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Neuronal Network Formation from Reprogrammed Early Postnatal Rat Cortical Glial Cells

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In the subependymal zone and the dentate gyrus of the adult brain of rodents, neural stem cells with glial properties generate new neurons in a life-long process. The identification of glial progenitors outside the neurogenic niches, oligodendrocyte precursors in the healthy brain, and reactive astrocytes after cortical injury led to the idea of using these cells as endogenous cell source for neural repair in the cerebral cortex. Recently, our group showed that proliferating astroglia from the cerebral cortex can be reprogrammed into neurons capable of action potential firing by forced expression of neurogenic fate determinants but failed to develop synapses. Here, we describe a maturation profile of cultured reprogrammed NG2+ and glial fibrillary acidic protein+ glia cells of the postnatal rat cortex that ends with the establishment of a glutamatergic neuronal network. Within 3 weeks after viral expression of the transcription factor neurogenin 2 (Ngn2), glia-derived neurons exhibit network-driven, glutamate receptor–dependent oscillations in Ca^{2+} and exhibit functional pre- and postsynaptic specialization. Interestingly, the Ngn2-instructed glutamatergic network also supports the maturation of a γ-aminobutyric acid (GABA)ergic input via $GABA_A$ receptors in a non-cell autonomous manner. The "proof-of-principle" results imply that a single transcription factor may be sufficient to instruct a neuronal network from a glia-like cell source.

Keywords: glutamatergic neurons, network activity, neural repair, reprogramming, synapse formation

Introduction

In rodents, postnatal and adult neurogenesis occurs in the ventricular subependymal zone (also called subventricular zone) and in the hippocampal subgranular zone of the dentate gyrus (Alvarez-Buylla et al. 2002; Doetsch 2003; Berninger et al. 2006; Nakafuku et al. 2008; Zhao et al. 2008). Local neuronal progenitors were also identified in the caudate nucleus of the adult rabbit (Luzzati et al. 2006). Neurogenic cells found in niches of adult neurogenesis share common features with radial glia cell, the major source of neurons in the embryonic forebrain (Doetsch et al. 1999; Malatesta et al. 2000, 2003; Seri et al. 2001; Doetsch 2003; Garcia et al. 2004; Merkle et al. 2004; Kriegstein and Ninkovic and Götz 2007; Pinto and Götz 2007; Alvarez-Buylla 2009). These observations support the concept that cells with glial properties can act as stem cells and/or neuronal progenitors during development and even in specialized niches of the adult mammalian brain (Doetsch et al. 1999; Seri et al. 2001, 2006; Doetsch 2003; Garcia et al. 2004; Mori et al. 2005).

The specialized character of glial-like neural stem cells is strikingly different from that of glial cells in non-neurogenic areas of the brain parenchyma (Doetsch 2003; Mori et al. 2005; Barres 2008). Most astroglial cells in the brain parenchyma are in a quiescent state and nondividing; however, they can resume cell division upon brain injury (Buffo et al. 2008). While reactive astrocytes remain within their lineage in vivo (Buffo et al. 2008, 2005), they exhibit self-renewal capacity and multipotency in vitro (Buffo et al. 2008). These cells therefore provide an exciting novel source of a stem cell pool for repair after injury. Another source for possible endogenous repair of degenerated neurons or oligodendrocytes upon a brain insult may be the endogenous dividing glial progenitor population, which is widespread throughout the adult brain parenchyma (Miller 2002; Ligon, Fancy, et al. 2006; Ligon, Kesari, et al. 2006; Dimou et al. 2008; Rivers et al. 2008). These progenitors divide even in the absence of injury and recently have been shown not only to express markers typically found in oligodendrocyte precursor cells (OPCs) during development (NG2, platelet-derived growth factor alpha receptor, Olig2) (Dawson et al. 2000) but also generate cells of the oligodendrocyte lineage in the adult brain (Dimou et al., 2008; Rivers et al., 2008). OPCs can be reverted to multipotent neural stem cells in vitro by using a defined sequence of extracellular signals, indicating a remarkable developmental potential of OPCs (Kondo and Raff 2000). Moreover, OPCs are also thought to exhibit a larger potential when exposed to a more neurogenic environment (Horner and Gage 2002; Belachew et al. 2003; Alonso 2005; Ligon, Kesari, et al. 2006). While NG2+ precursors fail to regenerate neurons following injury in vivo (Alonso 2005; Dimou et al. 2008), they can generate neurons in vitro (Belachew et al. 2003) or upon transplantation into neurogenic zones in vivo (Alonso 2005). Thus, there are 2 sets of dividing glial cells that may serve as a local pool for regenerating neurons after brain injury. One strategy of exploiting the remarkable potential of glial cells for neural repair, not only in the brain parenchyma (Buffo et al. 2005, 2008) but also in the spinal cord (Ohori et al. 2006), includes the ectopic expression of neurogenic transcription factors in a cellular state when glial cells have become dedifferentiated following injury. Indeed, as a "proof-of-principle" evidence that this is possible, we have shown previously that viral vector–mediated antagonism of Olig2 function or overexpression of paired box 6 protein (Pax6) in glial cells in the vicinity of a cortical injury site results in generation of significant numbers of immature neurons in vivo (Buffo et al. 2005).

The potential of astroglia to become neurons is mainly supported by in vitro assays. Cultured postnatal astroglia,

although non-neurogenic under control conditions, exhibit a remarkable degree of plasticity rendering them amenable to reprogramming to neurons by intrinsic or extrinsic factors (Laywell et al. 2000; Heins et al. 2002; Berninger, Costa, et al. 2007). In recent years, several transcription factors that are able to induce functional reprogramming of astroglia toward a neuronal identity in vitro have been identified. Starting from the observation that the transcription factor Pax6 (paired box gene 6), a key factor for embryonic cortical neurogenesis, is able to redirect postnatal mouse astrocytes toward a neuronal phenotype in vitro (Heins et al. 2002; Berninger, Costa, et al. 2007) and in vivo (Buffo et al. 2005), the downstream basic helix-loop-helix transcription factors neurogenin 2 (Ngn2) and mammalian achaete-schute homolog 1 (Mash1, also known as Ascl1) are more efficient to force astroglia to a neuronal fate (Berninger, Costa, et al. 2007; Berninger, Guillemot, and Götz 2007). In vitro, murine astroglia-derived neurons acquire remarkable physiological properties of true neurons, such as intrinsic excitability and the firing of action potentials. However, they failed to complete synaptic maturation (Berninger, Costa, et al. 2007), raising the question whether basic parameters of synapse formation (Craig et al. 2006) and especially the relevant process of presynaptic differentiation (Jin and Garner 2008) can develop in glia-derived reprogrammed neurons.

In the present study, we improved this in vitro reprogramming further, such that a functional neuronal network was established from rat postnatal glia cells. The proof-of-principle results presented here show that a single transcription factor may be sufficient to instruct the maturation of glia-like cells to functional neurons, which are part of an excitatory, glutamatergic network and are able to acquire a functional inhibitory input.

Materials and Methods

Cell Culture, Retrovirus Production, and Retroviral Transduction

For culturing postnatal rat glia, we modified a method described by Heins et al. (2002). A detailed and commented protocol is provided as supplementary information.

Cells were grown on poly-L-ornithine–coated flasks in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen), 3.5 mM glucose (Sigma), 10% fetal calf serum (Invitrogen), 5% horse serum (Invitrogen), penicillin/streptomycin (Invitrogen), B27 supplement (Invitrogen), 10 ng/ml epidermal growth factor (EGF) (Roche), and 10 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen). Cells were passaged after 5–6 days and plated on poly-D-lysine–coated coverslips. Retroviral transduction was performed 2 h after plating using vesicular stomatitis virus G protein–pseudotyped retroviruses. Controls, or FLAG-tagged Ngn2, were either expressed with pCLIG (Hatakeyama and Kageyama 2002), which directs expression of the cloned genes together with green fluorescent protein (GFP) from the long-terminal repeat (LTR) promoter (Hojo et al. 2000), or expressed under a chicken beta-actin promoter with cytomegalovirus (CMV) enhancer together with DsRed (Zhao et al. 2006). Viral particles were produced using helper-free packaging cells GPG (Ory et al. 1996). Then, cells were cultivated in serum-free differentiation medium (DMEM/F-12, 3.5 mM glucose, penicillin/streptomycin, and B27 supplement). From day postinfection (DPI) 9–11 on, we added 20 ng/ml brain-derived neurotrophic factor (Calbiochem or RD Systems) every fourth day during differentiation (DPI indicates day after retroviral transduction). Cells were cultured under 9% CO_2, resulting in a differentiation medium pH of ~7.2. Cells were used for either Ca^{2+} imaging experiments or immunofluorescene analysis at DPI 1–44.

