E,F,G, $n = 4$ mice for every regions). Taken together, our data make it very unlikely that reporter positive neurons exclusively observed in CNP-Cre:ROSA26/YFP mice are derived from postnatal NG2 cells and rather suggest that they are generated at the well known neurogenic time points during embryonic development.

Reporter Protein is Found in Radial Glial Cells of CNP-Cre:ROSA26/YFP

In search of the time point of Cre-mediated recombination in these YFP+ neurons or their precursors, we next asked whether they might already express the Cre reporter protein soon after their generation in the embryonic brain. Indeed, we found numerous YFP expressing cells exhibiting a neuronal morphology at E16 in the developing dorsal cortex of CNP-Cre:ROSA26/YFP mice (Fig. 6A, arrow heads). A double labelling for NG2 and YFP revealed that only a small fraction of 0.14 ± 0.03 of YFP+ cells ($n = 3$ mice) in this region expressed the oligodendroglial precursor marker NG2 (Fig. 6A, arrows), indicating that the vast majority of YFP+ cells, neurons and other cells, do not represent oligodendroglial cells. Furthermore, we found numerous YFP+ cells lined up at the ventricular wall (Fig. 6B). These cells displayed a polygonal or spindle-shaped cell body frequently in close proximity to the ventricular wall, and a solitary process extending towards the pial surface, properties reminiscent of radial glial cells (Malatesta et al., 2008). In fact, there was a substantial fraction of such YFP+ cells in the ventricular zone which co-expressed the radial glial

marker BLBP (Kurtz et al., 1994) (Fig. 6B, 0.28 ± 0.07 , $n = 3$ mice). In striking contrast to these observations in CNP-Cre:ROSA26/YFP mice, we did not detect GFP expressing cells in the developing cortex or the ventricular zone in the CNP-Cre:Z/EG line ($n = 4$ mice, Fig. 6C).

So far, our data suggest that in radial glial cells and basal progenitors (Malatesta et al., 2008) of CNP-Cre:ROSA26/YFP mice activation of the YFP reporter gene by Cre mediated genomic recombination has occurred during the embryonic period and that this genotype has been transferred to their neuronal progeny in the postnatal cortex.

Embryonic Induction of NG2creERTMBAC:ROSA26/ YFP Mice Also Yields Reporter Positive Radial Glial Cells and Neurons in the Postnatal Cortex

To check whether neuronal precursor cells also show activity of other oligodendroglial lineage cell promoters and to have temporal control of Cre expression we generated NG2creERTMBAC:ROSA26/YFP double transgenic mice which enabled us to temporally control Cre expression under the NG2 promoter by application of tamoxifen (Zhu et al., 2011). Subsequent to tamoxifen injection and Cre induction in NG2creERTMBAC:ROSA26/YFP mice at E16.5 we also observed numerous YFP expressing cells in the ventricular zone 3 days later at E19.5 (Fig. 7A,B). These cells rarely expressed NG2 (0.07 ± 0.04 , total of 283 YFP⁺ cells, $n = 3$ mice, Fig. 7A) and displayed a radial glial cell-like morphology and 0.16 ± 0.02 co-labeled with BLBP (total of 213 YFP⁺ cells, $n = 3$ mice, Fig. 7B). This finding suggests that radial glial cells indeed showed transient NG2 promoter activity, which resulted in the expression of enough Cre protein to sufficiently recombine the Cre reporter locus but not leading to detectable levels of the NG2 proteoglycan protein. Furthermore, already at this stage we observed YFP⁺ cells in the early dorsal cortex with a typical neuronal morphology (Fig. 7C, large cell bodies, arrowheads) in between numerous NG2⁺ oligodendroglial precursor cells (Fig. 7C, arrows).

