

CEND1 and NEUROGENIN2 Reprogram Mouse Astrocytes and Embryonic Fibroblasts to Induced Neural Precursors and Differentiated Neurons

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SUMMARY

Recent studies demonstrate that astroglia from non-neurogenic brain regions can be reprogrammed into functional neurons through forced expression of neurogenic factors. Here we explored the effect of CEND1 and NEUROG2 on reprogramming of mouse cortical astrocytes and embryonic fibroblasts. Forced expression of CEND1, NEUROG2, or both resulted in acquisition of induced neuronal cells expressing subtype-specific markers, while long-term live-cell imaging highlighted the existence of two different modes of neuronal trans-differentiation. Of note, a subpopulation of CEND1 and NEUROG2 double-transduced astrocytes formed spheres exhibiting neural stem cell properties. mRNA and protein expression studies revealed a reciprocal feedback loop existing between the two molecules, while knockdown of endogenous CEND1 demonstrated that it is a key mediator of NEUROG2-driven neuronal reprogramming. Our data suggest that common reprogramming mechanisms exist driving the conversion of lineage-distant somatic cell types to neurons and reveal a critical role for CEND1 in NEUROG2-driven astrocytic reprogramming.

INTRODUCTION

The mammalian adult cerebral cortex has a limited capacity to regenerate lost neural tissue after brain damage, due to the limited ability of the neural precursor cell (NPC) population residing in the two neurogenic niches to migrate and fully differentiate in response to injury-derived signals. To overcome this restriction, efforts have focused on the reprogramming of resident astroglial cells, initially *in vitro* and more recently *in vivo*, toward neurogenesis and the formation of functional synapse-forming neurons (Guo et al., 2014; Heinrich et al., 2010). Indeed, reactive astroglial cells isolated from non-neurogenic regions of the adult brain after local injury share hallmarks with NPCs and developmental radial glia (Sofroniew and Vinters, 2010), having the potential to be reprogrammed into induced neurons (Heinrich et al., 2010, 2011). Thus, forced expression of transcription factors are known to instruct neurogenesis in embryonic development, among which the basic-helix-loop-helix (bHLH) gene Neurogenin-2 (NEUROG2) or a combination of ASCL1, LMX1B, and NURR1 transcription factors direct reactive postnatal astrocytes *in vitro* toward generation of functional glutamatergic (Heinrich et al., 2011) or dopaminergic neurons (Addis et al., 2011), respectively. Furthermore, recent evidence demonstrates that the neuron-forming capacity of astro-

cytes is also active *in vivo* following neurodegeneration of the cortex and striatum (Guo et al., 2014; Magnusson et al., 2014; Niu et al., 2013; Torper et al., 2013), highlighting the existence of an endogenous cell source capable to restore connectivity and function following brain trauma.

Similarly, it has been shown that not only astrocytes but also somatic cells more distant in lineage to the CNS, such as fibroblasts, can be reprogrammed either into NPCs or directly into various types of neurons, including glutamatergic, dopaminergic, and spinal motor neurons, by different cocktails of transcription factors and neuron-specific microRNAs (Caiazzo et al., 2011; Vierbuchen et al., 2010; Wernig et al., 2008; Yoo et al., 2011) and may thus be used as an exogenous cell source to restore damage following neuronal loss.

The aim of this study is to explore the reprogramming potential of the neurogenic factor CEND1 in inducing the reprogramming of astrocytes and embryonic fibroblasts. CEND1 directs neural stem and precursor cells to acquire a neuronal phenotype both *in vitro* and *in vivo* (Georgopoulou et al., 2006; Katsimpardi et al., 2008; Politis et al., 2007). It forms part of the neuronal differentiation pathway(s) activated by proneural genes and is directly activated by the proneural genes Neurogenin-1 and 2 (Katsimpardi et al., 2008; Papadodima et al., 2005). Here, we

report a synergistic action of CEND1 and NEUROG2 in reprogramming of mouse postnatal cortical astrocytes and embryonic fibroblasts toward acquisition of a neuronal precursor and/or differentiated neuron phenotype. Additionally, we show that CEND1 expression is critical for the NEUROG2-driven reprogramming of astrocytes, suggesting the existence of a reciprocal feedback loop leading to neurogenesis.