Immunocytochemistry

Cultures were fixed in 4% phosphate-buffered saline–buffered paraformaldehyde and processed for antibody staining using standard procedures. A complete list of primary antibodies, their origin, and use is provided as supplementary information. Samples were incubated with primary antibodies at room temperature for 1–2 h and detected by secondary antibodies labeled with Alexa 488, Cyanine dye 3 (Cy3), or Cy5. Nuclei were labeled by 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI).

Cells were monitored either using an Olympus Fluoview 300 confocal system at an Olympus IX70 microscope, Olympus objectives (UPlanApo 20×/0.7; PlanApo 60×/1.4), or at an epifluorescence microscope setup (Olympus BX61 TRF microscope with an FView II Digital micro camera and Olympus objectives UplanSApo 20×/0.75; UPlan FLN 40×/1.3; PlanApoN 60×/1.42). In some experiments, a Zeiss Axiovert 100 microscope, equipped with a Fluoarc HBO100 excitation system and a Zeiss 10x/0.5 Fluar objective, was used. For quantitative cell count analysis, 5–15 randomly selected regions on 1 or 2 coverslips were counted per culture batch (=*n*).

Confocal Ca^{2+} Imaging

A 5 mM stock solution of Oregon Green 488 BAPTA-1-AM (OGB1) (Invitrogen; O6807), was prepared in 8.9 μl 20% Pluronic F-127 (Invitrogen) in dimethyl sulfoxide by means of a sonifier bath (Bandelin) for 2 min. Reprogrammed neurons were incubated with 5μM OGB1-AM in artificial cerebrospinal fluid (ACSF) solution. Cells were loaded with dye-containing ACSF in a cell culture incubator (37 °C, 9% CO_2) for 10–15 min. To enable a relatively fast stimulation of multiple cells by perfusion (ca. 20× volume exchange per minute), we constructed a perfusion chamber with a low buffer volume (~150 to 200 μl per 0.78 cm). Cells were imaged at 26–30 °C. Ligands (Ascent Scientific) were used at the following concentrations: bicuculline methiodide (10 μM), muscimol (10–50 μM), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM), D-2-Amino-5-phosphonopentanoic acid (10 μM), kainate (10 μM), tetrodotoxin (TTX; 500 nM), ATP (200 μM for glial cells), and glutamate (500 μM for glial cells). For confocal Ca^{2+} imaging (256 × 256 pixel, 2.16 Hz), an inverted confocal microscope (Olympus IX70, equipped with a Fluoview 300 laser scanning system) was used in combination with an Olympus UPlanApo 20×/0.7 objective. Transduced cells were identified by DsRed expression. Oregon Green–derived fluorescence was excited with a 488 nm laser line (emission filter: band pass 510/540 nm). DsRed was excited at 543 nm (emission filter: band pass 580 ± 40 nm). ACSF contained (in millimolar): 127 NaCl, 3 or 4.5 KCl, 2.5 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 23 $NaHCO_3$, and 25 D-glucose, bubbled with 95% O_2/5% CO_2. Ca^{2+}-free ACSF (in millimolar): 127 NaCl, 3 or 4.5 KCl, 2.5 NaH_2PO_4, 0.1 ethyleneglycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetra acetic acid, 4 $MgCl_2$, 23 $NaHCO_3$, and 25 D-glucose, bubbled with 95% O_2/5% CO_2.

Image Analysis

Images were analyzed using ImageJ software (WS Rasband, ImageJ, US National Institutes of Health, http://rsb.info.nih.gov/ij/, 1997–2006) and processed with Photoshop (Adobe Systems). *XY*-time Ca^{2+} imaging results were analyzed by a region-of-interest analysis (pixel intensity) in the extended TIFF format as described earlier (Rehberg et al. 2008).

Results

Efficient Reprogramming of Postnatal Cortical Glia Cells by Ngn2

First, we determined whether cultured glia cells of early postnatal rats can be reprogrammed by forced expression of the proneural genes, as described previously for astroglia cultured form postnatal day P5–P7 mice (Heins et al. 2002; Berninger et al. 2006; Berninger, Costa, et al. 2007). Routinely, cells were prepared from P5 to P8 rat cerebral cortex and cultured in the presence of serum, EGF, and bFGF. Cells were passaged once and transduced with moloney murine leukemia

virus (MMLV)–based retroviral vectors encoding Ngn2. Then cells were cultivated in serum-free, EGF-free, and bFGF-free medium containing B27 supplement (differentiation medium). To monitor transduced cells, all vectors carried a fluorescent marker protein (GFP or DsRed) under control of an internal ribosomal entry site 2 (IRES2). GFP alone– or DsRed alone–expressing vectors were used as control (Supplementary Fig. 1). Two types of MMLV-based vectors were used: one expressed the gene of interest via the intrinsic LTR promoter (Hojo et al. 2000) (Supplementary Fig. 1A) while the second type expressed the inserted genes under the control of an internal CMV enhancer/chicken-β-actin promoter (Zhao et al. 2006) (Supplementary Fig. 1B). At 7–8 DPI, cells were immunostained for the early neuronal markers doublecortin (DCX) and βIII-tubulin. In sharp contrast, in cells expressing GFP only, 2.6% of 2382 cells ($n_{\text{culture batch}} = 4$) expressed βIII-tubulin or DCX (Fig. 1*A*, yellow arrow; Supplementary Fig. 2). As described for mouse astrocytes (Berninger, Costa, et al. 2007), cells transduced with Ngn2 exhibited a neuronal morphology and were positive for both βIII-tubulin (89.7% of 1051 cells, $n_{\text{culture batch}} = 4$) and DCX (Fig. 1*B*). In transduced cultures and nontransduced controls, very few βIII-tubulin/DCX+ neurons appeared that did not express a transduction marker (Fig. 1*A*). These spontaneously appearing βIII-tubulin/DCX+ neurons were either found as single cells (34% of the cells) or seen in colonies of 2–10 cells (Supplementary Fig. 2; βIII-tubulin/DCX+ cell colonies are representatively documented). Since Ngn2, in contrast to GFP, forces cells to exit the cell cycle, the total number of nontransduced βIII-tubulin/DCX+ neurons was analyzed. In GFP- (mean = 1.04 cells per area) or Ngn2-transduced (mean = 1.52 cells per area) cultures ($n_{\text{culture batch}} = 4$, 52 areas), no significant difference in the numbers of nontransduced βIII-tubulin/DCX+ cells was observed (P value = 0.2, unpaired t-test). The induction of βIII-tubulin/DCX+ cells was successful and effective when cells were prepared from rats younger than P14.

At this early time point of differentiation (DPI 7–8), βIII-tubulin/DCX+ neurons did not express the isoforms of the γ-aminobutyric acid (GABA)–producing enzyme glutamic acid decarboxylase, GAD65 and GAD67, as verified by immunofluorescence labeling using 3 different, widely used GAD-specific antibodies (see supplementary antibody information). In addition, we did not observe the expression of the vesicular GABA transporter (vGAT), the vesicular glutamate transporter (vGluT1), or the synapse protein synapsin.

The Starting Cell Population Expresses Glial Properties

To identify the precise phenotype of the cells that were virally infected after cell seeding and which became immature βIII-tubulin/DCX-labeled neurons at DPI 7–8, immunocytochemical experiments were performed at 1–3 days after transduction. At this stage, neither GFP-alone cells nor Ngn2-transduced cells expressed βIII-tubulin or DCX. In addition, only anecdotal nontransduced βIII-tubulin or DCX+ cells were found (12 neurons on 10 coverslips, $n_{\text{culture batch}} = 5$). The prevailing fraction of transduced cells (expressing GFP under the LTR promoter) showed the morphological characteristics of astroglial cells and expressed glial fibrillary acidic protein (GFAP; Fig. 1*C*, Supplementary Fig. 3A,D), S100β (Supplementary Fig. 3A), glutamine synthetase (Supplementary Fig. 3C), and glial glutamate–aspartate transporter (GLAST, not shown). A second cellular fraction, which did not express GFAP, was positive for the proteoglycan NG2 (Fig. 1*C*, Supplementary Fig. 3B,D,E), normally found on the surface of OPCs (Nishiyama et al. 2009). Only some of the NG2+ cells also expressed O4, a protein observed in OPCs and immature oligodendrocytes (Supplementary Fig. 3B). For quantitative analysis of cells expressing GFP, we performed the analysis of quadruple-labeled cells (DAPI, GFP-postlabeled with chicken anti-GFP/Alexa 488, rabbit anti-NG2/Cy3, and mouse anti-GFAP/Cy5; Supplementary Fig. 3E). This revealed that transduced cells were either astroglia like and expressed GFAP (62 ± 1.1%; mean ± standard error of the mean [SEM]; $n = 4$; total number of 712 cells) or oligodendrocyte precursor like and expressed NG2 (38 ± 1.1%; mean ± SEM; $n = 4$) (Fig. 1*C*, graph: Supplementary Fig. 3D). Thus, neurons obtained after Ngn2 transduction were derived from either of these glial cell types.