To see whether these cells, expressing YFP during the embryonic period, also give rise to postnatal neurons as observed in CNP-Cre:ROSA26/YFP mice we induced Cre expression in another cohort of mice with tamoxifen at E16 and analyzed them at P30. Indeed, we detected numerous YFP positive neurons in dorsal, ventral and piriform cortices (Fig. 7D–F). The fractions of YFP positive cells labelled with NeuN were 0.74 ± 0.04 , 0.42 ± 0.1 , and 0.54 ± 0.04 for the three regions, respectively (total of 458, 264, 265 YFP⁺ cells, $n = 3$ mice).

On the other hand, analysis of animals administered tamoxifen at P6 did not reveal any YFP⁺ cells with neuronal morphology in adult NG2creERTMBAC:ROSA26/YFP mice (around P40, not shown), as reported previously (Zhu et al., 2011) reinforcing that postnatal NG2 cells do not generate neurons (also see Tsoa et al., 2014).

Discussion

In this study we use BrdU-based birth dating protocols, two different Cre-reporter mouse strains, CNP-Cre and inducible NG2CreER mice to show that *Cnp* activity in combination with a suitable reporter mouse can be used to define throughout the embryonic and postnatal

period a lineage-restricted subpopulation of NG2 cells, which we call late OPCs. This is in contrast to previous studies which report the generation of neurons when mapping the fate of postnatal OPCs defined by the activity of the *P1p*-promoter regulating another myelin component (Guo et al., 2009, 2010). In a highly sensitive reporter mouse line we also observed reporter positive neurons in several cortical regions. However, we could show that those neurons are generated embryonically from radial glial cells which we suggest to temporarily and at low-level express certain oligodendroglial genes.

We define “late OPCs” by the expression of GFP (not YFP) and NG2. “Late” refers to the stage of the OPC, close to differentiation into oligodendrocytes. In the corpus callosum for example the density of GFP and NG2 positive cells, “late OPCs” is highest at postnatal day 6 and strongly drops towards postnatal day 10 and 30 after the peak rate of differentiation of NG2 cells has occurred (P6: 3.1 ± 0.28 , P10: 2.5 ± 0.27 , P30: 1.55 ± 0.24 cells per $160 \mu\text{m}^2 \times 25 \mu\text{m}$). “Late OPCs” are still proliferative as we frequently observed pairs of GFP and NG2 positive cells (cf., Fig. 4E and 2B) which do not exist for long after mitosis (Kukley et al., 2008) and mitotic figures of pairs of positive cells (not shown). This is in agreement with a number of previous studies using the thymidine analogues BrdU/EdU to suggest that actually all NG2 cells are continuously, albeit slowly, cycling probably for the whole life (Clarke et al., 2012; Kang et al., 2010; Young et al., 2013). The following lines of evidence suggest to us that our “late OPCs” are closer to differentiation than the remaining GFP negative population of NG2 cells: First, “late OPCs” do not occur before birth, although NG2 cells are detectable (Fig. 6), which is consistent with the general timing of myelination of the forebrain happening in the postnatal period. Second, the density of NG2 and GFP double positive cells is highest just before myelination (see above). Finally, we could show that the population of NG2 cells which expresses GFP is more likely to additionally express low levels of GPR17 in their soma, a marker present at high levels in largely post-mitotic pre-myelinating oligodendrocytes (Boda et al., 2011), than GFP negative cells.

To date a systematic analysis addressing the question whether the effectiveness of Cre-mediated recombination of Cre reporter lines may affect the outcome of fate mapping has not been performed. We addressed this question by crossing the same Cre-driver line (CNP-Cre) with two different Cre reporter mouse strains, of which one (ROSA26/YFP) was more efficient than the other (Z/EG). We consistently observed a larger population of cells being labelled in the CNP-Cre:ROSA26/YFP line but the fraction of NG2 cells expressing GFP/YFP varied similarly across regions of the forebrain between the two reporter mouse strains (Fig. 1B). It therefore seems likely that the NG2 cells which are labelled in CNP-Cre:ROSA26/YFP mice include the smaller population of labelled cells observed in the CNP-Cre:Z/EG line. The population of cells labelled additionally in CNP-Cre:ROSA26/YFP mice clearly is qualitatively different from what we identified, and define above, as “late OPCs” because we found NG2 positive and YFP positive cells in the intermediate zone of the early dorsal cortex at embryonic day 16, which is almost 2 weeks earlier than the first appearance of myelin in this region. In addition, we even observed YFP expressing NG2 negative cells reminiscent of radial glial cells obviously violating our concept of “late OPCs.”

