Generation of subtype-specific neurons from postnatal astroglia of the mouse cerebral cortex

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Instructing glial cells to generate neurons may prove to be a strategy to replace neurons that have degenerated. Here, we describe a robust protocol for the efficient *in vitro* conversion of postnatal astroglia from the mouse cerebral cortex into functional, synapse-forming neurons. This protocol involves two steps: (i) expansion of astroglial cells (7 d) and (ii) astroglia-to-neuron conversion induced by persistent and strong retroviral expression of *Neurog2* (encoding neurogenin-2) or *Mash1* (also referred to as achaete-scute complex homolog 1 or *Ascl1*) and/or distal-less homeobox 2 (*Dlx2*) for generation of glutamatergic or GABAergic neurons, respectively (7–21 d for different degrees of maturity). Our protocol of astroglia-to-neuron conversion by a single neurogenic transcription factor provides a stringent experimental system to study the specification of a selective neuronal subtype, thus offering an alternative to the use of embryonic or neural stem cells. Moreover, it can be a useful model for studies of lineage conversion from non-neuronal cells, with potential for brain regenerative medicine.

INTRODUCTION

During early corticogenesis in the mouse, radial glia serve as a source for neurons and neurogenic progenitors, but at late embryonic neurogenesis (~E18), they lose their neurogenic potential and give rise to astroglia, either by direct transformation or through intermediate progenitors[1], concomitant with an epigenetic silencing of neurogenic genes[2]. Recent work has shown that cortical astroglia isolated at early postnatal stages (P5–P7) can be instructed by neurogenic fate determinants to become neurons[3–5]. Here, we provide a simple and robust protocol to selectively instruct the genesis of glutamatergic and GABAergic functional neurons from postnatal astroglia *in vitro*.

Astroglia are defined by the expression of glial fibrillary acidic protein (GFAP), which clearly distinguishes these cells from neurogenic radial glia, which in the mouse do not express GFAP[6,7]. Furthermore, astroglia express S100β, the L-glutamate/L-aspartate transporter (GLAST), glutamine synthetase, glutamate transporter 1, and aldehyde dehydrogenase 1 L1, and they are devoid of the dorsal telencephalic neurogenic fate determinant neurogenin-2 (refs. 3–5). Astroglia obtained from the early postnatal cerebral cortex (P5–P7) can be maintained in primary culture in serum-containing medium, under which conditions they proliferate as a monolayer. Although proliferating, astroglial cells remain within the glial lineage and do not give rise to neurons without manipulation, thus indicating the acquisition of a stable astroglial cell fate[2,4]. However, we have shown in previous studies that forced expression of the neurogenic fate determinant paired boxed gene 6 (*Pax6*) driven by retroviral vectors can induce a fate switch in cultured mouse cortical astroglia toward a neuronal identity, as revealed by the acquisition of a neuronal morphology and the gradual expression of neuronal markers[3]. Subsequently, it was shown that the effect of Pax6 encompasses the acquisition of a true neuronal identity, as reflected by the ability of the astroglia-derived neurons to fire action potentials[5]. Moreover, in this study, we found that retroviral overexpression of the proneural genes *Neurog2* or mammalian achaete-scute homolog 1 (*Ascl1*, referred to hereafter as *Mash1*) is even more effective to drive mouse cortical astroglia toward the generation of electrical excitable neurons, as revealed by electrophysiology using patch-clamp recordings[5]. However, no functional synaptogenesis was observed, as reflected by the absence of synaptic clustering of presynaptic proteins as well as the lack of spontaneous or evoked synaptic transmission, thus suggesting that astroglia-to-neuron conversion was incomplete[5]. Failure to complete neurogenesis may be because of the fact that the pCLIG-based retroviral vectors[8] used in this study were prone to silencing; hence, pro-neural gene expression had become gradually diminished. Indeed, stronger and prolonged expression of the neurogenic fate determinant using silencing-resistant pCAG-based retroviral constructs[9] allowed for the genesis of fully functional, synapse-forming neurons[4]. Importantly, by the selection of the respective transcription factor on basis of its known role in instructing the genesis of different neuronal subtypes in the developing telencephalon, we developed an efficient procedure to generate both glutamatergic (using *Neurog2*) or GABAergic (using *Mash1* and/or *Dlx2*) neurons from the same population of cortical astroglia[4].

For researchers interested in studying lineage reprogramming, recently an alternative procedure has also been described to convert embryonic and perinatal fibroblasts into glutamatergic neurons by forced coexpression of *Mash1*, *Pou3f2* (POU domain class 3, transcription factor 2, also referred to as *Brn-2*) and myelin transcription factor 1-like protein (*Myt11*)[10]. Besides the different cellular source, this procedure differs from our protocol in the use of lentiviral vectors for introducing the transcription factors. Moreover, maturation of neurons toward full functionality required three factors, while a single factor is sufficient for neuronal conversion of postnatal astroglia[4], possibly due to the closer lineage relationship of astroglia and neurons compared with mesoderm-derived fibroblasts. Lineage distance[11] may also contribute to the rather low efficiency of the reprogramming process elicited in fibroblasts (~20% (ref. 10)), compared with astroglia (~70% (ref. 4)). On the other hand, for researchers studying neuronal subtype specification, our system may provide an alternative experimental model to embryonic stem

(ES) cells, other pluripotent cell types[12] or even more restricted neural stem cells[13,14]. Specifically, protocols have been developed that allow for the selective generation of glutamatergic and GABAergic neurons from mouse ES cells[15–20]. Interestingly, ES cells cultured in the presence of a sonic hedgehog inhibitor recapitulate the sequential generation of distinct glutamatergic subtypes[21], therein mimicking the behavior of cultured cortical progenitors[22]. It is currently not clear whether astroglia under the influence of neurogenic fate determinants are capable of undergoing such sequential generation of distinct glutamatergic subtypes. The fact that astroglial cells undergoing neuronal conversion typically exit the cell cycle may in fact preclude this. Moreover, ES cells are easily amenable to gene targeting, which can be helpful in analyzing lineage decisions[23].

Potential applications of the protocol

Given that postnatal astroglia can be cultured at large scale and converted with high efficiency into neurons using the protocol presented here, this experimental system should be in principle amenable to biochemical analysis to decipher the molecular mechanisms underlying glia-to-neuron conversion and neuronal subtype specification. Further developments of the protocol should also examine the possibility to generate other types of neurons than glutamatergic or GABAergic neurons, such as, for instance, midbrain dopaminergic or spinal motor neurons. Furthermore, culturing astroglia from brain areas other than the cerebral cortex may expand the diversity of the neuronal phenotypes that can be obtained by forced expression of neurogenic fate determinants. Finally, with some modifications, the protocol presented here may be extendable to cells derived from the human brain in culture[24] to screen for transcription factors that convert non-neurogenic cells into neurons.

Limitations of the protocol

In its present form, the protocol warrants an efficient conversion of astroglial cells from the early postnatal cerebral cortex into glutamatergic or GABAergic neurons, depending on the transcription factors used. One major limitation of the protocol is related to the age of the experimental animals. Conversion of astroglial cells at later postnatal or even adult stages is hampered in first place by the rather limited yield of astroglial cells following culturing, and currently no protocols are available to routinely culture adult mouse astroglia. This is likely to be due to the limited proliferative potential of adult astroglia, which is unfavorable to large-scale expansion. However, following injury to the cerebral cortex, astroglia within the lesioned tissue resume proliferation, and when isolated *in vitro*, can give rise to self-renewing and multipotent neurospheres[25]. The protocol presented here can principally also be adapted for cells derived from adult astroglia-derived neurosphere cells and works with similar efficiency[4]. However, the molecular processes underlying the conversion of quiescent astroglia into sphere-forming astroglia are still largely unknown and require further characterization.

In addition, we observed marked differences in the potency of the respective transcription factors used with regard to their ability to induce a neuronal identity and to promote a complete maturation toward functional neurons[4]. Indeed, we always observed a lower yield of GABAergic neurons following astroglia-to-neuron conversion induced by *Mash1* or *Dlx2*, compared with the genesis of glutamatergic neurons by *Neurog2* (ref. 4). This can be overcome by combining more than one factor (*Mash1* and *Dlx2*). An alternative approach consists in the expansion of the astroglial cells under neurosphere conditions before astroglia-to-neuron conversion (see **Box 1**). Astroglial cells can indeed give rise to neurosphere cells until postnatal days 14–16 (C.H., M.G. and B.B., unpublished observations; see ref. 26). Prior expansion of astroglial cells under neurosphere conditions greatly enhanced neurogenesis induced by *Dlx2*. The higher susceptibility of astroglia-to-neuron conversion may be due in part to the higher expression of SRY-box containing gene 2 (*Sox2*) by astroglia under neurosphere conditions, but the precise molecular basis for this difference is not yet well understood[4].

Finally, another parameter likely to contribute to the outcome of the astroglia-to-neuron conversion is the astroglial origin. This protocol has been established for astroglia from the cerebral cortex, which may, hence, show a bias toward the generation of glutamatergic neurons. However, the fact that the very same cortical astroglia can give rise not only to glutamatergic but also to GABAergic neurons demonstrates that such restrictions are not insurmountable.

Experimental design

Overview of the procedure. The protocol presented here allows the *in vitro* generation of glutamatergic or GABAergic neurons from postnatal astroglia isolated from the mouse cerebral cortex, by the forced expression of distinct neurogenic fate determinants using a five-step procedure, as outlined below (**Fig. 1**):

- *Dissection of the cerebral cortex*: The cerebral cortex of postnatal mice (P5–P7) is dissected from the brain and mechanically dissociated to obtain a single-cell suspension.
- *Primary culture of astroglial cells*: Cells are placed into uncoated plastic flasks for expansion in medium containing 10% (vol/vol) FBS, 5% (vol/vol) horse serum, B27 supplement, epidermal growth factor and basic fibroblast growth factor.
- *Passaging and seeding of astroglial cells*: After 7 d, cultured cells are removed from the flask by trypsinization and seeded onto poly-D-lysine (PDL)-coated glass cover slips.
- *Retroviral transduction or plasmid transfection of astroglial cells*: At 2–4 h after seeding, the cells are transduced with retroviral vectors encoding neurogenic fate determinants such as *Neurog2*, *Mash1* or *Dlx2*. Alternatively, astroglia can be transfected with the corresponding retroviral vector plasmids.
- *Astroglia-to-neuron conversion*: Conversion of astroglia into neurons is a gradual process that requires from 7 d to 4 weeks until the astroglia-derived neurons reach full functionality, following the gene transfer of neurogenic fate determinants into astroglia. Whereas forced *Neurog2* expression induces a glutamatergic neuron identity, forced expression of *Mash1* and/or *Dlx2* induces a GABAergic fate.