To follow the physiological maturation of glia-derived reprogrammed neurons over time, we performed Ca^{2+} imaging experiments of Ngn2-transduced cells. Glial cells were transduced with retroviral vectors carrying $Ngn2^{IRES}$DsRed and used at different days after transduction. As a Ca^{2+}-sensitive probe, we used Oregon Green 488 BAPTA-1 (OGB1) in its acetoxymethyl-derivatized, membrane-permeable form. This dye has a dissociation constant of ~170 nM, allowing the detection of small changes in $[Ca^{2+}]$, and its emission spectrum can be separated from DsRed marking Ngn2-transduced cells. At 1 DPI, cells transduced with $Ngn2^{IRES}$DsRed exhibited sufficiently high levels of DsRed expression for live imaging experiments. Both transduced cells, identified by DsRed/OGB1 colabeling, and nontransduced cells, labeled by OGB1 only, exhibited glial-like morphology (Supplementary Fig. 4). Upon stimulation with 200 μM ATP for 5 s to activate purinergic receptors that subsequently activate Ca^{2+} release from the endoplasmic reticulum, all transduced cells showed a raise in cytosolic Ca^{2+} and many cells exhibited nonsynchronous Ca^{2+} oscillations lasting up to 1 min (Supplementary Fig. 4B; 4 representative transients of single transduced cells are shown). Next, cells were stimulated with a high concentration of glutamate (500 μM) that induced nonsynchronous Ca^{2+} oscillations in the majority of cells. Removing extracellular Ca^{2+} did not block the glutamate-induced response but reduced the duration of the oscillatory behavior. Transduced and nontransduced cells behaved identically and showed a physiological response typically found in astroglia and NG2+ glia (Cornell-Bell et al. 1990; Wigley et al. 2007; Agulhon et al. 2008).

These data confirm the glial nature of the cell population undergoing neuronal reprogramming. In the following, we refer to these cells as glia-derived neurons (GdNs).

GdNs Transiently Acquire Functional Attributes Typical of Immature Neurons

At DPI 5–8, when 90% of all Ngn2-transduced cells expressed DCX (Fig. 1*B*), indicating an immature neuronal phenotype (Brown et al. 2003; Couillard-Despres et al. 2005), we determined whether the onset of DCX expression is also accompanied by the acquisition of responsiveness to the neurotransmitter GABA, which typically arises early during neuronal differentiation (Ben-Ari 2002; Ge et al. 2007). In immature neurons, GABA-mediated activation of $GABA_A$ receptors can cause an outflow of Cl^-, which then elicits an inflow of

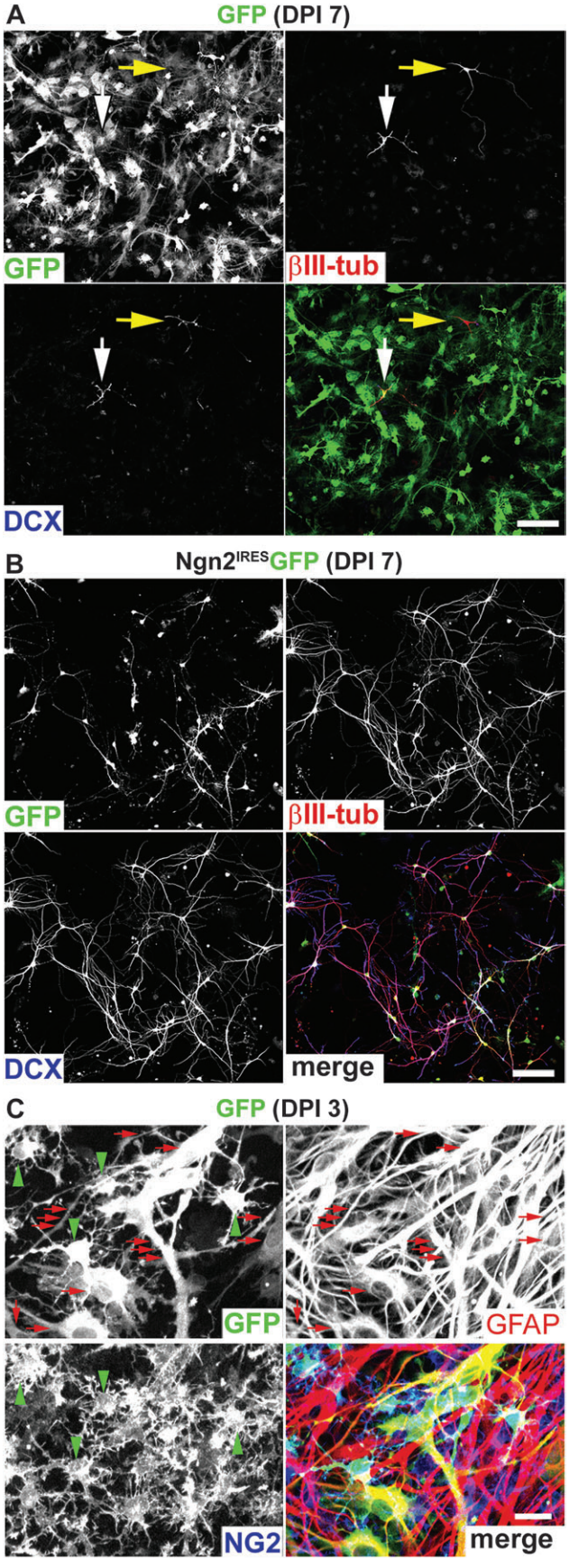

Figure 1. Ngn2 has a strong potential to reprogram postnatal cortical glia of the rat to a neuronal fate. Immunostaining of rat cortical glia cells after transduction with retroviral vectors expressing GFP alone (*A*) or Ngn2 together with GFP (*B*) at DPI 7. Antibody staining for GFP (green), the early neuronal marker βIII-tubulin (βIII-tub, red), and DCX (blue) are shown. White arrow: βIII-tub/DCX+ cell expressing GFP; yellow arrow: GFP-deficient βIII-tub/DCX+ cell. Bar, 100 μm. (*C*) Example of rat glia at DPI 3, transduced with GFP alone. Cells expressing GFP are positive either for GFAP (red arrows) or for NG2 (green arrows). Bar, 25 μm. (For quantitative analysis, see text and Supplementary Fig. 3E.)

Ca^{2+} (Owens et al. 1996; Ben-Ari 2002; Yuste and Bonhoeffer 2004; Ge et al. 2007). To establish whether this physiological state can be observed in Ngn2-reprogrammed glia, we applied 100 μM GABA by fast perfusion. Ngn2-expressing cells exhibited a GABA-evoked rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Fig. 2*B*,*B*′) as well as a response to kainate (10 μM for 5 s; Fig. 2*B*,*B*′). Kainate is a potent agonist of ionotropic non-N-Methyl-D-aspartate (NMDA) glutamate receptors and causes influx of $[Ca^{2+}]$ (Pinheiro and Mulle 2006). In nontransduced, glia-like cells, neither GABA nor kainate showed an agonist-evoked Ca^{2+} signal (Fig. 2*A*,*A*′). These results revealed that within 5 days after transduction, GdNs had acquired a physiological phenotype typically found in immature neurons and expressed GABA receptors and ionotropic glutamate receptors.

A rise in intracellular Ca^{2+} was also obtained when muscimol, a selective $GABA_A$ receptor agonist, was applied at concentrations of 10–50 μM (Fig. 2*C*, here average graphs are shown). This effect was blocked when extracellular Ca^{2+} was removed from the ACSF perfusion buffer and was reversed by re-adding extracellular Ca^{2+} (Fig. 2*C*′,*C*″). In addition, when GABA receptors were preblocked with 25 μM bicuculline, a selective $GABA_A$ receptor antagonist (Fig. 2*D*,*D*′), 10 μm muscimol was no longer effective. This effect was reversed by washing out bicuculline for at least 10 min (Fig. 2*D*″, for comparison with the experiment in Fig. 2*C* shown with 50 μM muscimol). This pharmacology is consistent with an excitatory action of GABA through $GABA_A$ receptors (Carleton et al. 2003; Tozuka et al. 2005). In 4 series of experiments, 127 out of 164 Ngn2-transduced cells (DPI 6–8) showed a rise in $[Ca^{2+}]_{cyt}$ upon stimulation with either GABA or muscimol. Muscimol and kainate were without effect in Ngn2-transduced cells at DPI 1 (not shown).