How can the qualitative difference of cells being labelled in the two reporter strains be explained? The probability of a recombination event at a given reporter gene locus likely depends on the concentration of and the exposure time to Cre-recombinase (Ringrose et al., 1998). If the reporter gene is located in a genomic region with high recombination efficiency, it will be recombined at a smaller product of concentration and time. Therefore, when a subpopulation of NG2 cells slowly starts to monotonically increase their *Cnp* promoter activity, reporter expression in this population of cells commences earlier if they contain an easily recombinable reporter gene. Therefore, at any given time a larger fraction of NG2 cells will be found reporter protein positive until all NG2 cells show a long-lasting *Cnp* promoter activity sufficient to also recombine the less efficient reporter gene. A permanent qualitative difference in the population of cells labeled by the reporter protein can arise if there are peaks in the expression of Cre-recombinase and the enzyme is expressed only transiently. Cells transiently expressing a low concentration of Cre-recombinase may activate an easily recombinable reporter gene and become and remain positive for the associated reporter protein but can remain unlabelled and negative for a reporter protein whose gene is difficult to recombine. In this way NG2 cells which only transiently express relatively low levels of Cre-recombinase may activate the reporter gene in CNP-Cre:ROSA26/YFP but never in CNP-Cre:Z/EG mice. This can explain the qualitative differences in the mapped progeny described above.

YFP-reporter positive progenitors (being NG2 negative) detected in embryonic brains of the CNP-Cre:ROSA26/YFP line provide evidence for out of lineage oligodendroglial promoter activation, i.e., that general precursor cells and/or radial glial cells weakly or transiently activate the *Cnp* promoter. Fate mapping studies reported that NG2-glia are one of the main populations originating from radial glia (Malatesta et al., 2003) and it is conceivable that those radial glial cells which give rise to oligodendrocyte precursor cells transiently switch on oligodendroglial genes. Olig-2 represents another example oligodendroglial gene, which has been found to be activated in radial glial cells (Masahira et al., 2006). Nestin-positive and NG2-negative cells generating neurospheres *in vitro* also activate the *Cnp* promoter as evidenced by a transgenic CNP-GFP mouse line (Belachew et al., 2003). Further, we observed reporter-positive radial glial cells when embryonically inducing Cre-activity under the *Ng2* promoter which is similar to the findings by (Tsoa et al., 2014) who described reporter activation in ventricular zone precursor cells under the *Ng2* promoter. Finally, the occurrence of YFP-positive neurons in the CNP-Cre:ROSA26/YFP line was not random but distinct and reproducible in certain regions of the forebrain suggesting that there is a specific temporal and/or regional window when certain general progenitor cells switch on oligodendroglial genes or promoters.

In both the CNP-Cre:ROSA26/YFP and CNP-Cre:Z/EG line there is an unusual long delay between activation of the *Cnp* promoter and generation of the CNPase protein. With the caveats mentioned above the presence of reporter positive cells (CNP-Cre:ROSA26/YFP and CNP-Cre:Z/EG) at postnatal day six suggests that the *Cnp* gene is transcribed at least 10 days earlier than the CNPase protein is found in corpus callosum (Scherer et al., 1994). Early transcription of the *Cnp* gene is in line with previous studies reporting embryonic expression of *Cnp* based on *in situ* hybridization (Yu et al., 1994). The physiological significance of the long delay before the mRNA is translated into protein is at present

unclear but it seems likely that specific mechanisms, such as micro-RNAs or RNA binding proteins, must be negatively regulating the translation of *Cnp* mRNA.