The protocol described here has been optimized for the successful glia-to-neuron conversion of early postnatal astroglia derived from the cerebral cortex of C57BL/6J mice[3–5], and has also been successfully applied to the conversion of astroglia from the postnatal rat cerebral cortex[27].

Choice of the gene delivery method. Both retroviral transduction as well as plasmid transfection allow for conversion of postnatal cortical astroglia toward full functional neurons. The choice of gene delivery will mainly depend on the costs, availability of biosafety facilities and the experimental question to be addressed. For instance, although retroviral transduction was the method of choice for most experiments in Berninger *et al.*[5] and Heinrich *et al.*[4], transfection was used to demonstrate that cell division is not required for astroglia-to-neuron

BOX 1 | PREPARATION OF NEUROSPHERES FROM ASTROGLIA FROM THE MOUSE POSTNATAL CORTEX ● TIMING 5–7 D

The first steps dealing with cortical tissue dissection are identical to those described in the PROCEDURE section (see Steps 1–4 of PROCEDURE). This protocol is mainly adapted from reference 37.

1. Mince the dissected cortices, with two blades, into small tissue pieces and transfer them into a 15-ml conical tube containing 5 ml of prewarmed (37 °C) dissociation medium. Incubate at 37 °C for 15 min.
2. Gently triturate the solution containing tissue pieces a few times (~10 times) up and down with a 5-ml disposable pipette.
3. Incubate the solution containing now partially dissociated cells and remaining tissue pieces at 37 °C for an additional 15 min.

▲ **CRITICAL STEP** The total incubation time in the dissociation solution should not exceed 30 min.

4. Add 5 ml of ice-cold neurosphere solution III to 5 ml dissociation medium to block trypsin activity. Gently mix with a 5-ml disposable pipette.
5. Pass the obtained cellular suspension through a 70-µm cell strainer to remove the tissue debris and collect the cells into a fresh 15-ml conical tube.
6. Centrifuge at 200*g* (1,300 r.p.m.) for 5 min at 4 °C.
7. Remove the supernatant and resuspend the cells in 10 ml of ice-cold neurosphere solution II.
8. Centrifuge at 450*g* (2,000 r.p.m.) for 10 min at 4 °C.
9. Carefully remove the supernatant and resuspend the cells in 2 ml of ice-cold neurosphere solution III.
10. Fill a new 15-ml conical tube with 12 ml of ice-cold neurosphere solution III.
11. Gently apply 2 ml of cell suspension obtained in step 9 to the top of the 12 ml of ice-cold neurosphere solution III prepared in step 10.
12. Centrifuge at 250*g* (1,500 r.p.m.) for 7 min at 4 °C.
13. Carefully remove the supernatant and resuspend the cell pellet in an appropriate volume (~1 ml) of prewarmed (37 °C), CO_2-equilibrated neurosphere medium.
14. Count the cells in a hemocytometer, using Trypan blue to exclude dead cells, as previously described in ref. 35. Dilute the cells by adding prewarmed (37 °C), CO_2-equilibrated neurosphere medium to obtain 10 cells per µl of medium (i.e., clonal density).
15. Transfer the cell suspension to a 25-cm^2 (or 75-cm^2) tissue culture flask and incubate at 37 °C with 5% CO_2 for 5–7 d. We usually obtain neurospheres within this time frame.

▲ **CRITICAL STEP** If neurospheres are allowed to grow for too long time, partial differentiation may occur.

16. Collect the medium containing the astroglia-derived neurospheres in a 15-ml conical tube.
17. Centrifuge at 120*g* (1,000 r.p.m.) for 5 min at room temperature.
18. Carefully remove the supernatant and add 750 µl of 0.05% (wt/vol) trypsin/EDTA to the neurosphere pellet and incubate the mixture at 37 °C for 2 min.
19. Gently triturate the mixture up and down with a 200-µl filter tip and incubate for 2 min at 37 °C.
20. Dissociate the neurospheres by slowly pipetting the mixture up and down with a 200-µl filter tip.
21. Add 750 µl of neurosphere solution III and gently mix.
22. Centrifuge at 250*g* (1,500 r.p.m.) for 1 min at room temperature.
23. Remove the supernatant and resuspend the pellet in 500 µl of fresh neurosphere medium. Count the cells.
24. Seed 50,000–60,000 cells onto poly-D-lysine-coated glass cover slips in 500 µl of fresh, prewarmed (37 °C), CO_2-equilibrated neurosphere medium in 24-well tissue culture plates and incubate at 37 °C with 5% CO_2.

Thereafter, follow the protocol described in the PROCEDURE section for retroviral transduction and cultivation of transduced cells (starting from Step 17).

conversion. Of note, gene delivery through transfection is subject to a higher degree of variability than retroviral transduction, once the viral titer is determined. In addition, the relative efficacy of glia conversion (i.e., percentage of neurons per reporter-positive cells) by transfection may be slightly overestimated. Indeed, astroglia successfully undergoing neuronal conversion maximally undergo one round of cell cycle division, become postmitotic and, therefore, keep high reporter levels over time, suggesting persistent expression, whereas astroglia failing to respond to the neurogenic fate determinants often continue proliferating and thus lose episomal plasmids more rapidly. In contrast, on genomic integration of the retroviral genome, the transgene is faithfully transmitted to all progeny, independent of whether cells leave the cell cycle or continue proliferating and, thus, over time, the relative proportion of astroglia failing to undergo neuronal conversion may increase as a result of their prolonged proliferation.

We have used different retroviral constructs to drive *Neurog2*, *Mash1* and *Dlx2* expression. By using retroviral backbones with native long terminal repeat–driven expression without an internal promoter such as pCLIG, we noted a gradual decrease of reporter fluorescence over time following transduction[5,14]. In contrast, when forced expression of neurogenic fate determinants was driven by self-inactivating vectors, due to the deletion of the 3′ U3 enhancer[28], that had been developed for a persistent expression *in vivo* such as the pCAG retroviral vector (**Fig. 2**)[9,29], we did not detect any overt decrease in reporter-fluorescence[4] or nuclear staining for the transcription factor (C.H. and B.B., unpublished data). Accordingly, we obtained more efficient astroglia-to-neuron conversion using the latter retroviral vectors. Of note, despite the continued expression of transcription factors that are typically switched off following cell cycle exit during development, we did not observe signs of aberrant differentiation or toxicity[4]. Finally, we have not yet assessed astroglia-to-neuron conversion by lentiviral vectors. However, given the successful reprogramming of embryonic and perinatal fibroblasts[10], lentiviral gene delivery may prove to be a viable alternative

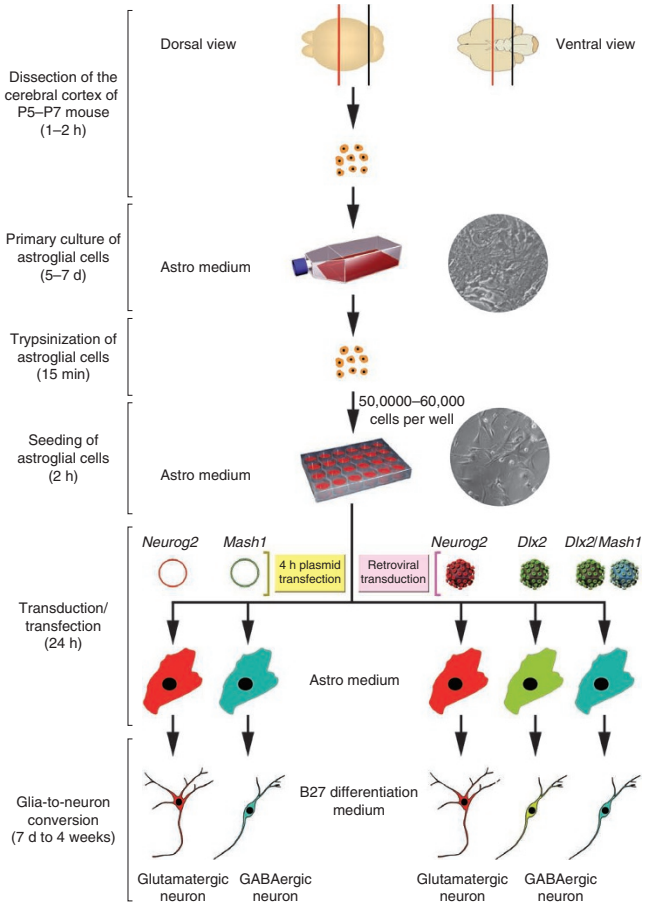

Figure 1 | This schematic diagram illustrates the major steps of the protocol for direct conversion of postnatal astroglia into synapse-forming glutamatergic or GABAergic neurons.

to retroviral delivery for neuronal conversion of astroglia. Moreover, lentiviral vectors would also allow targeting nonproliferating cells within the culture. Of note, proliferation was found not to be a prerequisite for astroglia-to-neuron conversion[4].

Controls. The protocol involves two types of controls. First, it is important to ascertain the astroglial identity of the cells that will be converted into neurons. To this aim, we transduced astroglial cultures with a retroviral vector encoding the expression of DsRed only (pCAG-IRES-*DsRed*). Immunocytochemistry revealed that cells targeted by the retrovirus coexpress GFAP 1 d post infection (d.p.i.), thus confirming their astroglial identity[5]. An additional control for the astroglial identity of the converted cells can be provided by genetic fate mapping, as described below. Second, it is necessary to ensure that the astroglial culture contains few, if any, cells with intrinsic neurogenic potential. To this aim, we transduced astroglial cultures with pCAG-IRES-*DsRed* retrovirus and performed immunocytochemistry to assess the expression of neuronal markers such as βIII tubulin at different time periods following transduction (7, 11 and 32 d.p.i.). Consistent with their non-neurogenic nature, >98% of astroglial cells prepared from the P5–P7 cerebral cortex and transduced with pCAG-IRES-*DsRed* were found to be negative for βIII tubulin[4]. Moreover, astroglial cells transduced with a control retrovirus exhibited passive electric properties and did not fire action potentials[5].