In Ngn2-transduced cultures at DPI 9–11, DCX started to disappear from somatic cell areas of βIII-tubulin+ cells. At DPI 15, GdNs expressed the neuronal marker βIII-tubulin while DCX was downregulated, and only a minority of cells still showed DCX immunoreactivity (Supplementary Fig. 5). It is known that DCX is transiently expressed in developing neurons and is downregulated when neurons become mature (Brown et al. 2003; Couillard-Despres et al. 2005). The finding that DCX disappears from Ngn2-instructed neurons is consistent with an ongoing neuronal maturation of GdNs in vitro.

Neurons Derived from Rat Glia Form Functional Synapses

At DPI 18, when DCX was downregulated and Ngn2-expressing cells still showed a strong expression of the neuronal proteins βIII-tubulin and microtubule-associated protein 2 (MAP2), Ca^{2+} measurements were performed to monitor the process of neuronal maturation at a physiological level (Fig. 3*A*–*C*″′). When cells were treated with 40 mM K^+ in the perfusion buffer for 5 s, repetitive increases in $[Ca^{2+}]_i$ appeared in many Ngn2-transduced cells (Fig. 3*A*, single cells labeled by arrows in red,

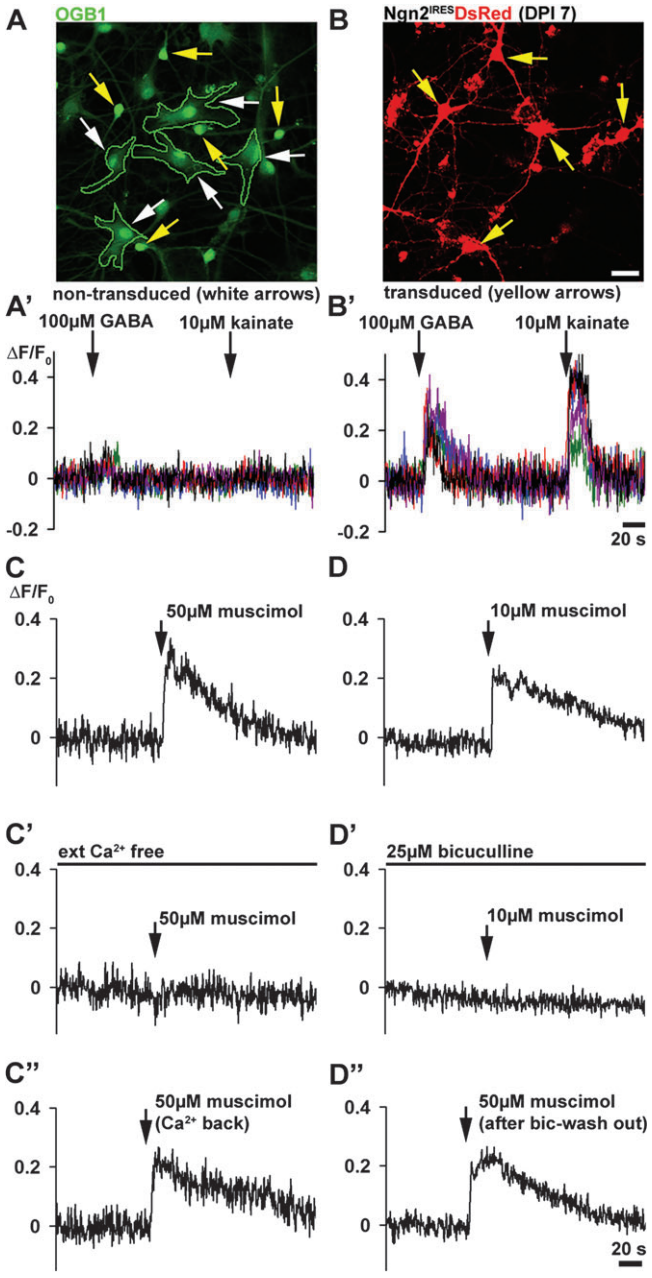

Figure 2. At DPI 7, Ngn2-expressing cells show physiological properties of immature neurons. (*A*) Cells labeled with the Ca^{2+} indicator OGB1 are shown. (*A′*) Glia-like cells (*A*, analyzed regions of interest are indicated) do not respond with a rise in $[Ca^{2+}]_i$, either to GABA or to kainate (*A′*, single traces). (*B*) $Ngn2^{IRES}$DsRed-expressing cells show a rise in $[Ca^{2+}]_i$ after stimulation with GABA and kainate (*B′*). Yellow arrows in (*A*, *B*) indicate cells analyzed in (*B′*). (*C*) Muscimol elicits a rise in $[Ca^{2+}]_i$ in Ngn2-expressing cells. (*C′*) This effect is lost when extracellular Ca^{2+} is removed during muscimol stimulation and returns after re-addition of extracellular Ca^{2+} (*C″*). (*D*) The muscimol-mediated rise in $[Ca^{2+}]_i$ disappears in the presence of bicuculline (*D′*) and reappears after removal of bicuculline (*D″*). $\Delta F/F_0$: change in fluorescence emission divided by baseline fluorescence. (*C*, *D*) series: average graphs of 9 $Ngn2^{IRES}$DsRed-transduced cells. Arrows: time of stimulation. Bar: 25 μm.

blue, and yellow correspond to the graphs shown in Fig. 3*B*). In Figure 3*C*, the average signal measured in 9 GdNs reveals the high synchronicity of the Ca^{2+} transients. These depolarization-dependent, synchronous Ca^{2+} oscillations were blocked by perfusion with 500 nM TTX, a potent blocker of voltage-gated sodium channels and, consequently, action potential firing in neurons (Fig. 3*C′*). Under resting conditions, cells were perfused with ACSF with a slightly increased extracellular $[K^+]$ (4.5 mM instead of 3 mM) to support spontaneous synaptic activity in neurons (Rehberg et al. 2008). However, no spontaneous Ca^{2+} signals were detectable in GdNs (Fig. 3*C′′*). Continuous perfusion with 10 μM bicuculline to block potential inhibitory GABAergic influence was also without effect (Fig. 3*C′′′*).

Since the stimulus with 40 mM K^+ was relatively short (5 s), but synchronous Ca^{2+} oscillations were long lasting (~1 min) and TTX sensitive, we suspected the presence of synapses in the reprogrammed neurons. Therefore, we fixed the coverslip used for Ca^{2+} imaging and immunostained with antibodies against the presynaptic proteins synapsin and bassoon (De Camilli et al. 1983; tom Dieck et al. 1998). This staining revealed the presence of clustered synapsin and bassoon in Ngn2-transduced cells at DPI 18 (Supplementary Fig. 6).

Neurons Derived from Rat Glia Are Able to Form a Synchronized Neuronal Network

At DPI 24–28, $Ngn2^{IRES}$DsRed-expressing neurons formed a complex network (Fig. 3*D*). To our surprise, these cells showed only rare spontaneous Ca^{2+} transients. However, when perfused with the $GABA_A$ receptor blocker bicuculline, the reprogrammed neurons exhibited fully synchronous, rhythmic Ca^{2+} transients (supplementary Video 1) of defined shape and comparable amplitude (compare average graph of 15 neurons shown in black, in Fig. 3*F*, with transients observed in single cells shown in red, blue, and yellow, Fig. 3*E*). The shape of these oscillatory Ca^{2+} transients was virtually identical to that described in 3-week-old cultured rat cortical neurons and recorded in the presence of picrotoxin, another inhibitor of GABAergic transmission (Murphy et al. 1992). The bicuculline-induced Ca^{2+} oscillations were fully blocked by TTX (Fig. 3*E′*,*F′*).

A Network of GdNs with Synaptic Glutamatergic and GABAergic Transmission

The foregoing led us to suspect that synchronous network activity in Ngn2-reprogrammed neurons is mediated via synaptic glutamate release. As shown in Ngn2-transduced cells (Fig. 3*G*–*J′′* series), bicuculline-induced synchronous Ca^{2+} transients were fully blocked by 10 μM CNQX, a competitive antagonist of ionotropic glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate family (Fig. 3*J*–*J′′′*). This pharmacology strongly points to synaptic, glutamatergic transmission. Nontransduced cells in the neighborhood of Ngn2-transduced cells remained non-responsive to this stimulation protocol (Fig. 3*I*–*I′′′*).