Our concept of “late OPCs” suggests that there is a stage through which all OPCs pass on their way to differentiate into oligodendrocytes and in which those differentiating cells switch on the *Cnp* promoter and activate expression of the reporter protein. This scenario is largely consistent with the finding that almost all oligodendrocytes are positive for the reporter protein. However, in both reporter mouse lines we identified a small fraction (0.13 in CNP-Cre:Z/EG and 0.05 in CNP-Cre:ROSA26/YFP, P30) of CC1+ oligodendrocytes which did not express GFP or YFP. Thus, these oligodendrocytes must be derived from reporter negative precursor cells, i.e. from cells, which did not go through the “late OPC” stage. At present we do not have evidence what may be the source or the significance of OPCs without (sufficient) *Cnp* promoter activity. These OPCs may represent a specific subpopulation which could be related to different waves of generation of OPCs known to occur during embryonic and postnatal periods (Kessaris et al., 2006). Alternatively, some OPCs may differentiate much more rapidly into oligodendrocytes than others such that they express CC1 before the Cre-reporter protein (YFP or GFP) is detectable.

Our results suggest that the choice of a Cre-reporter mouse line can be critical for the interpretation of the results. For fate mapping studies the goal is typically to track the fate of cells defined by the presence of a specific marker protein such as NG2. Therefore, depending on the Cre-expression level of the Cre-driver line a reporter line should be chosen in which expression of the reporter is strictly limited to cells identified by the marker protein. If this specificity cannot be reached or shown, the results of fate mapping studies are difficult to interpret. The choice of an appropriate Cre-reporter line to visualize those cells in which a floxed gene of interest has been deleted by Cre-mediated recombination is more difficult because the efficacy of deletion of the floxed gene and how it compares to the recombination of the reporter locus is a priori unknown. Additional experiments on the single cell level employing immunohistochemistry, physiology, genotyping or other similarly sensitive assays have to be performed to verify an acceptable correlation between gene deletion and reporter expression.

Do NG2 cells generate astrocytes? We did not observe reporter positive astrocytes in our CNP-Cre:Z/EG line indicating that the postnatally occurring “late OPCs” do not generate astrocytes. This is in line with previous studies inducing Cre expression in different mouse lines under the *Ng2* or *Pdgfra* promoter during the postnatal period which also did not detect positive astrocytes (Kang et al., 2010; Rivers et al., 2008; Zhu et al., 2011). The study by (Guo et al., 2009) tracking the progeny of *Plp*-expressing precursors represents an exception and does report astrocytes. However, as the authors have not ruled out that those astrocytes already occurred immediately after tamoxifen application (i.e., within ~2 days) it may be that reporter positive astrocytes sporadically expressed Cre themselves and are not derived from *Plp*-expressing NG2 positive precursor cells.

We did observe large numbers of reporter-positive astrocytes in the ventral cortex when we either induced Cre expression by embryonic tamoxifen application or when we analyzed mice of the CNP-Cre:ROSA26/YFP line. In both cases we also detected reporter positive

radial glial cells which raises the possibility that postnatal astrocytes in the ventral cortex are direct descendants of those radial glial cells and are not derived from embryonic NG2 cells. However, we still think that it is very likely that these astrocytes are indeed generated from embryonic NG2 cells for the following reasons. First, astrocytes were observed in the ventral cortex but the majority of reporter-positive radial glial cells was found in the dorsal ventricular zone. Further, we did not find astrocytes in the dorsal cortex where they would have been expected according to the localization of the majority of reporter positive radial glial cells. Second, in an earlier study using the same NG2-Cre line used in the present study but crossbred with Z/EG mice no radial glial cells were labeled after embryonic tamoxifen injection but postnatal astrocytes in the ventral cortex were still observed (Zhu et al., 2011).