Genetic characterization of cellular identity. A central aspect of any lineage reprogramming study involves the precise characterization of the cellular identity of the lineage-converted cells. Although immunocytochemical analysis allows for the assessment of the identity of the majority of cells to be converted, genetic fate mapping can provide definitive evidence for the conversion of one cell type into another. In our previous studies, we employed a combination of immunocytochemical and genetic tools to ascertain the astroglial nature of the cells undergoing astroglia-to-neuron conversion[4,5]. To this aim, we used GLAST::CreERT2/Z/EG mice[30], expressing a tamoxifen-inducible form of the Cre recombinase under the control of the astroglia-specific GLAST promoter, crossed with a Z/EG reporter mouse line[31]. We showed that when recombination is induced between P2 and P7 by administration of tamoxifen to the pups through the mother's milk, reporter-positive cells give rise to 98.7 ± 0.7% of GFAP-positive astroglia and to a limited number of NG2-positive cells. Of crucial importance, in the absence of forced expression of neurogenic fate determinants, no βIII tubulin-positive neurons are generated[4], indicating the gliogenic nature of the cells labeled by this fate-mapping strategy. Other fate-mapping tools in which an inducible Cre recombinase is expressed under the control of an astroglia-specific promoter may be employed to the same aim. However, it is important to keep in mind to activate recombination of the reporter locus when neurogenesis is completed[30]. Indeed, when recombination is induced by E18, very few (<4%) reporter-positive neurons are observed in the cerebral cortex[30]. Thus, fate mapping should be induced at postnatal stages. Finally, single-cell tracking of genetically fate-mapped astroglial cells by time-lapse video microscopy allows for the direct visualization and inference of the time course of the glia-to-neuron conversion[4].

MATERIALS

REAGENTS

Experimental animals

- C57BL/6J wild-type mice at postnatal day 5–7 ▲ **CRITICAL** The protocol is developed for the efficient culturing and neuronal conversion of astroglia prepared from the cerebral cortex between postnatal day 5 and 7. ! **CAUTION** All experiments should be performed in accordance with all relevant governmental and institutional regulations regarding the use of animals for research purposes.

Cultures of postnatal cortical astroglia

- Hanks' balanced salt solution with $CaCl_2$ and $MgCl_2$ (10× HBSS; Invitrogen, cat. no. 14065-049)
- Hanks' balanced salt solution with $CaCl_2$ and $MgCl_2$ (1× HBSS; Invitrogen, cat. no. 24020-091)
- Dulbecco's modified Eagle's medium: Nutrient Mixture Ham's F-12 without L-glutamine (DMEM/Ham's F-12; PAA, cat. no. E15-012)
- Earle's balanced salt solution without $CaCl_2$ and $MgCl_2$ (EBSS; Invitrogen, cat. no. 14155048)
- Reduced-serum medium with L-glutamine and HEPES (Opti-MEM; Invitrogen, cat. no. 11058-021)
- Dulbecco's phosphate-buffered saline without $CaCl_2$ and $MgCl_2$ (10× PBS; Invitrogen, cat. no. 14200-067)
- HEPES buffer solution (1 M; Invitrogen, cat. no. 15630-056)

Figure 2 | Schematic structure of the recombinant retroviral vector of pCAG-*Neurog2*-IRES-*DsRed*. RV, retrovirus; CMV_{IE}, immediate early enhancer element of the cytomegalovirus (CMV) promoter; LTR, long-terminal repeat; ψ^+, extended retroviral packaging signal; CAG, compound promoter containing the minimum enhancer sequence of CMV, chicken β-actin promoter and a synthetic intron 29; *Neurog2*, cDNA encoding neurogenin-2; IRES, internal ribosomal entry site; PPT, polypurine tract; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; SIN, self-inactivating (ΔU3). EcoRI, SfiI, PmeI and NotI are restriction sites of the restriction enzymes.

- Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)
- D-(+)-glucose solution (45% in H_2O; Sigma-Aldrich, cat. no. G8769)
- D-(+)-saccharose (Carl Roth, cat. no. 4621)
- Heat-inactivated FBS (Invitrogen, cat. no. 10106-169) ▲ **CRITICAL** Inactivate the serum by incubation at 56 °C for 30 min; prepare appropriate aliquots and store at −20 °C for up to 6 months. ▲ **CRITICAL** As serum may contain unknown or variable concentrations of growth factors that promote astroglial differentiation, it is important to test different sera in case of unsuccessful attempts of astroglia-to-neuron conversion. This point raises the important technical caveat that, in contrast to neuronal cultures, there is currently no serum-free defined medium available for the growth of astroglia.
- Heat-inactivated horse serum (Invitrogen, cat. no. 16050-122). ▲ **CRITICAL** Inactivate the serum by incubation at 56 °C for 30 min; prepare appropriate aliquots and store at −20 °C for up to 6 months.
- B27 serum-free supplement (Invitrogen, cat. no. 17504-044) ▲ **CRITICAL** Store in appropriate aliquots at −20 °C for up to 1 year ▲ **CRITICAL** We noticed some variability in the quality of B27 for long-term maintenance of neurons; for practicability, we recommend the use of B27, but in case of inconsistent results, the use of an alternative defined supplement medium may be indicated[32].
- GlutaMAX-I supplement (200 mM; Invitrogen, cat. no. 35050-038) ▲ **CRITICAL** Store in appropriate aliquots at −20 °C for up to 6 months.
- Recombinant human epidermal growth factor (EGF; Invitrogen, cat. no. PHG0311; see REAGENT SETUP) ▲ **CRITICAL** Dilute and store all the growth factors in adequate aliquots according to the manufacturer's recommendations at −20 °C for up to 6 months.
- Recombinant human basic fibroblast growth factor (bFGF; Invitrogen, cat. no. 13256-029; see REAGENT SETUP) ▲ **CRITICAL** Dilute and store all the growth factors in adequate aliquots according to the manufacturer's recommendations at −20 °C for up to 6 months.
- Recombinant human brain-derived neurotrophic factor (BDNF; Calbiochem, cat. no. 203702; see REAGENT SETUP) ▲ **CRITICAL** Dilute and store all the growth factors in adequate aliquots according to the manufacturer's recommendations at −20 °C for up to 6 months.
- Trypsin/EDTA (0.25% (wt/vol); Invitrogen, cat. no. 25200-056)
- Trypsin from bovine pancreas (Sigma-Aldrich, cat. no. T9201)
- Hyaluronidase from bovine testes (Sigma-Aldrich, cat. no. H3884)
- Albumin from bovine serum (BSA; Sigma-Aldrich, cat. no. A2153)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)
- Hydrochloric acid (1 M; AppliChem, cat. no. A1434)
- Acetone (Carl Roth, cat. no. 9372.4)
- Ethanol (≥ 99.8%; Carl Roth, cat. no. 9065.4)
- Poly-D-lysine hydrobromide (PDL; Sigma-Aldrich, cat. no. P0899)
- Ultrapure water (used for the preparation of the aqueous solutions, dilution of buffers and cover slip cleaning and rinsing)
- Trypan blue stain (Invitrogen, cat. no. 15250-061)

Immunocytochemistry
- BSA (Sigma-Aldrich, cat. no. A2153)
- Paraformaldehyde (PFA; Sigma-Aldrich, cat. no. P6148) ! **CAUTION** As a formaldehyde-releasing agent, PFA is a suspected carcinogen. Do not inhale and handle under a laminar flow hood.
- Triton X-100 (Sigma-Aldrich, cat. no. T9284)
- Antibodies (See **Table 1**)

Electrophysiological recordings
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S3014)
- Potassium chloride (KCl; Merck, cat. no. 104938)
- Calcium chloride, dihydrate ($CaCl_2$, $2H_2O$; Merck, cat. no. 208291)
- Magnesium chloride, hexahydrate ($MgCl_2$, $6H_2O$; AppliChem, cat. no. A3618)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- D-(+)-Glucose (Sigma-Aldrich, cat. no. G8270)
- Potassium D-gluconate (Sigma-Aldrich, cat. no. G4500)
- Ethylene glycol-*bis* (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; Sigma-Aldrich, cat. no. E3889)
- Amphotericin B (Calbiochem; cat. no. 171375)
- (+)-Bicuculline (Tocris Bioscience, cat. no. 0130)
- 6-Cyano-7-nitroquinoxaline-2,3-dion (CNQX; Tocris Biosciences, cat. no. 0190)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D5879)

pH adjustment
- NaOH, 1N (Merck, cat. no. 1091371000)
- KOH, 0.1N (Merck, cat. no. 1099210001)
- HCl, 1N (Merck, cat. no. 1090571000)

Retroviral vectors
- Retroviral vectors expressing the neurogenic fate determinants (see REAGENT SETUP)
- pSKSP shuttle vector (Stratagene)

EQUIPMENT

Microdissection instruments
- ! **CAUTION** All instruments are sterilized by autoclaving
- Dumont no. 5 forceps (Fine Science Tools, cat. no. 11251-20)
- Dumont no. 5SF forceps (Fine Science Tools, cat. no. 11252-00)
- Surgical scissors (8.5 cm; Fine Science Tools, cat. no. 14084-08)
- Extra-fine spring scissors (8.5 cm; Fine Science Tools, cat. no. 15003-08)
- Disposable surgical blades (Schreiber Instrumente, cat. no. SM 11-0020-22)

Cell Culture
- Tissue culture dishes (60 mm; Greiner Bio-One, cat. no. 628160)
- Tissue culture flasks (25 cm^2, Greiner Bio-One, cat. no. 690175)
- Tissue culture flasks (75 cm^2, 250 ml; Greiner Bio-One, cat. no. 658175)
- Tissue culture plates (24 well; Orange Scientific, cat. no. 5530305)
- Cover slips (diameter 12 mm; Menzel, cat. no. CB00120RA1)

TABLE 1 | List of primary antibodies for immunocytochemistry.