To verify this, patch-clamp experiments were performed, as described in other studies (Berninger, Costa, et al. 2007; Berninger, Guillemot, and Götz 2007). As shown in Supplementary Figure 7A, under whole-cell patch-clamp conditions, long-lasting inward currents were recorded. In addition, individual synaptic inward currents were detectable during the complete recording phase. Next, dual-recording patch-clamp experiments were performed (Supplementary Fig. 7B). When a series of action potentials was elicited in a presynaptic neuron under current-clamp conditions, the corresponding postsynaptic neuron responded with a series of postsynaptic potentials (Supplementary Fig. 7B′). Under voltage-clamp conditions, a suprathreshold step depolarization of the presynaptic neuron

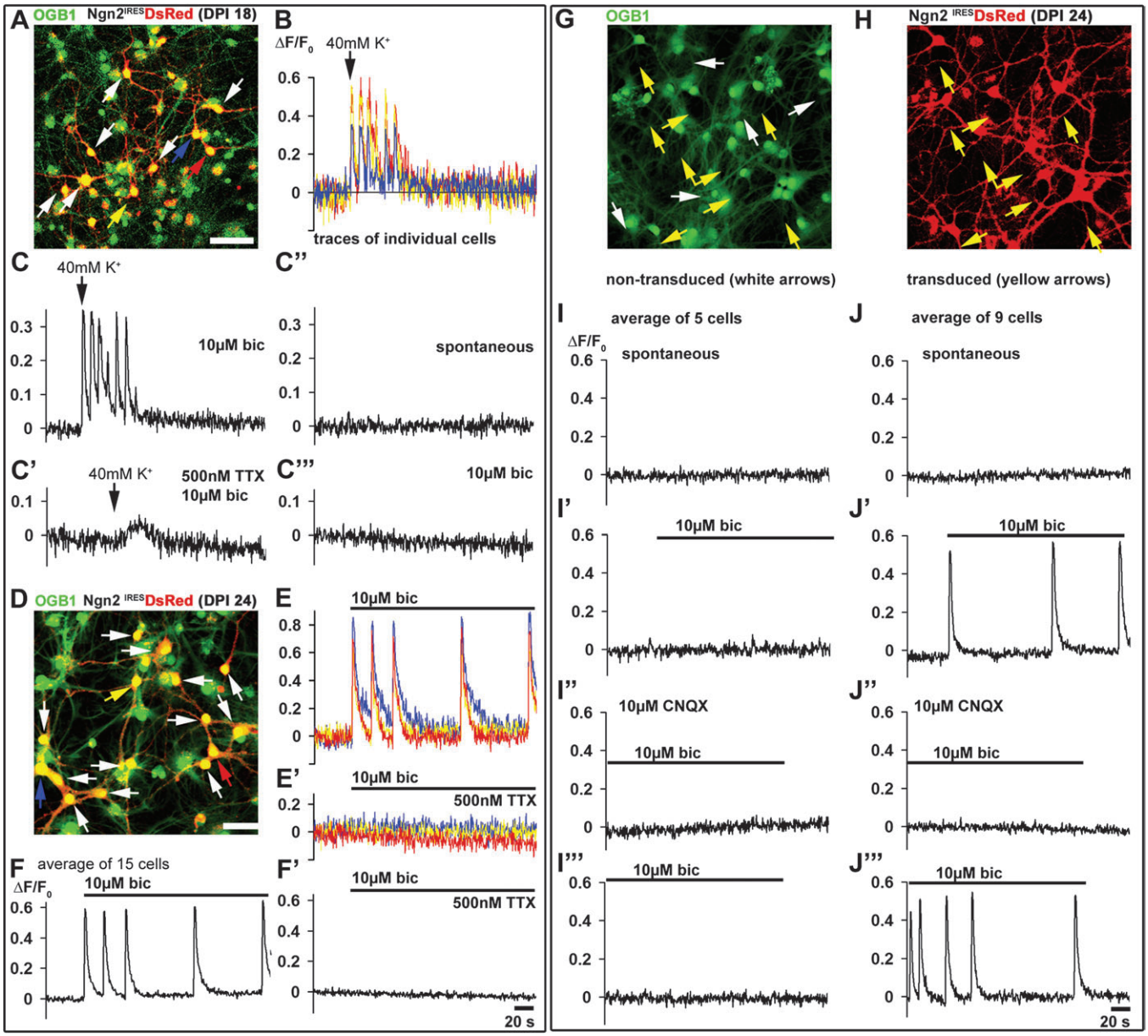

Figure 3. (*A–C″′*) At DPI 18, Ngn2-expressing cells show TTX-sensitive, synchronous oscillations in $[Ca^{2+}]_i$ after strong depolarization with high K^+. (*A*) DsRed fluorescence of Ngn2^{IRES}DsRed-expressing cells is merged with the OGB1 signal. Arrows indicate cells contributing to the average graph in (*C*). (*B*) Ca^{2+} transients in 3 single cells, corresponding to red, blue, and yellow arrows in (*A*), after depolarization with 40 mM KCl in extracellular ACSF. (*C–C″′*) Average graphs of changes in $[Ca^{2+}]_i$ in cells indicated in (*A*). (*C*) Cells show a synchronous oscillation in $[Ca^{2+}]_i$ after depolarization. (*C′*) TTX blocks the oscillation in $[Ca^{2+}]_i$ after depolarization. (*C″*) Cells exhibit no spontaneous activity. (*C″′*) Perfusion with bicuculline to block inhibitory transmission via $GABA_A$ receptors has no effect (compare with *F*,*J′*,*J″′*). Bar, 100 μm. Cells used for this Ca^{2+} imaging experiment express the synaptic proteins synapsin and bassoon (Supplementary Fig. 6). (*D–F′*) At DPI 24, Ngn2-expressing cells show bicuculline-induced, TTX-sensitive, synchronous oscillations in $[Ca^{2+}]_i$. (*D*) DsRed fluorescence of Ngn2^{IRES}DsRed-expressing cells is merged with the OGB1 signal. (*E*) Ca^{2+} transients in 3 single cells, corresponding to red, blue, and yellow arrows in (*D*), after addition of 10 μM bicuculline (upper graph). These oscillations in $[Ca^{2+}]_i$ are blocked by the sodium channel blocker TTX (*E′*). (*F*) Average graph of rise in $[Ca^{2+}]_i$ after bicuculline addition and after TTX block (*F′*) of all cells indicated in (*D*). Bar, 50 μm. (*G–J″′*) Mature GdNs are synaptically connected via glutamate and get a GABAergic input. Left panels (*G–I″′*): nontransduced. Right panels (*H–J″′*): Ngn2^{IRES}DsRed-transduced (*G*) OGB1 fluorescence. White arrows indicate nontransduced cells, analyzed in the C-series. Yellow arrows indicate GdNs analyzed in the D-series. (*H*) DsRed label of Ngn2^{IRES}DsRed-expressing cells. (*I–I″′*) Nontransduced, glia-like cells do not contribute to bicuculline-induced, synchronous oscillations in Ca^{2+}. Average graph of 5 cells. (*J–J′*) GdNs require the block of $GABA_A$ receptors by bicuculline before exhibiting synchronous oscillations in $[Ca^{2+}]_i$. (*J″–J″′*) These oscillations can be blocked reversibly by CNQX, a potent blocker of ionotropic AMPA/kainate glutamate receptors. Ca^{2+} transients are highly synchronous, as shown in the average graph representing 9 cells. Bar, 50 μm. $\Delta F/F_0$: change in fluorescence emission divided by baseline fluorescence.

caused an inward current in the postsynaptic cell (Supplementary Fig. 7B″, red line). The postsynaptic current was completely blocked by the AMPA receptor blocker CNQX (Supplementary Fig. 7B″, black line). These data confirm the presence of functional glutamatergic synapses in Ngn2-reprogrammed GdNs by an independent experimental approach.