In conclusion, our study shows that postnatal NG2 cells enter a stage in which they can self-renew and are committed to an oligodendroglial lineage. Further, our study demonstrates that fate mapping studies can yield strikingly different results depending on the choice of Cre-reporter lines and provides further evidence against the generation of neurons from NG2 cells in the healthy brain. It has previously been proposed that mature neurons in the postnatal and possibly adult brain may transiently activate promoters of oligodendroglial genes such as *Ng2* (Huang et al., 2014; Nishiyama et al., 2014), *Plp* (Delaunay et al., 2008), or *Pdgfra* (Vignais et al., 1995). Our study now suggests that transient transcriptional activity of oligodendroglial genes can even be found in radial glial cells and other embryonically existing progenitors.

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FIGURE 1:

Reporter expression by NG2 cells in the CNP-Cre:Z/EG and CNP-Cre:ROSA26/YFP lines.

A: Illustration of postnatal day 6 murine brain partial frontal section with anatomical structures analyzed in this study. D. Ctx, Dorsal Cortex; V. Ctx, Ventral Cortex; Piri. Ctx, Piriform Cortex; CA1, Hippocampal Cornus Ammonis Region 1; CC, Corpus Callosum; Fim, Fimbriae. B: Fraction of NG2 cells being GFP+ or YFP+ at P6 in the given gray and white matter regions. CNP-Cre driven GFP and YFP expression by a subpopulation of NG2 cells. Note that in the CNP-Cre:ROSA26/YFP line, a significantly greater proportion of

reporter positive cells among NG2⁺ OPCs exists as compared to the CNP-Cre:Z/EG line (Two-way Anova, Bonferroni post-hoc test, $\alpha = 0.05$). C–D: Single optical section confocal scan of the Dorsal Cortex of CNP-Cre:Z/EG (C) and CNP-Cre:ROSA26/YFP (D) mice at P6 co-immunolabeled for GFP (green), NG2 (red) and Nissl deep red (blue). All arrowheads point to GFP[−]/NG2⁺ cells. All arrows point to GFP⁺/NG2⁺ cells. E–F: Coronal sections through the Corpus Callosum of CNP-Cre:Z/EG (E) and CNP-Cre:ROSA26/YFP (F) mice at P6 double immunolabeled for GFP (green), NG2 (red) and Nissl deep red (blue). All arrowheads point to GFP[−]/NG2⁺ cells. All arrows point to GFP⁺/NG2⁺ cells. All scale bars: 10 μ m. G: A larger fraction GFP⁺ NG2⁺ cells co-labeled with anti-GPR17 antibodies when compared to NG2 cells being GFP[−]. White arrows indicate NG2 cells, red arrow marks GPR17 positive cells and green asterisks denote GFP⁺ cells. NG2 cells (cells with consistent labeling of their membrane at least around the nucleus) only showed GPR17 labeling within the soma. Cells showing GPR17 positive processes were found to be NG2[−]. Scale bar: 10 μ m. H: As above but instead of the GFP channel the nuclear counter stain is shown in green for clearer cell identification.

FIGURE 2:

CNP-Cre driven GFP and YFP expression in mature oligodendrocytes. A: Fraction of GFP⁺ cells that are NG2⁺ (black bars) in Dorsal Cortex and Corpus Callosum of CNP-Cre:Z/EG mice at P10 and P30. Red bars show the fraction of GFP⁺ cells representing CC1⁺ oligodendrocytes at P30. B-C: Single optical section confocal scan of the Dorsal Cortex (B) and Corpus Callosum (C) of P10 and P30 (upper and lower row, respectively) CNP-Cre:Z/EG mice immunolabelled for GFP/NG2 or GFP/CC1. D: Fraction of YFP⁺ cells that are NG2⁺ (black bars) and CC1[±] (red bars) in the P10 and P30 Dorsal Cortex and Corpus

Callosum of CNP-Cre:ROSA26/YFP mice. E–F: Single optical section confocal scan of the Dorsal Cortex (E) and Corpus Callosum (F) of P10 and P30 CNP-Cre:ROSA26/YFP mice immunolabelled for GFP/NG2 or GFP/CC1. Asterisks indicate large YFP+ cell bodies being NG2– and CC1– seen only in CNP-Cre:ROSA26/YFP mice. All arrowheads point to GFP1/NG2– or GFP1/CC1–cells. All arrows point to GFP1/NG2+ or GFP+/CC1+ cells. All scale bars: 10 μ m.