Antibody	Host	Dilution	Supplier, cat. no.
βIII tubulin	Mouse (IgG2b)	1:500	Sigma-Aldrich, T8660
CamKIIα	Mouse (IgG1)	1:200	Abcam, ab2725
GFAP	Rabbit	1:2,000	Dako Cytomation, Z0334
GFP	Chicken	1:1,000	Aves Labs, GFP-1020
MAP2	Mouse (IgG1)	1:200	Sigma-Aldrich, M4403
DsRed/red fluorescent protein (RFP)	Rabbit	1:500	Chemicon, AB3216
DsRed/RFP	Rat	1:500	Chromotek, 5F8
Tbr1	Rabbit	1:1,000	Millipore, AB9616
Tbr2	Rabbit	1:500	Millipore, AB9618
vGaT	Guinea pig	1:200	Synaptic Systems, 131004
vGluT1	Rabbit	1:1,000	Synaptic Systems, 135302

- Polypropylene conical tubes (15 ml; Greiner Bio-One, cat. no. 188271)
- Polypropylene conical tubes (50 ml; Greiner Bio-One, cat. no. 277261)
- Cell strainers (70 µm; Falcon, cat. no. 352350)
- Cotton-plugged glass Pasteur pipettes (230 mm; VWR International, cat. no. 612-1702; sterilized by autoclaving)
- Syringe filters (0.2-µm pore size; Starstedt, cat. no. 831826001)
- Bottle-top filter systems (0.22-µm pore, 500 ml; Millipore, cat. no. SCGPT05RE)
- Improved Neubauer hemocytometer (0.1-mm depth, 0.0025 mm^2; Hecht-Assistent, cat. no. 191844272)
- Refrigerated centrifuge and swing-out rotor with adapters for 15 ml and 50 ml tubes (Universal 320R; Hettich Lab Technology, cat. no. 1406)
- Humidified cell culture incubator (set at 37 °C, 5% CO_2; Binder, cat. no. CB210)
- Humidified cell culture incubator (set at 37 °C, 10% CO_2; Binder, cat. no. CB210)
- Microcentrifuge (Eppendorf, cat. no. 5452-000-018)
- Water bath at 37 °C
- Laminar flow hood
- Inverted tissue culture microscope with phase contrast and epifluorescence equipped with ×10 and ×40 objectives (Axiovert 40 CFL, Zeiss)
- Stereomicroscope (MZ6, Leica)

Electrophysiological recordings

- Custom-built Faraday cage with front side open
- TMC 65-560 anti-vibration table (Technical Manufacturing Corporation)
- Axon Axopatch 200B (Molecular Devices)
- Axon Digidata 1322A (Molecular Devices)
- Headstage CV203BU (Molecular Devices)
- SM-5 Controller for six axes (Luigs & Neumann, cat. no. 200-100 900 2621)
- Display for controller (6 × Luigs & Neumann, cat. no. 200-100 910 0030)
- LN-Mini 25 manipulator block XYZ (MRE) (Luigs & Neumann, cat. no. 210-100 000 0010)
- Headstage holder for Axopatch (Luigs & Neumann, cat. no. 100-350-20004-AP)
- Remote control for six axes (Luigs & Neumann, cat. no. 200-100 900 9010)
- Axioskop 2 upright microscope with bright-field and epifluoresence illumination equipped with ×4 (Achroplan 440020) and ×40 (Achroplan 440090) immersion objectives (Zeiss)
- Filter set 43 HE Cy3 shift free (Zeiss, cat. no. 489043-9901-000)
- Glass micropipettes (Inner diameter 1.0 ± 0.05 mm; outer diameter 1.5 ± 0.05 mm; Garner Glass Company, cat. no. KG33)
- PP-830 Glass Microelectrode Puller (Narishige)
- Minipuls 3 Peristaltic Pump (Gilson)
- pCLAMP electrophysiology data acquisition and analysis software (Molecular Devices)
- Clampfit 10 software (Molecular Devices)

Immunocytochemistry

- Epifluorescence microscope BX61 (Olympus)
- Confocal microscope LSM710 (Zeiss)

pH adjustment

- Laboratory pH meter InoLab pH 720 (WTW, Wissenschaftlich-Technische Werkstätten)
- pH-sensitve electrode Sentix 61 (WTW)

REAGENT SETUP

Dissection medium To prepare the dissection medium, add 5 ml of HEPES (1 M) (final concentration, 10 mM) to 500 ml of HBSS 1×. Store the dissection medium at 4 °C for up to 2 weeks.

Dissociation solution To prepare 5 ml of dissociation solution, dissolve 3.4 mg of trypsin powder and 3.5 mg of hyaluronidase powder in 5 ml of neurosphere solution I (see below). ▲ **CRITICAL** This solution must be prepared fresh shortly before use.

Basic medium To prepare basic medium, add 5 ml of 45% D-(+)-glucose and 5 ml of penicillin/streptomycin to 500 ml of DMEM/Ham's F-12. Store the basic medium at 4 °C for up to 2 weeks.

Astro medium To prepare 50 ml of astro medium, add 5 ml of heat-inactivated FBS (final concentration, 10%), 2.5 ml of heat-inactivated horse serum (final concentration, 5%), 1 ml of B27 supplement, EGF at a final concentration of 10 ng ml^{-1}, bFGF at a final concentration of 10 ng ml^{-1} and GlutaMAX at a final concentration of 2 mM; bring to a final volume of 50 ml with basic medium. ▲ **CRITICAL** This medium should be freshly prepared before use.

B27 differentiation medium To prepare 50 ml of B27 medium, combine 1 ml of B27 supplement with GlutaMAX at a final concentration of 2 mM and bring to a final volume of 50 ml with basic medium. ▲ **CRITICAL** This medium should be freshly prepared before use.

Neurosphere solution I (HBSS-glucose) To prepare 500 ml of neurosphere solution I, mix 50 ml of HBSS 10× with 6 ml of 45% D-(+)-glucose and 7.5 ml of HEPES (1 M); bring to a final volume of 500 ml with pure water. Adjust the pH to 7.5 with NaOH (1N) and a lab pH meter. Filter-sterilize the solution, prepare appropriate aliquots and store at −20 °C for up to 6 months.

Neurosphere solution II (saccharose-HBSS) To prepare 500 ml of neurosphere solution II, combine 25 ml of HBSS 10×, and D-(+)-saccharose at a final concentration of 0.9 M (154 g); bring to a final volume of 500 ml with pure water. Adjust the pH to 7.5 with NaOH (1N) and a lab pH meter. Filter-sterilize the solution, prepare appropriate aliquots and store at −20 °C for up to 6 months.

Neurosphere solution III (BSA-EBSS-HEPES) To prepare 500 ml of neurosphere solution III, combine 10 ml of HEPES (1 M) and 4% (wt/vol) BSA; bring to a final volume of 500 ml with EBSS. Adjust the pH to 7.5 with NaOH (1N) and a lab pH meter. Filter-sterilize the solution, prepare appropriate aliquots and store at −20 °C for up to 6 months.

Neurosphere medium To prepare 50 ml of neurosphere medium, mix 1 ml of B27 supplement with 0.4 ml of HEPES (1 M), 0.5 ml of penicillin/streptomycin, EGF at a final concentration of 20 ng ml^{-1}, and bFGF at a final concentration of 20 ng ml^{-1}; bring to a final volume of 50 ml with DMEM/Ham's F-12. ▲ **CRITICAL** This medium should be freshly prepared before use.

Epidermal growth factor Centrifuge the recombinant human EGF vial briefly before opening to bring the contents to the bottom. Reconstitute the lyophilized EGF (100 µg) with 10 ml of basic medium. The final concentration of the EGF is 10 µg ml^{-1}. Prepare 100-µl aliquots and store them at −20 °C for up to 6 months. Avoid freeze/thaw cycles.

Basic fibroblast growth factor Centrifuge the recombinant human bFGF vial briefly before opening to bring the contents to the bottom. Reconstitute the lyophilized bFGF (10 µg) with 1 ml of basic medium. The final concentration of the bFGF is 10 µg ml^{-1}. Prepare 100-µl aliquots and store them at −20 °C for up to 6 months. Avoid freeze/thaw cycles.

Brain-derived neurotrophic factor Centrifuge the recombinant human BDNF vial briefly before opening to bring the contents to the bottom. Reconstitute the lyophilized BDNF (10 µg) in 1 ml of basic medium. The final concentration of BDNF is 10 µg ml^{-1}. Prepare 50-µl aliquots and store them at −20 °C for up to 6 months. Avoid freeze/thaw cycles.

PDL stock solution Dissolve 50 mg of PDL powder in pure water at a concentration of 1 mg ml^{-1}, and then filter-sterilize the solution. Prepare 1-ml aliquots and store them at −20 °C for up to 6 months.

PDL working solution Add 1 ml of the PDL stock solution to 50 ml of sterile PBS, filter-sterilize the solution and store it at 4 °C for up to 2 weeks.

PDL coating of the glass cover slips Before coating, the glass cover slips need to be cleaned according to the following procedure. Put the glass cover slips in a beaker, cover them with a solution of HCl (0.1 M) and shake for 1 h. Afterward, transfer them into acetone, place the beaker for the first 20 min in an ultrasonic bath and then shake (~50 r.p.m.) for 1 h. Then transfer the cover slips in a 70% (vol/vol) ethanol solution and shake (~50 r.p.m.) for 1 h. Finally, rinse them in 100% ethanol solution and let them dry on a paper towel under the laminar airflow.

Add 500 µl of PDL working solution to each well of a 24-well tissue culture plate, each well containing a glass cover slip. Incubate the plate for at least 2 h or overnight at 37 °C. Wash thoroughly three or four times with pure water, and let the cover slips dry in a laminar airflow. Store coated glass cover slips at 4 °C for no longer than 1 week. ▲ **CRITICAL** The best adherence of the plated cells is obtained on fully dried glass cover slips.

Paraformaldehyde (4% (wt/vol)) Add 40 g of PFA powder to 500 ml of PBS (1×) and heat to 60 °C while stirring the solution. Wait until the solution becomes clear. Cool down the solution on ice. Bring to a final volume of 1 liter with PBS (1×). Filter the solution and adjust the pH to 7.4 using HCl (1N) and a lab pH meter. Prepare 10-ml aliquots and store at −20 °C for up to 6 months. ! **CAUTION** Because of the toxic nature of PFA, this procedure should be carried out under a flow hood. Do not inhale PFA fumes.

Extracellular solution To prepare 1 liter of extracellular solution, mix 150 ml of NaCl (1 M) with 3 ml of KCl (1 M), 3 ml of $CaCl_2$ (1 M), 2 ml of $MgCl_2$ (1 M), 10 ml of HEPES (1 M) and 5 ml of D-glucose (1 M); bring to a final volume of 1 liter with pure water. Adjust the pH to 7.4 with NaOH (1N) and a lab pH meter. The final osmolarity of the solution should be 310 ± 10 mOsm. ▲ **CRITICAL** Store the freshly prepared solution at 4 °C for up to 1 week.