Deblockade of activity upon bicuculline application observed by calcium imaging suggested the potential presence of a spontaneous GABAergic input to the neuronal network. Therefore, we performed immunofluorescence analysis to determine the number of GABAergic neurons in comparison to the number of glutamatergic neurons. For this, we analyzed

the reprogramming experiments and analyzed Ngn2-infected cultures at DPI 7, DPI 14, and DPI 24 (Supplementary Fig. 7C). At DPI 7, we could find neither vGlut cells nor vGAT+ cells. At DPI 14, when Ngn2-instructed neurons are already synapsin+, a mean value of 76 ± 10.9 cells was vGluT+ (n = 30 regions of interest (ROIs) of 3 mm^2; mean ± standard deviation; 2 coverslips of 113 mm^2), while only 0.63 ± 0.8 cells (n = 30) were vGAT+. Interestingly, the amount of vGAT+ cells increased over time. At DPI 24, the amount of vGluT+ cells remained constant (n = 27; 81.3 ± 21.4), while we now counted 5.3 ± 3.4 vGAT+ neurons (n = 29). Next, we performed electrophysiological experiments to get more evidence for GABAergic transmission. Consistent with the low number of mature GABAergic neurons in these cultures, patch-clamp recording of spontaneous synaptic activity revealed the virtual absence of GABAergic events. In cultures exhibiting spontaneous activity, in only 1 out of 8 cells, we found evidence for inhibitory synaptic activity (Supplementary Fig. 7D). Thus, albeit our Ca^{2+} experiments suggest the presence of GABA signaling in these cultures, they contain very few, if any, mature GABAergic neurons.

Pre- and Postsynaptic Proteins in GdNs

The key experiments described above suggested that GdNs were undergoing a physiological maturation of synapses, including functional pre- and postsynaptic structures. In order to establish functional glutamatergic synapses, GdNs would also need to express proteins of excitatory pre- and postsynaptic compartments.

To test this, we performed a detailed analysis of synaptic structures in Ngn2-expressing cultures at DPI 24–30. Ngn2-reprogrammed neurons showed strong staining for bassoon (tom Dieck et al. 1998), which is known to be part of the presynaptic active zone in axons (Schoch and Gundelfinger 2006), as well as for the postsynaptic protein ProSAP/Shank (Bockmann et al. 2002), which is involved in the organization of the postsynaptic density (Gundelfinger et al. 2006) (Fig. 4*A*). High amounts of presynaptic bassoon and postsynaptic ProSAP/Shank were found in close proximity, indicating mature synaptic contacts in vitro (Supplementary Fig. 8A,B). In addition, Ngn2-reprogrammed neurons also expressed the vGluT, a marker for glutamatergic neurons, which was enriched in close proximity to bassoon (Fig. 4*B*). vGluT+ and ProSAP/Shank+ cells were never found in cultures transduced with GFP or DsRed alone (not shown).

Ngn2 is a principal transcription factor for the generation of glutamatergic neurons (Schuurmans et al. 2004; Brill et al. 2009) and can be used to instruct a glutamatergic neuronal subtype from mouse astroglia, expanded adult neuronal stem cells (Berninger, Costa, et al. 2007; Berninger, Guillemot, and Götz 2007; Heinrich et al. 2010), and postnatal cortical glia of the rat (this study). In more than 5000 cells, collected from 9 experiments, $Ngn2^{IRES}$Dsred- or $Ngn2^{IRES}$GFP-instructed GdNs were all positive for vGlut. Given the evidence for GABA signaling in these cultures, we performed immunocytochemistry for the β-subunit of the $GABA_A$ receptor. Indeed, GdNs also show a clustering of $GABA_A$ receptors in the same subcellular regions where bassoon is detectable (Fig. 5*A*). A colocalization of bassoon with $GABA_A$ receptors is a hallmark normally observed in neurons undergoing synaptogenesis (Zhai et al. 2000) and is consistent with a physiological role of GABA in Ngn2-reprogrammed GdNs. The cultures were then labeled

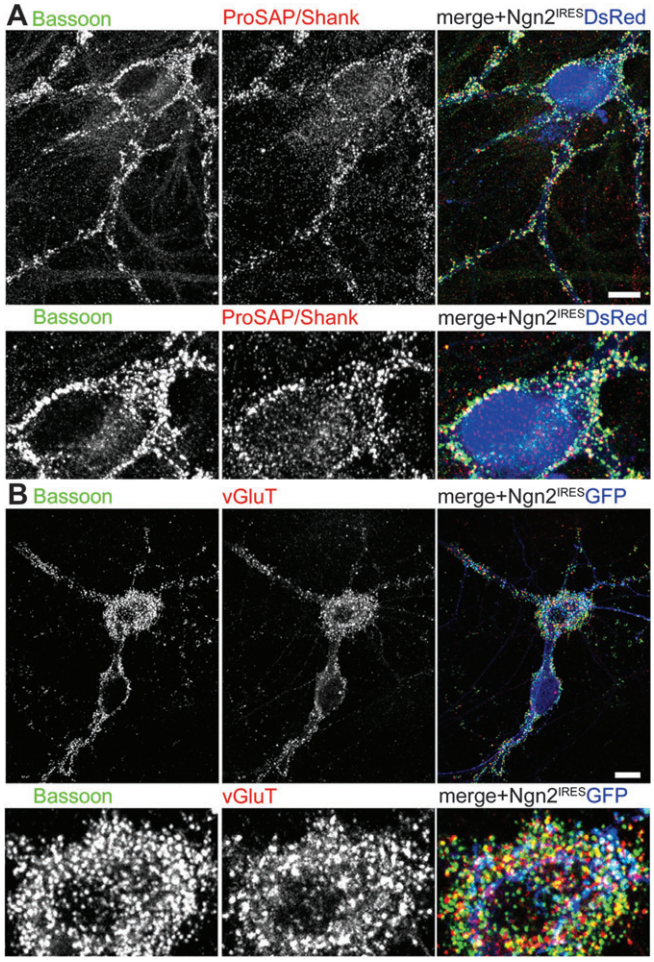

Figure 4. Pre- and postsynaptic proteins are expressed in mature GdNs. (*A*) GdNs showing bicuculline-induced oscillations in $[Ca^{2+}]_i$ express the presynaptic marker bassoon (left panels) and the postsynaptic marker ProSAP/Shank (middle panels). Bassoon and ProSAP/Shank immunoreactivity is often found in close proximity (right panel). Bar, 10 μm. High-resolution examples are also shown in Supplementary Figure 8. (*B*) GdNs express vGluT, a marker protein for glutamatergic neurons, in close proximity to bassoon. Bar, 10 μm. Anti-GFP and intrinsic DsRed labels are pseudocolored to blue (right panels).

with an antibody against vGAT. Indeed, we found single cellular processes positive for this marker of GABAergic neurons (Fig. 5*B*). In striking difference to the vGlut+ synapses, vGAT positive boutons were weakly developed on the soma of the GdNs (Fig. 5*B*,*C*). This is in striking contrast to that what is seen in neuronal cultures of the rat cortex or hippocampus, where glutamatergic neurons receive hundreds of GABAergic synapses. Interestingly, the staining indicated that these cells did not express retroviral Ngn2, since they were negative for the transduction marker DsRed (Fig. 5*B*,*C*). In control cultures at DPI 24–30, infected with GFP/DsRed-alone vectors and kept in differentiation medium, we hardly found any cells positive for neuronal markers MAP2 or βIII-tubulin, and we could not find a single cell expressing vGAT, vGluT, ProSAP, or showing colocalization of ProSAP and bassoon. A vGAT+, Ngn2-instructed GdN has not yet been found. This suggests that the GABAergic neurons formed spontaneously from the founder cell source and were able to mature in the presence of Ngn2-instructed glutamatergic neurons.