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FIGURE 3:

CNP-Cre driven YFP, but not GFP expression in astrocytes. A–D: Single optical section confocal scan of the Dorsal Cortex of P30 CNP-Cre:Z/EG (A) and CNP-Cre:ROSA26/YFP (B) mice double immunolabelled for GFP (green) and GFAP (red). (C) and (D) as before but for the Ventral Cortex of P30 mice. In the CNP-Cre:Z/EG line no astrocytes were GFP positive. On the other hand, YFP positive cells in the ventral cortex of the CNP-Cre:ROSA26/YFP line very frequently expressed GFAP (arrow) and represented 0.12 ± 0.02 of all YFP+ cells in the ventral cortex (total of 179 YFP+ cells, $n = 4$ mice). Note the

overlap of GFP and GFAP expression in the primary and secondary processes of the astrocyte (D). All arrowheads point to GFP+/GFAP-cells. scale bars: 20 μ m. E: Fraction of GFP+/YFP+ cells that are GFAP+ in the given gray and white matter regions of P30 CNP-Cre:Z/EG ($n = 4$ mice) and CNP-Cre:ROSA/YFP mice ($n = 4$ mice). Student's unpaired t test was used to indicate significant difference, * indicates $P < 0.05$. D.Ctx, Dorsal Cortex; CC, Corpus Callosum; V.Ctx, Ventral Cortex.

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FIGURE 4:

Widespread CNP-Cre driven YFP expression in neurons. A: Overview scan of YFP/NeuN double labelling showing the typical distribution of YFP positive cells in CNP-Cre:ROSA26/YFP mice. Note the dense group of YFP+ and NeuN+ large cells in layer II of the medial part of the dorsal cortex (dashed line). B–D: Single optical section confocal scan of the Dorsal Cortex (B) Ventral Cortex (C) and Piriform Cortex (D) of P30 CNP-Cre:ROSA26/YFP mice double immunolabelled for YFP (green) and NeuN (red). Arrows point to YFP1/NeuN+ cells. Arrowheads point at YFP+/NeuN-cells, likely representing

OPCs. Note the numerous NeuN positive large somata. 0.55 ± 0.07 (total of 607 YFP+ cells, $n = 4$ mice), 0.37 ± 0.08 (total of 188 YFP1 cells, $n = 4$ mice) and 0.7 ± 0.05 (total of 353 YFP+ cells, $n = 4$ mice) of YFP+ cells that were NeuN+ mature neurons in the dorsal, ventral and piriform cortices, respectively. E: As above but dorsal cortex of P30 CNP-Cre:Z/EG mice. All scale bars: 20 μ m. F: Fraction of GFP+ or YFP+ positive cells that are NeuN+ in the Dorsal, Ventral and Piriform Cortices of P30 CNP-Cre:Z/EG and CNP-Cre:ROSA/YFP mice. Two-way Anova with Bonferroni post-hoc test was used to test significant difference. D.Ctx, Dorsal Cortex; V.Ctx, Ventral Cortex; Piri.Ctx, Piriform Cortex.