Intracellular solution To prepare 100 ml of intracellular solution, mix 3.1975 g of potassium gluconate with 583 µl of KCl (3 M), 300 µl of NaCl (3 M), 50 µl of $MgCl_2$ (2 M), 100 µl of HEPES (1 M) and 100 µl of EGTA (0.2 M); bring to a final volume of 100 ml with pure water. Adjust the pH to 7.4 using KOH (0.1 N) and a lab pH meter. The final osmolarity of the solution should be 300 ± 10 mOsm. ▲ **CRITICAL** Store the solution in 15-ml aliquots at −20 °C for up to 6 months. When thawed, an aliquot can be used for up to 1 week.

Amphotericin B stock solution Dissolve 4 mg of amphotericin B in 80 µl DMSO, vortex thoroughly (5 min), sonicate (5 min), vortex once more (5 min) and aliquot at 2 µl volume. Store aliquots at −20 °C for up to 1 month. The final concentration of the stock solution is 50 mg ml^{-1}. ▲ **CRITICAL** Amphotericin B is light sensitive; therefore, use light-protected microtubes.

Amphotericin B working solution Add 500 µl of intracellular solution to a freshly prepared or thawed aliquot of amphotericin B stock solution (2 µl at 50 mg ml^{-1}). ▲ **CRITICAL** Amphotericin B is light sensitive; therefore, use light-protected microtubes.

Retroviral vectors Retroviruses pseudotyped with VSV-G (vesicular stomatitis virus-glycoprotein) encoding *Neurog2*, *Dlx2* or *Mash1* were used as described previously[4]. *Neurog2*, *Dlx2* or *Mash1* are expressed under the regulatory control of an internal compound CAG promoter[29] together with DsRed or GFP (as reporter proteins) followed by an internal ribosomal entry site (IRES) to allow for simultaneous reporter gene expression (**Fig. 2**). The *Neurog2*-coding cDNA was isolated from the pCLIG-*Neurog2* (ref. 8) and subcloned into the EcoRI site of the pSKSP shuttle vector (Stratagene). From there, it was then placed between the 5′-SfiI and 3′-PmeI restriction sites within the pCAG retroviral vector. The resulting construct is referred to as pCAG-*Neurog2*-IRES-*DsRed*. Likewise, the *Mash1*-coding cDNA was subcloned from pCLIG-*Mash1* (ref. 8) to generate pCAG-*Mash1*-IRES-*DsRed*. Following the same strategy, *Dlx2*-coding cDNA was subcloned from the pMXIG-*Dlx2* (ref. 33) and inserted into the pCAG retroviral vector to generate pCAG-*Dlx2*-IRES-*DsRed*. For control experiments, we use a virus encoding *DsRed* behind the IRES driven by the same compound CAG promoter (pCAG-IRES-*DsRed*).

To generate the VSV-G-pseudotyped viral particles, we used a clonal derivative of the retroviral packaging cell line 293GPG[34]. These HEK293-derived cells stably express the *gag-pol* genes of murine leukemia virus and *vsv-g* under the control of a tet/VP16 transactivator (Tet-off). Alternatively, HEK293T cells can be co-transfected with the recombinant viral vector containing neurogenic fate determinants and the plasmids encoding *gag-pol* genes and *vsv-g*. For a detailed protocol for retrovirus preparation and titration see reference 29. ▲ **CRITICAL** We obtained the best efficiency of astroglia-to-neuron conversion with strong and persistent expression of the neurogenic fate determinants driven by the silencing-resistant pCAG promoter. ▲ **CRITICAL** Prepare appropriate aliquots of the concentrated viral suspension and store at −80 °C until use. Avoid repeated freeze/thaw cycles. ! **CAUTION** Production, concentration and handling of VSV-G-pseudotyped retroviruses must be done in accordance with the local biosafety guidelines of the institution and under S2/BL2 conditions.

PROCEDURE

Preparation of primary culture of adherent astroglia from the mouse postnatal cortex ● TIMING ~7d

1| Decapitate one (or several) mouse (mice) at the age of postnatal days 5–7 (P5–P7). Isolate the head and extract the whole brain.

! **CAUTION** Handling of experimental animals must be performed in accordance with all relevant governmental and institutional regulations regarding the use of animals for research purposes.

2| Transfer the brain into a 60-mm tissue culture dish containing 10 ml of ice-cold dissection medium.

3| Remove the cerebellum with a blade and discard it (as depicted in **Fig. 1**, black line). Cut the brain coronally into two equal parts at the level of the optic chiasm and discard the anterior part (as depicted in **Fig. 1**, red line). Cut the rest of the brain along the longitudinal fissure to separate it into two equal hemispheres.

▲ **CRITICAL STEP** We discard the anterior part of the brain to eliminate any risk of possible contamination of the astroglia culture by progenitor cells residing in the subependymal zone.

4| Carefully remove the meninges using fine forceps. Separate the cortical gray matter tissue of both hemispheres from the diencephalon, hippocampal formation and the developing white matter (containing myelinating oligodendrocytes and the first myelin sheets).

▲ **CRITICAL STEP** Carefully remove the potentially remaining developing white matter from the dissected cortical gray matter tissue to ascertain the purity of the culture.

5| Using two blades, mince the dissected cortices into small tissue pieces and transfer them into a 15-ml conical tube containing 2 ml of ice-cold dissection medium (see also **Box 1** for the preparation of neurospheres from cortical astroglia that can be used as an alternative procedure at this step).

6| Pipette the mixture up and down a few times (approximately ten times) with a fire-polished glass Pasteur pipette to dissociate the tissue mechanically and achieve a single-cell suspension, and then add 13 ml of ice-cold dissection medium.

▲ **CRITICAL STEP** Avoid generating air bubbles when triturating the tissue, as this will reduce the viability of the cells.

7| Centrifuge the cell suspension at 120*g* (1,000 r.p.m.) for 5 min at 4 °C, aspirate the supernatant and resuspend the cells with 6 ml of prewarmed (37 °C) and CO_2-equilibrated astro medium by gently triturating the cellular pellet. Transfer the

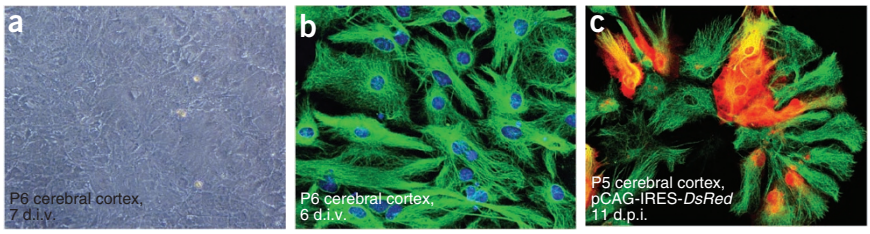

Figure 3 | Primary culture of adherent astroglial cells from the mouse postnatal cerebral cortex. (**a**) Bright-field (BF) micrograph depicting live-imaged, adherent astroglial cells isolated from the cerebral cortex of postnatal day 6 (P6) mice at the end of the expansion phase in the tissue culture flask (after 7 d *in vitro* (d.i.v.)). (**b**) Double-immunostaining for GFAP (green) and DAPI (blue) reveals that the vast majority of the cultured cells are GFAP-positive astroglia. Cells isolated from the cerebral cortex of P6 mice shown in **a** were passaged, seeded onto glass cover slips and stained at day 6 *in vitro* (d.i.v.) after seeding. (**c**) Astroglia from the cerebral cortex of postnatal day 5 (P5) mice transduced with a control retrovirus (pCAG-IRES-*DsRed*, red) remain in the astroglial lineage, as revealed by maintenance of GFAP expression (green). Immunostaining was performed at 11 d post infection (d.p.i.).

cell suspension into a 25-cm^2 (or 75-cm^2, in which case resuspend the cells with 12 ml of medium) tissue culture flask and incubate at 37 °C with 5% CO_2 for 5–7 d.

8| After 3 d, remove the medium and add 6 ml of fresh basic medium. Gently shake the tissue culture flask and remove the basic medium. Add 6 ml of fresh, prewarmed (37 °C) and CO_2-equilibrated astro medium. We usually achieve a monolayer of astroglial cells with 70–80% confluence within 5–7 d in culture (**Fig. 3a**).

Passaging and plating of astroglial cells ● TIMING ~2 h

9| When the cells become confluent (generally after 5–7 d), remove the astro medium from the flask and wash the cell monolayer with 5 ml of PBS.

▲ **CRITICAL STEP** A few contaminating oligodendrocyte precursor cells may have grown typically on top of the astroglia monolayer. Remove these cells by brusquely shaking the culture flask several times.

10| Remove the PBS and the floating cells and discard them. Add enough (0.05% (wt/vol)) trypsin/EDTA to cover the cells. Tilt the flask to distribute the trypsin over the cells. Incubate for 5 min at 37 °C. Gently tap the flask on its side from time to time to dislodge all cells.

11| Once all the cells have detached from the flask, collect the cells in the trypsin solution and transfer the cell suspension to a 15-ml conical tube. Add 5 ml of fresh, prewarmed (37 °C) and CO_2-equilibrated astro medium and mix by gently tapping.

12| Centrifuge at 120*g* (1,000 r.p.m.) for 5 min at room temperature (20–25 °C).

13| Remove the supernatant and resuspend the cells in an appropriate volume (~1 ml for a confluent 25-cm^2 tissue culture flask or ~3 ml for a confluent 75-cm^2 tissue culture flask) of fresh astro medium by slowly pipetting the mixture up and down a few times using a 5-ml disposable pipette, until the cell suspension looks homogenous.

▲ **CRITICAL STEP** Avoid generating air bubbles when triturating the cell suspension, as this will reduce the viability of the cells.

14| Count cells in a hemocytometer, using Trypan blue to exclude dead cells, as previously described[35]. Usually, we obtain ~1.0- to 1.5 × 10^6 cells from one confluent 25 cm^2 tissue culture flask. Dilute the cells by adding fresh, prewarmed (37 °C) and CO_2-equilibrated astro medium to obtain 50,000–60,000 cells per 100 µl of medium.

15| Seed 100 µl of cell suspension directly onto each of the air-dried, PDL-coated glass cover slips in a 24-well tissue culture plate (see REAGENT SETUP), and incubate for 1 h at 37 °C with 5% CO_2.