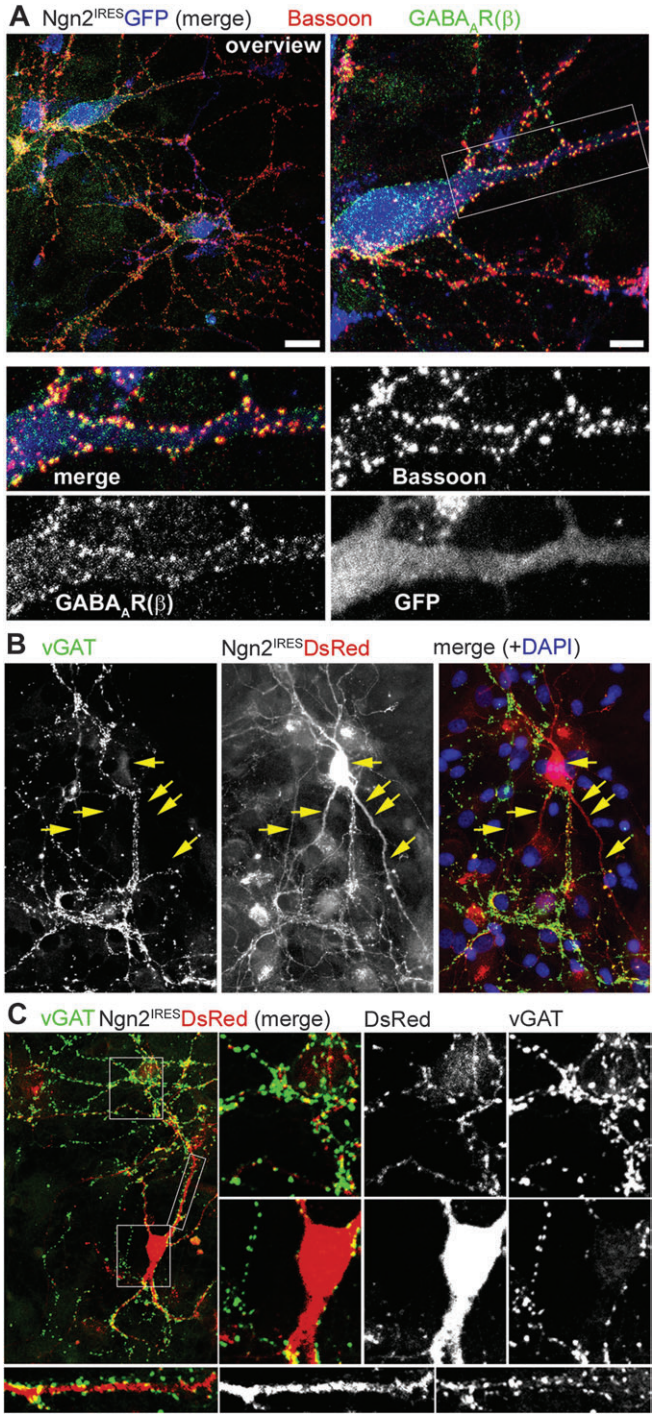

Figure 5. (*A*) Mature GdNs express clustered $GABA_A$ receptors. Immunostaining of clustered $GABA_A$ receptors (β-subunit) (green) in $Ngn2^{IRES}GFP$-expressing cells, in close proximity to the synaptic marker bassoon (red). Anti-GFP labels are pseudocolored to blue. Bar (left): 25 μm; bar (right): 5 μm. (*B*, *C*) GABAergic neurons with synaptic properties are nontransduced. (*B*) Ngn2-expressing GdNs exist in close proximity to nontransduced neurons expressing vGAT, a marker of GABAergic neurons (yellow arrows, neurites of transduced cell, and epifluorescence image). (*C*) vGAT+ cell (green) in contact with a DsRed-expressing cell (red). Three regions of interest (ROIs) are indicated. (Upper ROI) Concentration of vGAT+ labels in close proximity, but not within a DsRed+ neurite deriving from a neighbored Ngn2-DsRed–expressing cell. (Middle ROI) A DsRed+ cell soma is contacted by a vGAT+ neurite. (Lower ROI): vGAT+ signals are sitting on but not within DsRed+ neurites. DsRed labels are enhanced by anti-red fluorescent protein immunostaining.

This observation opened the question whether a small amount of neural progenitors positive for the proneural transcription factor Mash 1 (Ascl1) and the marker GAD67, indicating a GABAergic lineage, can be observed in our starting cell population. By using a monoclonal antibody against Mash1, combined with a rabbit anti-GAD67, in uninfected cells at days 1–3 in culture, we could not find a single cell positive for both markers (not shown, 2 experimental series, 6 coverslips). In summary, we have no evidence for a substantial amount of DCX+ neuroblasts or Mash1/GAD67+ neuronal progenitors.

Discussion

This study provides experimental evidence that a single transcription factor is sufficient to instruct a functional, synaptically connected neuronal network from glia in vitro. The process of reprogramming of glia cells to neurons follows a physiological and cellular maturation profile in which a glial state is followed by a defined immature, synapse-deficient neuronal state, before neurons enter a mature state with pre- and postsynaptic specializations and spontaneous network activity.

Maturation Profile of Reprogrammed Glia-Derived Neuronal Networks Partly Recapitulate Neurogenesis

The process of reprogramming of glia cells into functional neurons follows a typical maturation profile (Fig. 6). GdNs reach defined sequential differentiation states that are also found during neurogenesis starting from glia-like neuronal stem cells (Alvarez-Buylla et al. 2002; Doetsch 2003; Götz and Huttner 2005; Guillemot 2005; Ge et al. 2007; Pinto and Götz 2007). In this study, reprogramming to excitatory glutamatergic neurons was started from 2 different glia cell populations: one expressed GFAP and S100β, as well as other proteins typically found in astroglia (e.g., glutamine synthetase and GLAST), while the second population was negative for GFAP but expressed NG2, a proteoglycan expressed on the surface of oligodendrocyte progenitors (polydendrocytes) (Nishiyama et al. 2009). We have no direct proof that indeed both cell types contribute to the formation of the neuronal network. While studies from our group have already described the potential of postnatal astroglia in reprogramming to neurons of the mouse model (Heins et al. 2002; Berninger, Costa, et al. 2007; Heinrich et al. 2010), the potential of NG2+ cells after such reprogramming remains unclear so far. However, we have also shown recently that purified NG2+ oligodendrocyte precursors can acquire neurogenic potential in vitro (Dimou L, Laugwitz L, Götz M, in preparation). Lack of appropriate animal models in the rat makes it difficult to solve the question of whether astroglia-like cells, oligodendrocyte precursor–like cells, or both contribute to the formation of the glia-derived neuronal network.

At DPI 5–11, reprogrammed cells exhibited clear physiological and morphological hallmarks of immature neurons. A substantial percentage of all cells showed a $GABA_A$ receptor–dependent Ca^{2+} influx from the extracellular space, which argues for an excitatory action of GABA during this maturation stage (Yuste and Katz 1991; Ben-Ari 2002; Zhao et al. 2006; Ge et al. 2007). In addition, the cells transiently expressed DCX but no synaptic proteins, again indicating their immature state (Brown et al. 2003; Carleton et al. 2003; Couillard-Despres et al. 2005; Zhao et al. 2006). At DPI 18, when DCX was no longer detectable in Ngn2-reprogrammed neurons, the appearance of

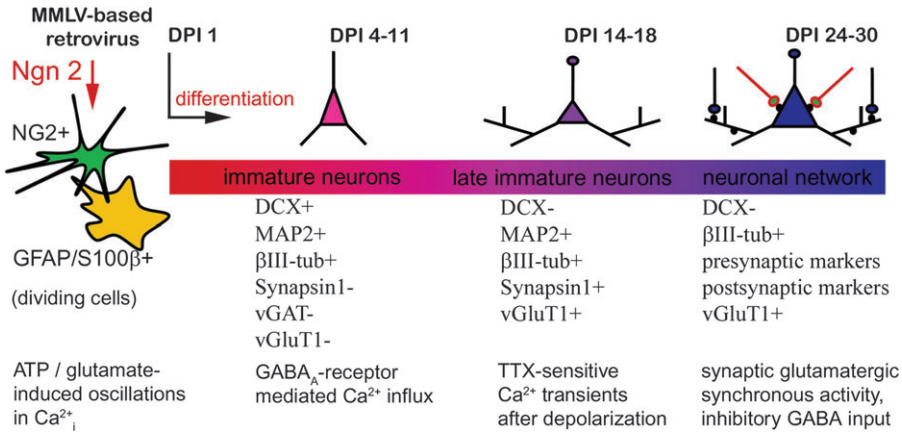

Figure 6. Maturation profile of Ngn2-reprogrammed neuronal networks originating from a glial founder population. Details are found in the text.

functional synapses marked ongoing neuronal maturation. This finding is also in accordance with the observation that newborn glutamatergic granule neurons in the dentate gyrus lose their immunoreactivity to DCX during maturation to functional neurons (Lledo et al. 2006; Bergami et al. 2008; Toni et al. 2008).