FIGURE 5:

Embryonic but not postnatal BrdU pulse-chase labels YFP+ neurons. A: BrdU administration strategy to E12.5 CNP-Cre:ROSA26/YFP mice via oral gavage, followed by analysis at P30. B–C: Single optical section confocal scan of the Ventral Cortex (B) and Piriform Cortex (C) of embryonic BrdU administered animals at P30 double immunolabelled for YFP (green) and BrdU (red). Arrows point to YFP+/BrdU+ neurons. D: BrdU administration strategy to postnatal CNP-Cre:ROSA26/YFP mice via i.p injections from P10-P13, followed by analysis at P32. E–F: Single optical section confocal scan of the

ventral cortex (E) and piriform cortex (F) of postnatal BrdU injected animals at P32 double immunolabelled for YFP (green) and BrdU (red). Arrowheads point to YFP⁺/BrdU-neurons. All scale bars: 20 μ m. G: Fraction of YFP⁺ neurons that are BrdU⁺ at both postnatal and embryonic BrdU administration time points in the ventral and piriform cortices. D.Ctx, Dorsal Cortex; V.Ctx, Ventral Cortex; Piri.Ctx, Piriform Cortex.

FIGURE 6:

Embryonic CNP-Cre driven reporter expression in radial glial cells, neurons and NG2 cells exclusively in ROSA26/YFP Cre reporter mice. **A:** Overview confocal scan of the dorsal cortex of E16 CNP-Cre:ROSA26/YFP mice double immunolabelled with GFP (green) and NG2 (red). Arrowheads point at NG2 negative YFP+ cells in the cortical plate, intermediate cortical layer and ventricular neuroepithelium, likely representing neurons, general progenitors and radial glia cells, respectively. Arrows point at NG2+ YFP+ cells which we only found in the intermediate cortical layer. Only the small fraction of 0.14 ± 0.03 of YFP

cells in this region expressed NG2 (total of 762 YFP+ cells, $n = 3$ mice). B: Single optical section confocal scan of the ventricular zone of E16 CNP-Cre:ROSA26/YFP mice double immunolabeled with GFP (green) and BLBP (red). BLBP identifies a fraction of 0.28 ± 0.07 (total of 435 YFP+ cells, $n = 3$ mice) of YFP+ cell located in the ventricular neuroepithelium as radial glia cells (arrows). Arrowheads point at BLBP negative YFP+ cells. C: GFP-NG2 double labelling of the E16 cortical plate of CNP-Cre:Z/EG mice. In this and other regions of the embryonic brain only vascular cells were positive for GFP and NG2. All scale bars: 20 μm .

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FIGURE 7:

Radial glia-like cells display *Ng2* promoter activity and generate neurons in the embryonic and adult dorsal cortex. A: Single optical section confocal scan of the ventricular zone of E19.5 NG2creERTM:ROSA26/YFP administered tamoxifen at E16.5 double immunolabelled for YFP (green) and NG2 (red). Arrowheads point at YFP+/NG2-cells. Clear YFP positivity of radial glia-like cell indicates activity of *Ng2* promoter and lack of NG2 immunoreactivity suggests transient nature of this activity. B: Single optical section confocal scan of the ventricular zone of E19.5 NG2creERTM:ROSA26/YFP administered tamoxifen at E16.5

double immunolabelled for YFP (green) and BLBP (red). Arrows point at YFP⁺/BLBP⁺ cells. BLBP labelling demonstrates a radial glia-like phenotype of most YFP⁺ cells. Arrowhead points at YFP⁺/BLBP⁺-cells. C: In the early dorsal cortex of the same mice large YFP⁺ and NG2-cells are observed which likely represent pyramidal neurons of the dorsal cortex (arrowheads). Note the smaller and brighter YFP⁺ cells (arrows) representing NG2⁺ cells seen at significant number at this age. D–F: Single optical section confocal scan of the dorsal cortex (D) ventral cortex (E) and piriform cortex (F) of P30 NG2creERTM;ROSA26/YFP administered tamoxifen at E16.5 and double immunolabelled for YFP (green) and NeuN (red). Arrows point to YFP⁺/NeuN⁺ cells. Upon embryonic induction of Cre-recombinase, widespread reporter expression is observed in NeuN⁺ neurons. All scale bars: 20 μ m.