RESULTS

Reprogramming of Mouse Postnatal Cortical Astrocytes by CEND1 and NEUROG2 to Induced Neurons with Subtype-Specific Identity

The aim of the study was to explore the action and possible synergy of CEND1 and NEUROG2 in inducing reprogramming of postnatal cortical astrocytes toward the neuronal lineage. The expression of NEUROG2 ceases with the onset of the gliogenic period during the late embryonic stage (Ozen et al., 2007). On the other hand, CEND1 is expressed in embryonic and adult NPCs and more strongly in differentiated neurons (Katsimpardi et al., 2008; Politis et al., 2007). It is not expressed in cortical astrocytes either in vivo (Koutmani et al., 2004) or in acute cortical cultures (Figures S1A and S1B). However, we observed that its expression was elevated upon astrocytic activation occurring after a short time in culture (Figures S1C and S1D), indicating that activated astrocytes acquire NPC characteristics in agreement with previous reports (reviewed in Robel et al., 2011). Overexpression of CEND1 and NEUROG2 was achieved in postnatal day 5 (P5) cortical astrocytes under the control of chicken β -actin promoter (CAG)—along with expression of the fluorescent proteins GFP and dsRed, respectively—using the pCAG-Cend1-IRES-Gfp and pCAG-Neurog2-IRES-dsRed retroviral vectors (Figure 1). Forced expression of CEND1, NEUROG2, or both resulted in the appearance of morphologically distinct, bipolar, elongated GFAP⁺ cells resembling embryonic radial glia, strongly expressing the radial glial marker GLAST, 24 and 48 hr following transduction (Figures 1B–1H, arrows). The appearance of an elongated radial glia phenotype amounted to 20%–40% of the total cell population depending on the transgene(s) expressed and was enhanced with time in culture (Figures 1I–1L).

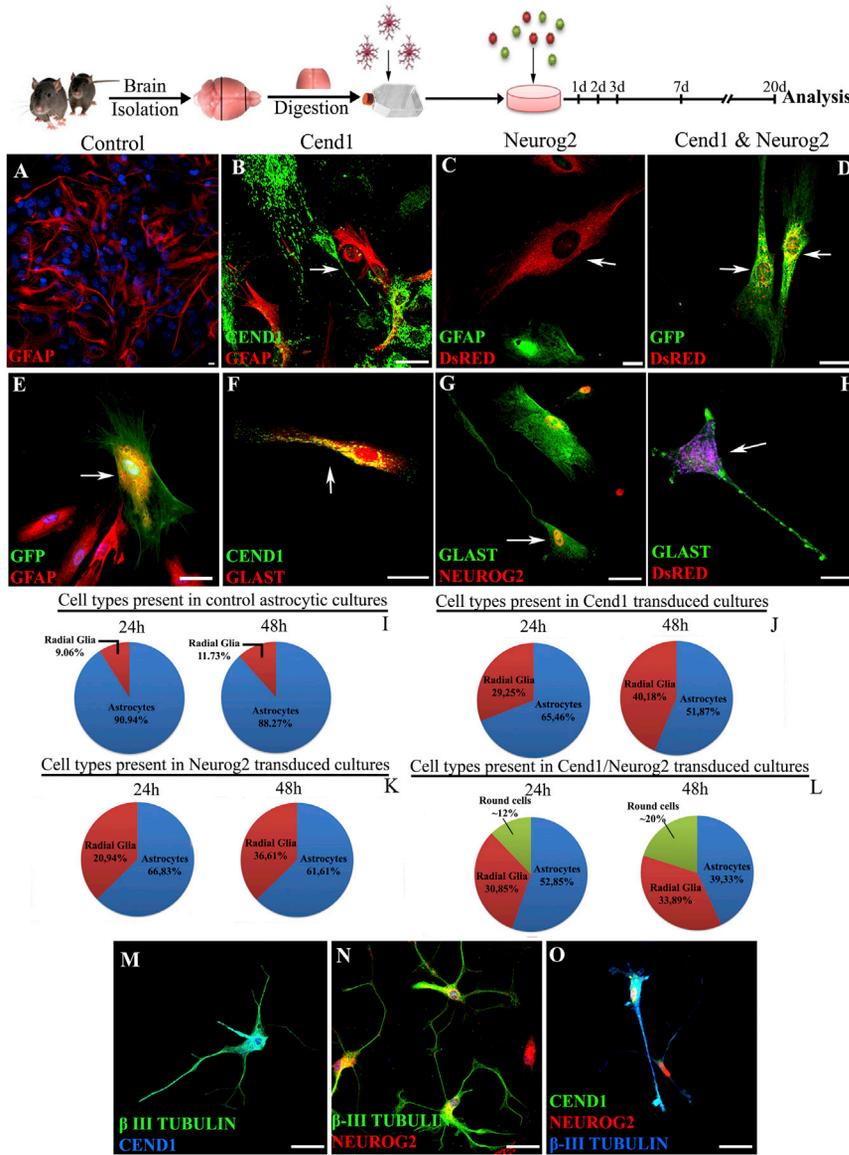
48 hr following transduction, cells with neuronal morphology emerged (Figures 1M–1O), while their number and level of differentiation was significantly higher after 72 hr (Figures 2A and 2L). All cells with neuronal morphology and β III-TUBULIN expression corresponded to either single- or double-transduced cells with CEND1 and/or NEUROG2. After 72 hr in culture, in addition to their differentiated branched morphology and β III-