Neurons from Reprogrammed Glia Form Functional Synapses

Starting from postnatal cortical glia cells of the rat and the forced expression of Ngn2, a functional neuronal network was formed within 24 days after transduction. This neuronal network expresses the basic hallmarks of neuronal cultures from cortical or hippocampal tissue (Craig et al. 2006). Ngn2-reprogrammed GdNs express the presynaptic marker bassoon and the postsynaptic marker ProSAP/Shank (ProSAP1 and ProSAP2), and both markers are found in close proximity to each other and to the synaptic markers synapsin and vGluT (tom Dieck et al. 1998; Bockmann et al. 2002; Garner et al. 2002; Opitz et al. 2002; Craig et al. 2006; Di Biase et al. 2009). Functional analysis verified that these morphological criteria reflect functional synaptic contacts between GdNs rather than clustering and concentration of proteins within intracellular vesicular membranes of the trafficking system. At a late stage in maturation, Ngn2-reprogrammed neurons develop synchronous oscillations in cytosolic Ca^{2+}, dependent on TTX-sensitive sodium channels involved in action potential firing, driven by the transmission of the excitatory transmitter glutamate, mediated by ionotropic glutamate receptors of the AMPA/kainate type, and, in many experiments, inducible by blockade of $GABA_A$ receptors. NMDA receptors are not primarily responsible for the rise in Ca^{2+}, as treatment with an NMDA receptor blocker alone was without effect (not shown), while the AMPA/kainate blocker CNQX was sufficient to block the synchronous rise in cytosolic Ca^{2+} completely. This pharmacological profile of the coordinated or synchronous activity during inhibition of GABAergic transmission is consistent with a synaptically connected network found in older (~15 days in vitro) primary neuronal cultures of the cortex or the hippocampus (Ogura et al. 1987; Murphy et al. 1992; Wang and Gruenstein 1997; Turrigiano et al. 1998; Ben-Ari 2001; Hardingham et al. 2001; Opitz et al. 2002; Voigt et al. 2005; Rehberg et al. 2008).

The phenomenon of synchronous network activity in neurons per se is not a stringent proof of the existence of a mature, synaptically connected neuronal network. However, the pharmacological profile of the "older" (DPI 24–44) GdNs distinguishes them from spontaneous Ca^{2+} oscillations known to coincide with a depolarizing action of GABA and a blocking action of GABA antagonists. These oscillations in cytosolic Ca^{2+} are known to occur in immature neuronal networks both in vivo and in vitro (Garaschuk et al. 2000; Ben-Ari 2001; Opitz et al. 2002; Voigt et al. 2005) but were not observed here.

The morphological analysis of GABAergic synapses on the soma of glutamatergic GdNs is puzzling. On one hand, clustered GABA receptors are detectable on GdNs (Fig. 5*A*), and the physiological bicuculline sensitivity of neuronal activity is obvious. However, merely few vGAT+ boutons are associated with somata of GdNs (Fig. 5*C*), and electrophysiological analysis could not confirm a dominant synaptic GABAergic influence (Supplementary Fig. 7E). To find out whether the Ngn2-instructed GdNs are limited in their ability to form non-glutamatergic synapses, future experiments are needed, preferentially in vivo.

GABAergic Input to Ngn2-Instructed Glutamatergic Neurons

During development, Ngn2 endows neurons with a glutamatergic fate (Schuurmans et al. 2004; Guillemot 2005), and during reprogramming of mouse astroglia, which failed to develop synapses, ectopic Ngn2 expression had been shown previously to upregulate the T-box transcription factor Tbr1 (Berninger, Costa, et al. 2007). Tbr1 is known to be an early marker during the development of telencephalic glutamatergic neurons (Hevner et al. 2006). Thus, the detection of a functional GABAergic input to a glutamatergic, reprogrammed network raised the question of the origin of the GABAergic neurons. Two scenarios are most likely. In one scenario, typical Mash1/GAD67+ neural progenitors are maintained under gliogenic culture conditions and give rise to GABAergic neurons in our serum-free differentiation medium. However, a careful screen has not given us any hint for the presence of this cell type. Based on our data, we favor the hypothesis that these cells are derived from nontransduced dividing cells with glial properties that undergo spontaneous neurogenesis, acquire attributes of GABAergic neurons, and get a maturation support by glutamatergic GdNs. In nontransduced cultures, at days 1–3 after plating, out of several thousands of cells per coverslip, we

found only 12 DCX+ and/or βIII-tubulin+ cells (10 coverslips in 5 experiments). However, after 7–8 days, 10–100 DCX+/βIII-tubulin+ cells were found per coverslip. Gene transfer by MMLV-based retroviruses occurs only in cells that are actively replicating at the time of transduction, a feature that makes these vectors useful for the specific targeting of dividing cells within a neuronal lineage (Roe et al. 1993; Breunig et al. 2007). When glial cells were transduced with a retrovirus, about 3% of all transduced cells became DCX+/βIII-tubulin+, and many of them were part of isolated clones of 4–10 cells. This indicates that they were replicating at the time of transduction and suggests that they had entered a neuronal lineage. Under control conditions, these neurons barely survived DPI 20 in culture, and if so, they did not express hallmarks of mature neurons. However, in coculture with Ngn2-reprogrammed glutamatergic neurons, spontaneously formed neurons acquired attributes of GABAergic neurons (Fig. 5*B*,*C*). In neuronal cultures of the rat cortex, long-term blockade of glutamatergic network activity has been reported to strongly impair maturation and survival of a subtype of GABAergic interneuron (de Lima et al. 2004). Given this, it is possible that glutamatergic neuronal activity provides an environment in which GABAergic neurons survive and can develop synaptic hallmarks. More importantly in this context, our data show that GdNs are able to develop functional excitatory synaptic maturation and are able to acquire an inhibitory input.

Neurons from Reprogrammed Glia Form a Neuronal Network

Over the last decade, it has become increasingly clear that adult neural stem cells in the mammalian brain of rodents generate functional and synaptically integrated neurons throughout life (Carleton et al. 2003; Bergami et al. 2008; Faulkner et al. 2008; Grubb et al. 2008; Toni et al. 2008). This observation stimulated attempts to identify endogenous cell sources for the repair of degenerating neurons or the replacement of lost neurons (Buffo et al. 2005, 2008). Indeed, proof-of-principle evidence revealed a neurogenic potential of proliferating glial cells in the adult mouse neocortex after stab wound injury (Buffo et al. 2005, 2008).

At least in vitro, enormous progress in the reprogramming of glia cells toward neurons has recently been achieved. By using various transcription factors with a strong neurogenic potential, such as Ngn2 and Mash1, neuronal maturation of reprogrammed mouse astrocytes in vitro ended before synaptogenesis was completed (Berninger, Costa, et al. 2007). This study now shows that the reprogramming of postnatal rat glial cells can end in a synaptically interconnected network in vitro. Interestingly, this approach to instruct glial cells to a neuronal fate can also be extended to astroglial cells isolated from the mouse cerebral cortex upon injury and even enables subtype specification, as shown recently (Heinrich et al. 2010). Recently, Vierbuchen et al. (2010) described the induction of excitatory, glutamatergic neurons from mouse embryonic fibroblasts and early postnatal tail tip fibroblasts in vitro. In these experiments, reprogramming was effective when 3 transcription factors Ascl1 (Mash1) and Brn2 were combined with either Myt1l or Zic1. These studies (Heinrich et al., 2010; Vierbuchen et al., 2010; this study) independently verify the possibility to instruct excitatory neurons from a postnatal non-neuronal cell source from different cell systems or species that do not require an embryonic stem cell intermediate.

Synaptic connectivity is one of the most important prerequisites, making endogenous cell sources an attractive target for neuronal repair strategies. A functional network of neurons consisting of excitatory and inhibitory activities exhibits synergistic properties that regulate not only gene expression (Hardingham et al. 2001) but also neuronal maturation (Ben-Ari 2001; Voigt et al. 2005), survival (de Lima et al. 2004), synaptogenesis (Lüscher et al. 2000; Voigt et al. 2005), and even spinogenesis (Engert and Bonhoeffer 1999; Yuste and Bonhoeffer 2004). Even though the molecular mechanisms are not yet fully elucidated, there is striking evidence that neuronal network activity forms an extrinsic environment required for the tuning and scaling of neuronal communication (Turrigiano et al. 1998; Nelson and Turrigiano 2008; Turrigiano 2008). Consequently, establishment of a neuronal network by reprogramming of glia cells may be sufficient to start a self-autonomous program of neuronal wiring during neuronal repair.

Conclusion

This study provides evidence that glial cells may be reprogrammed into a functional, synaptically interconnected neuronal network. Future experiments have to be focused on in vivo experiments to see whether the cellular plasticity of glial cells in vitro can be repeated with dividing glial cells in vivo, especially during neuronal repair strategies in the cerebral cortex. In these experiments, it will be crucial to show that new neurons will be integrated into the somatotopic organization of the cortex and do not cause uncontrolled neuronal activity. These studies need to include a better understanding of the cellular mechanisms of reprogramming, a physiological verification of coordinated network activity and the behavioral monitoring of the affected cortical area.

Supplementary Material

Supplementary material can be found at http://www.cercor.oxfordjournals.org/.

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Notes

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