▲ **CRITICAL STEP** Make sure that the cell suspension stays on the cover slips, and no cells are wasted by direct adherence to the well outside the glass cover slip. Avoid generating air bubbles when seeding the cells.

16| After 1 h, when the vast majority of the cells have attached to the cover slips, add 400 µl of fresh, prewarmed (37 °C) and CO_2-equilibrated astro medium to each well. Incubate the cells at 37 °C with 5% CO_2 for 2–4 h. Immunocytochemistry for GFAP (**Table 1**) may be performed to ensure the purity of the astroglial cultures, as described in **Box 2** (wait for an additional ~24 h before performing the immunostaining) (**Fig. 3b–c**; see also ref. 5).

Retroviral transduction or plasmid transfection of the astroglial cells

17| At this point, the cells can either be transduced with retroviral vectors using option A or transfected with retroviral plasmids using option B.

BOX 2 | IMMUNOCYTOCHEMISTRY ● TIMING 1 H 30 MIN ON DAY 1 AND 2 H 30 MIN ON DAY 2

Day 1:
1. Remove the medium from the cells (from Step 16, 18 or 21) by aspiration and discard it. Wash the cells three times with 1 ml of PBS.
2. Fix the cells with 500 µl of 4% (wt/vol) paraformaldehyde in PBS for 15 min at room temperature.
3. Wash the cells three times with 1 ml of PBS.
4. Pretreat the cells with 0.5% (vol/vol) Triton X-100 in 50–80 µl PBS for 30 min.
5. Incubate the cells with 50–80 µl PBS containing 2% (wt/vol) BSA and 0.5% (vol/vol) Triton X-100 for 30 min.
5. Add primary antibodies (see **Table 1**) diluted in 50–80 µl PBS containing 2% (wt/vol) BSA and 0.5% (vol/vol) Triton X-100 and incubate overnight at 4 °C.

Day 2:
6. Remove the primary antibody solution by aspiration and discard it. Wash the cells three times with PBS (5 min each time).
7. Add the appropriate species- or subclass-specific secondary antibodies diluted in PBS and incubate for 2 h in the dark at room temperature.
8. Wash the cells three times with PBS (5 min each time).
9. Mount the glass cover slips onto microscope glass slides with anti-fading mounting medium.
10. Analyze the cover slips using an epifluorescence microscope (BX61, Olympus) or a confocal microscope (LSM 710, Zeiss).

(A) Transduction of the astroglial cells with retroviral particles ● TIMING ~24 h

(i) At 2–4 h after seeding the cells on the cover slips, add 1 µl of the appropriate concentrated retroviral vector (pCAG-*Neurog2*-IRES-*DsRed*, pCAG-*Dlx2*-IRES-*DsRed* or pCAG-*Mash1*-IRES-*DsRed*—see REAGENT SETUP for further details; titer ranging from: 1×10^6 to 1×10^8 colony-forming units (c.f.u.) ml^{-1}) to 500 µl of astro medium and gently shake the tissue culture plate to ensure the homogenous distribution of the viral particles. For details of titration of the retroviral particles please refer to ref. 29. Transduce astroglial cells with vectors encoding only *DsRed* behind an IRES sequence as a control (pCAG-IRES-*DsRed*).

▲ **CRITICAL STEP** The best rate of transduction of astroglial cells is typically obtained with high-titer (concentrated) retroviral pseudotyped particles ($>1 \times 10^7$ c.f.u. ml^{-1}).

! CAUTION When working with viral particles, you should refer to the local biosafety guidelines of your institution and handle the virus under S2/BL2 conditions.

? TROUBLESHOOTING

(ii) Incubate the cells for 24 h at 37 °C with 5% CO_2.

? TROUBLESHOOTING

(B) Transfection of the astroglial cells with expression plasmids ● TIMING ~24 h

(i) Transfer 50 µl of Opti-MEM into a 2-ml tube.

(ii) Transfer 0.5 µl of Lipofectamine 2000 directly into the Opti-MEM prepared in Step 17B(i), mix gently by tapping and incubate for 5 min at room temperature.

(iii) Transfer 50 µl of Opti-MEM into a 2-ml tube, add 0.5 µg of the appropriate retroviral plasmid DNA (pCAG-*Neurog2*-IRES-*DsRed*, pCAG-*Dlx2*-IRES-*DsRed*, pCAG-*Mash1*-IRES-*DsRed* or pCAG-IRES-*DsRed*—see REAGENT SETUP for further details)—and mix gently by tapping.

(iv) Add 50 µl of Lipofectamine 2000–Opti-MEM mixture prepared in Step 17B(ii) dropwise to the plasmid-containing tube prepared in Step 17B(iii), mix gently by tapping and incubate for 20 min at room temperature.

(v) Remove the astro medium from the cells from Step 16 and collect it as conditioned medium for later reuse. Add 300 µl of fresh and prewarmed (37 °C) Opti-MEM to the cells.

(vi) After 20 min incubation, add the DNA/Lipofectamine 2000 complexes prepared in Step 17B(iv) (total volume 100 µl) dropwise onto the 300 µl of Opti-MEM covering the cells and gently move the tissue culture plate for homogeneous distribution of the DNA/Lipofectamine 2000 complexes.

(vii) Incubate for 4 h at 37 °C with 5% CO_2.

(viii) Remove the transfection medium and add 250 µl of the prewarmed (37 °C) and CO_2-equilibrated, conditioned astro medium collected in Step 17B(v) plus 250 µl of prewarmed (37 °C) and CO_2-equilibrated fresh astro medium.

? TROUBLESHOOTING

(ix) Incubate the cells for 20 h at 37 °C with 5% CO_2.

? TROUBLESHOOTING

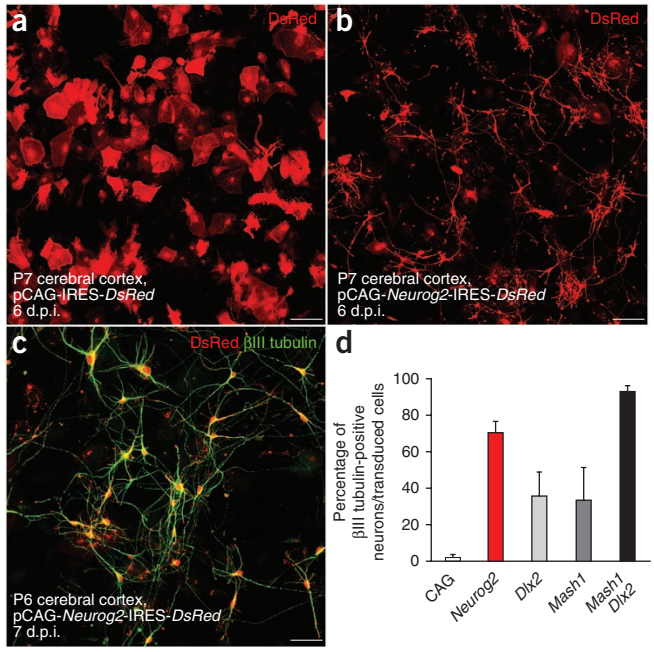

Figure 4 | Postnatal cortical astroglia differentiate into βIII tubulin-positive neurons on forced expression of neurogenic fate determinants. (**a**) The micrograph depicts astroglial cells 6 d post infection (d.p.i.) with a control retroviral construct encoding *DsRed* only (pCAG-IRES-*DsRed*). Note that the cells exhibit typical astroglial morphology. (**b**) Representative example of astroglial cells 6 d.p.i. with a retrovirus encoding *Neurog2* (pCAG-*Neurog2*-IRES-*DsRed*). Note that the vast majority of the reporter-positive cells show an immature neuron morphology extending one or two long processes. (**c**) Double immunocytochemistry for DsRed (red) and βIII tubulin (green) reveals that the majority of the transduced astroglial cells are converted into βIII tubulin-positive neurons following forced expression of *Neurog2*, as shown 7 d.p.i. (**d**) The graph shows the percentage of βIII tubulin-positive neurons among all transduced cells following infection with retroviruses encoding *DsRed* only (CAG; white bar), *Neurog2* (red bar), *Dlx2* (gray bar), *Mash1* (dark gray bar) or *Mash1* and *Dlx2* together (black bar). Cultures were analyzed at 7.3 ± 1.0, 9.8 ± 3.1 and 10.7 ± 2.0 d following transduction with a control vector (CAG), *Neurog2* and *Dlx2-Mash1-Mash1/Dlx2*, respectively. The error bars represent the s.e.m. of the percent of neurons among reporter positive cells. This panel has been adapted with permission from ref. 4. Astroglia were isolated between postnatal days 6–7 (P6–P7) as indicated in the fluorescence micrographs. Scale bars in (**a**,**b**), 120 μm; Scale bar in **c**, 60 μm.

Astroglia-to-neuron conversion of astroglial cells ● **TIMING ~7 d to 4 weeks**

18| At 24 h after completion of retroviral transduction or plasmid transfection, remove the astro medium from the cells and add 1 ml of fresh, prewarmed (37 °C) and CO_2-equilibrated B27-differentiation medium. To estimate transduction or transfection efficiency, one to three cover slips containing astroglial cultures transduced or transfected in parallel to the experimental group can be fixed after 48 h to allow expression of the reporter gene and immunostained for the expression of DsRed and GFAP (see **Box 2**). Count the number of DsRed-positive cells among all GFAP-positive cells.

19| Maintain the cells in culture in B27 differentiation medium at 37 °C with 10% CO_2 until the emergence of astroglia-derived neurons. Immature astroglia-derived neurons can usually be observed 5–7 days post infection (d.p.i.), with the retrovirus encoding the expression of neurogenic fate determinants. They will further develop over time in culture and become functional neurons after 3–4 weeks.

▲ **CRITICAL STEP** We obtained the best efficiency of astroglia-to-neuron conversion, as well as the most advanced neuronal maturation and synapse formation by incubating the cells in a 10% CO_2 atmosphere. We had the best experience with no change of medium throughout the course of astroglia-to-neuron conversion and neuronal differentiation, apart from adding BDNF (see below).

20| Add BDNF at a final concentration of 20 ng ml^{-1} every fourth day during the differentiation period (starting on day 5 following transduction or transfection) in order to promote the maturation of the astroglia-derived neurons and synapse formation.

21| Characterize the cells for their astroglia-to-neuron conversion by immunocytochemistry (see **Box 2**) to monitor the loss of astroglial markers such as GFAP; the acquisition of neuronal markers such as βIII tubulin or microtubule-associated protein 2 (MAP2) (**Fig. 4**); and the expression of T-box brain 1 protein (Tbr1), T-box brain 2 protein (Tbr2), vesicular glutamate transporter 1 (vGluT1), Ca^{2+} calmodulin-dependent kinase II alpha (CamKIIα) for the glutamatergic sublineage and vesicular GABA transporter (vGaT) for the GABAergic sublineage (**Table 1**) (**Fig. 5a–d,f**). In addition, electrophysiological recordings (see **Box 3**) allow assessment of the proper acquisition of electrical neuronal properties and the formation of glutamatergic or GABAergic autapses or synapses (**Fig. 5e–h**; see also refs. 4,5). Astroglial cells transduced with control retrovirus remain in the glial lineage, show an astroglial morphology and express GFAP (**Figs. 3c** and **4a**).

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
17A(i)	Low transduction efficiency	Low-titer retrovirus	Use high-titer retrovirus; avoid repeated freeze/thaw cycles of viral vector aliquots
17A(ii)	Cell death after retrovirus transduction	Endotoxin-related toxicity	Use endotoxin-free or cesium chloride plasmid DNA preparations for retrovirus production; clean the viral suspension by several washing steps and centrifugation
17B(viii)	Low transfection efficiency	Low quality of the plasmids	Use high-quality plasmids (endotoxin-free plasmid purification). Avoid repetitive freeze/thaw cycles of plasmid aliquots to prevent DNA nicking and degradation
		Non-optimal DNA/Lipofectamine 2000 complex formation	Optimize the ratio of DNA/Lipofectamine 2000 by decreasing the amount of DNA compared with that of Lipofectamine 2000
17B(ix)	Cell death after plasmid transfection	Endotoxin-related toxicity	Use endotoxin-free or cesium chloride plasmid DNA preparations for transfection
		Poor survival of the transfected cells	Reduce Lipofectamine 2000 amount. Increase (approximately double) the serum proportion in astro medium following transfection
21	No neuronal reprogramming	Non-optimal culture conditions; cell death of the transduced cells; cell death of the reprogrammed cells	Ensure that culture conditions allow for the survival of neurons (for instance, change the B27 lot or try alternative supplement medium)
		Low expression of neurogenic fate determinant	Increase the expression of neurogenic fate determinants using silencing-resistant vectors
21	No synapse formation after *Neurog2* overexpression	Non-optimal culture conditions	Ensure that culture conditions allow the long-term survival of neurons (for instance, change the B27 lot or try alternative supplement medium)
		Partial reprogramming	Increase the expression of neurogenic fate determinants using silencing-resistant vectors

● TIMING

Steps 1–8, preparation of primary culture of adherent astroglia from the mouse postnatal cortex: 7 d
Steps 9–16, passaging and plating of astroglial cells: 2 h
Step 17, retroviral transduction or plasmid transfection of astroglial cells: 24 h
Steps 18–21, astroglia-to-neuron conversion of astroglial cells: 7 d to 4 weeks

ANTICIPATED RESULTS

With this protocol, postnatal astroglia isolated from the mouse cerebral cortex can be directed *in vitro* by overexpression of neurogenic fate determinants toward the generation of functional neurons establishing synapses after 3–4 weeks. Importantly, astroglia can be converted into different neuronal subtypes by selective expression of distinct neurogenic transcription factors. Whereas forced *Neurog2* expression induces a glutamatergic neuron identity, encompassing the cascade of transcription factor expression Neurog2→Tbr2→Tbr1, forced expression of *Mash1* and/or *Dlx2* induces a GABAergic neuron fate. There are important differences in the efficiency of the respective factors to induce a neuronal fate and to promote differentiation toward full functionality.

Efficiency of glia-to-neuron conversion

The overall proportion of astroglial cells converted into neurons depends on various parameters. First, paramount to a high degree of astroglia-to-neuron conversion is a high transduction or transfection efficiency (see Step 18). Second, successful neuronal conversion typically requires high expression levels of the neurogenic fate determinants. This dependence varies with the transcription factor used. For instance, we found that *Dlx2* expression driven from a weak and silencing-prone retroviral vector (pMXIG; compared with the pCAG-vector) resulted in nearly negligible neurogenesis in contrast to *Dlx2* encoded

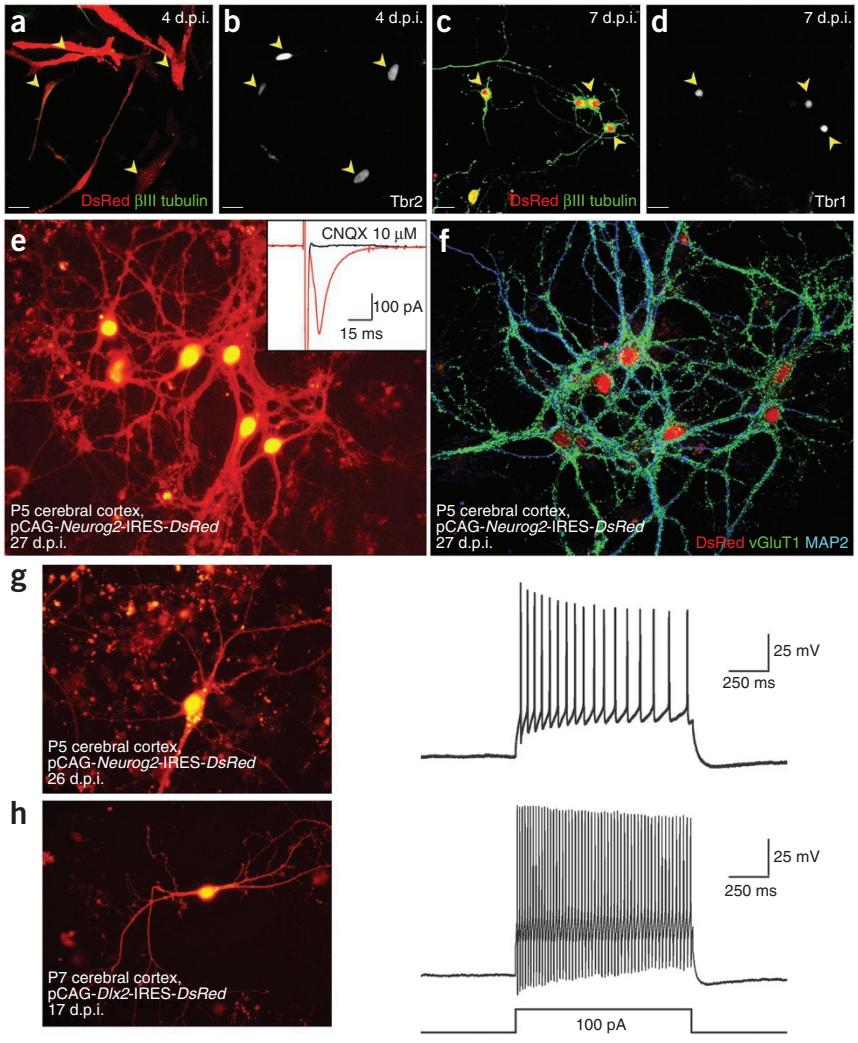

Figure 5 | Postnatal cortical astroglia give rise to distinct types of neurons following forced expression of *Neurog2* or *Dlx2*. (**a**–**d**) Astroglia-to-neuron conversion induced by *Neurog2* involves the sequential upregulation of Tbr2 and Tbr1. (**a**) At 4 d post infection (d.p.i.) βIII tubulin expression is barely detectable. (**b**) Upregulation of Tbr2 (white) by 4 d.p.i. (arrowheads in **a** and **b**). (**c**) Up-regulation of βIII tubulin 7 d.p.i. following forced *Neurog2* expression. (**d**) Immunostaining for Tbr1 (white) reveals the up-regulation of Tbr1 at 7 d.p.i. (arrowhead in **c** and **d**). (**e**) A cluster of live-imaged, astroglia-derived neurons at 27 d.p.i. following forced *Neurog2* expression. The inset shows that step-depolarization evokes an autaptic response (red trace) that is abolished in the presence of the AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate)/kainate receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dion; 10 μM, black trace). (**f**) Triple immunostaining for DsRed (red), MAP2 (blue) and vGluT1 (green) reveals that astroglia-derived neurons, following forced expression of *Neurog2*, show a dense labeling of vGluT1-positive puncta outlining their soma and their MAP2-positive processes. This panel has been adapted with permission from ref. 4. (**g**) A live-imaged astroglia-derived neuron following forced *Neurog2* expression. The trace depicts repetitive firing of action potentials at moderate frequency with frequency adaptation, consistent with a pyramidal neuron-like identity. Step-current injection, 100 pA. (**h**) A live-imaged astroglia-derived neuron at 17 d.p.i. with *Dlx2*-encoding retrovirus. The trace shows repetitive firing of action potentials at high frequency with little frequency adaptation, characteristic for a subclass of GABAergic interneurons. Step-current injection, 100 pA. Astroglia were isolated between postnatal days 5–7 (P5–P7) as indicated in the fluorescence micrographs. Scale bar (**a**–**d**), 20 μm.

by a strong and silencing-resistant vector (pCAG; **Fig. 2**). However, in the case of *Neurog2*, the dependence is less obvious, as similar numbers of astroglia-derived neurons can be obtained using silencing-prone (pCLIG) or silencing-resistant (pCAG) vectors. Yet, the degree of functionality of the neurons obtained by using silencing-resistant vectors is markedly higher. Third, in contrast to astroglia undergoing neuronal conversion[4,5], astroglia that do not respond to neurogenic cues often continue proliferating. Therefore, the relative proportion of astroglia failing to undergo neuronal conversion may increase over time in culture. Finally, single-cell tracking with time-lapse video microscopy (see **Supplementary Video 1**) reveals that during the process of astroglia-to-neuron conversion, a not yet quantified level of cell death occurs, which is likely to result from a catastrophic conflict of cell fate signals. For details on the single-cell tracking method see reference 36.

Morphological changes

As conversion of postnatal astroglia into neurons by forced expression of neurogenic fate determinants is a gradual process, distinct maturational steps can be distinguished[27]. Direct observation by single-cell tracking[36] suggests that the onset of astroglia-to-neuron conversion is not much delayed compared with the onset of forced expression of the respective fate determinants, as assessed by reporter expression (**Supplementary Video 1**). Once astroglia-to-neuron conversion commenced, an immature neuronal morphology is typically acquired between 5–7 d after transduction or transfection (**Figs. 4b**,**c** and **5c** and **Supplementary Video 1**; see also ref. 4). In case of the *Neurog2*-induced glia-to-neuron conversion, single-cell tracking shows that astroglia subject to astroglia-to-neuron conversion often undergo a sequence of distinct morphological transitions from a multipolar to a mono- or bipolar stage, resembling the morphological transitions of cortical precursors during development (**Supplementary Video 1**). At later stages, astroglia-derived neurons acquire a morphology that is different, depending on their glutamatergic or GABAergic subtype identity. Indeed, neurons derived from astroglia following forced expression of *Neurog2* exhibit a large soma size with more than three primary dendrites (**Fig. 5e**,**g**), whereas neurons derived from

BOX 3 | ELECTROPHYSIOLOGICAL RECORDINGS

The steps delineated here follow standard procedures for whole-cell perforated patch-clamp recordings of cultured neurons[38,39] following astroglia-to-neuron conversion. Recording of single neurons allows the assessment of neuronal electrical properties such as repetitive action potential firing as well as the presence of autaptic connections (i.e., synaptic connections of a given neuron onto itself). For the assessment of synaptic connections between two neurons following astroglia-to-neuron conversion by dual recording, the setup must be adapted for the simultaneous recording of a second cell. Electrical signals are sampled at 10 kHz and filtered at 5 kHz with Axopatch 200B patch-clamp amplifiers coupled to DIGIDATA 1322A using pCLAMP 10 software. Data are subsequently analyzed with Clampfit 10 software. For a more detailed protocol for voltage-clamp and current-clamp patch-clamp recordings using Axopatch 200 B patch-clamp amplifiers, see Cummins *et al.*[40]. For further details on whole-cell recording using the amphotericin B perforated patch-clamp technique see Lippiat[41].

1. Transfer cover slips containing cells following astroglia-to-neuron conversion (from Step 21) into a perfusion chamber mounted on an Axioskop 2 microscope placed on an anti-vibration table surrounded by a Faraday cage. The perfusion chamber should be constantly perfused at room temperature at 0.5 ml min^{-1} with extracellular solution containing 150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES and 5 mM glucose (pH 7.4) at an osmolarity of 310 mOsm.
2. Select transduced cells for patch-clamp recording on basis of their DsRed-fluorescence using a ×40 immersion objective (**Fig. 5e,g,h**).
3. Prepare micropipettes from borosilicate glass capillaries using a microelectrode glass puller. When placed into extracellular solution, the micropipettes should have resistances of 2–2.5 MΩ.
4. Tip-fill the micropipette with an intracellular solution consisting of 136.5 mM K-gluconate, 17.5 mM KCl, 9 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES and 0.2 mM EGTA (pH 7.4) at an osmolarity of 300 mOsm and back-fill the micropipette with intracellular solution containing an additional 200 μg ml^{-1} amphotericin B for perforation.
5. Place the filled micropipette onto the recording headstage, coupled to an Axopatch 200B patch-clamp amplifier.
6. Move filled micropipette close to a selected cell under bright-field illumination using micromanipulators.
7. Place the micropipette slowly in direct contact with the plasma membrane of the cell, which can be monitored by a reduction of the current induced by a square voltage pulse (5 mV).
8. Establish a GΩ seal onto the selected neuron by gentle aspiration. Wait for 5 min for the perforation to occur.
9. To assess whether a given cell is capable of (repetitive) action potential firing, stimulate the cell under current clamp condition at a holding potential of −70 mV by depolarizing step-current injection (lasting for 1,000 ms).
10. To assess the presence of autaptic connections[42], step-depolarize the cell in voltage-clamp for 1 ms from −70 mV to + 30 mV at a frequency of 0.05 Hz.
11. If autaptic responses can be detected, assess whether they can be abolished in the presence of either the $GABA_A$ receptor antagonist bicuculline methiodide (5–10 μM) or the AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate)/kainate receptor antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dion, 10 μM) to determine the neurotransmitter released at the autapse.

astroglia following forced expression of *Dlx2* or *Mash1* are considerably smaller in size and often bipolar (**Fig. 5h**). As revealed by immunoreactivity for Ca^{2+} calmodulin-dependent kinase II α, neurons derived from *Neurog2*-transduced astroglia exhibit spine-like structures covering their dendritic processes by 4 weeks after transduction[4].

Immunocytochemical changes

Astroglia-to-neuron conversion is accompanied by the rapid loss of GFAP expression. In the case of *Neurog2*-induced astroglia-to-neuron conversion, we observed a transient up-regulation of Tbr2 as one of the earliest signs of successful astroglia-to-neuron conversion in 20.7 ± 1.9% of the DsRed-positive cells by 4 d.p.i., preceding the expression of the neuronal marker βIII tubulin (**Fig. 5a,b**). Subsequently, *Neurog2*-transduced cells start to express Tbr1 reaching up to 48.2% at 7 d after transduction (**Fig. 5c,d**), and many converted astroglial cells maintain Tbr1 expression thereafter. However, neither Tbr2 nor Tbr1 are detected in all *Neurog2*-transduced cells, indicating that their expression is either transient or restricted to a subpopulation of cells. At 10 d post transduction, 70.2 ± 6.3% (at 9.8 ± 3.1 d.p.i.) of the *Neurog2*-transduced cells express βIII tubulin, indicating the acquisition of a neuronal identity (**Fig. 4c–d**). By 2–3 weeks after transduction, neurons derived from *Neurog2*-transduced astroglia acquire MAP2 immunoreactivity as a sign of dendritic maturation (**Fig. 5f**). Along with the establishment of functional glutamatergic connections, by 4 weeks after transduction, 85.4 ± 5.0% of the *Neurog2*-transduced cells express the vesicular glutamate transporter 1 (vGluT1; at 26.3 ± 2.2 d.p.i., **Fig. 5f**). It is important to be aware that vGluT1-immunopositive puncta label presynaptic terminals and do not necessarily originate from the neuron covered by the puncta. Thus, they cannot serve to characterize the neurotransmitter identity of the cell. However, as all neurons in these cultures are derived from astroglia, vGluT1-positive terminals must also originate from astroglia-derived neurons. Moreover, neurons tend to form autaptic connections *in vitro* (see below), and therefore some of the vGluT1-positive terminals often originate from the same neuron the terminals impinge onto. Finally, consistent with their glutamatergic identity, neurons derived from *Neurog2*-transduced astroglia exhibit Ca^{2+}-calmodulin-dependent kinase II α immunoreactivity.

As in *Neurog2*-induced astroglia-to-neuron conversion, *Mash1* or *Dlx2* induce a rapid loss of GFAP expression, and upregulation of βIII tubulin or MAP2. However, although using the same vector backbones to drive expression, only ~40% of the *Mash1*- or *Dlx2*-transduced astroglia generate neurons (*Mash1*: 33.5 ± 17.8%, *Dlx2*: 35.9 ± 13.0% at 10.7 ± 2.0 d.p.i.; **Fig. 4d**). Consistent with their GABAergic identity, 33.7 ± 3.6% of the astroglia-derived neurons following *Dlx2* transduction express the vesicular γ-aminobutyric acid (GABA) transporter vGaT at 22.0 ± 0.6 d.p.i. This rather low number of vGaT-positive neurons may reflect the overall lower degree of functionality of neurons derived from *Dlx2*-transduced astroglia compared with *Neurog2*. Of note, combining *Mash1* and *Dlx2* increases astroglia-to-neuron conversion efficiency up to 93.0 ± 3.1%, as assessed by βIII tubulin immunoreactivity at 10.7 ± 2.0 d.p.i. (**Fig. 4d**). In addition, prior expansion of astroglial cells under neurosphere conditions greatly enhances the efficiency of *Dlx2*-mediated astroglia-to-neuron conversion (94.7 ± 0.3% versus 35.9 ± 13.0% in neurosphere cells versus adherent astroglia, respectively).

Electrophysiological changes

Along with the morphological and immunocytochemical metamorphosis of astroglia into neurons, the electrical properties of the cells also change over the course of astroglia-to-neuron conversion (see **Box 3**). At 4 d after *Neurog2* transduction, cells are largely unexcitable[5]. Subsequently (6–10 d), cells undergoing astroglia-to-neuron conversion show progressively increasing tetrodotoxin-sensitive Na^+-currents in response to step depolarization[5]. Accordingly, responses to step-current injection in current clamp typically range from small spikelets to single mature action potentials. With maturation proceeding, astroglia-derived neurons acquire spiking characteristics of mature neurons that also correspond to the neuronal subtype identity (**Fig. 5g,h**). At the same time, input resistance values decrease, presumably reflecting an increase in neuronal cell body size as well as ion channel density[4,5].

Synapse formation

We observed that synapse formation by astroglia-derived neurons, as assessed by immunocytochemistry (see **Box 2**) or electrophysiology (see **Box 3**), highly depends on the overall expression level of the neurogenic fate determinant. When *Neurog2* was expressed from a silencing-prone retroviral vector construct (pCLIG), we failed to observe the establishment of synaptic connections, although astroglia-derived neurons were capable of receiving synaptic input from co-cultured embryonic cortical neurons[5]. In contrast, when a stronger and more persistent *Neurog2* expression is induced by using a silencing-resistant retroviral construct (pCAG), a substantial number of astroglia-derived neurons (58.3% at 24.6 ± 0.9 d.p.i.) form autaptic or synaptic connections that are exclusively of a glutamatergic nature (**Fig. 5e**, inset). In contrast, *Dlx2* induces the generation of GABAergic synapses, although at drastically lower rates (6.1% of the *Dlx2*-expressing neurons recorded at 26.9 ± 1.4 d.p.i.).

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AUTHOR CONTRIBUTIONS C.H. contributed to protocol design, generation and characterization of the astroglia-derived neurons and preparation of the manuscript; S.G. to the design of the transfection procedure and single-cell tracking by time-lapse video microscopy; G.M. to the characterization of the astroglia-derived neurons; A.L. and R.S. to viral vector design; T.S.-E. to astroglia culture and immunocytochemistry; and T.S. to single-cell tracking by time-lapse video microscopy. M.G. pioneered this protocol for astroglia-to-neuron conversion and contributed to the current protocol design and the manuscript; B.B. contributed to protocol design, electrophysiological characterization of the astroglia-derived neurons and preparation of the manuscript.

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