TUBULIN expression, reprogrammed astrocytes started to express the neuronal subtype-specific markers GABA (Figures 2B, 2H, and 2M) or Tyrosine Hydroxylase (TH) (Figures 2I and 2M), depending on the neurogenic factor undergoing forced expression. In particular, $34\% \pm 2\%$ of the β III-TUBULIN⁺ neurons were GABA⁺ upon CEND1 overexpression (Figures 2B and 2M), while in NEUROG2-transduced cultures, $27\% \pm 2\%$ of β III-TUBULIN⁺ neurons were TH⁺ (Figure 2M). In double-transduced cultures, both GABA⁺ and TH⁺ neuronal subtypes were produced (Figures 2H, 2I, and 2M). 1 week after CEND1 and/or NEUROG2 overexpression, the pre- and post-synaptic markers synapsin and PSD95 appeared (Figures 2C, 2D, 2F, 2G, 2J, and 2K), indicating further maturation of the induced neurons. After 20 days in culture, glutamatergic neurons appeared only upon NEUROG2 overexpression (Figure 2E), as already reported (Heinrich et al., 2010), amounting to $8\% \pm 1\%$ of β III-TUBULIN⁺ neurons.

Reprogrammed neurons cultured for more than 1 week exhibited a highly differentiated morphology with multiple and complex processes emanating from their small cell somas (Figure S2A). Quantification of the total neurite length in the three different neuronal subtypes produced (GABA⁺, TH⁺, GLUTAMATE⁺) indicated that total neurite length was $158 \pm 0.5 \mu\text{m}$ in the CEND1-overexpressing GABA⁺ neurons and $287 \pm 1 \mu\text{m}$ in the NEUROG2-overexpressing TH⁺ neurons, whereas NEUROG2-overexpressing glutamatergic neurons exhibited the highest neurite length of $540 \pm 0.7 \mu\text{m}$. Additionally, NEUROG2-derived neurons (both TH⁺ and GLUT⁺) and, to a lesser extent, CEND1-derived neurons exhibited a higher ratio of total neurite length to cell body perimeter and more neurites sprouting (Figures S2A and S2B), while neurons formed by double-transduced astrocytes (Figures 2I and 2L) exhibited a less differentiated morphology with fewer processes, reduced total neurite length, and larger cell bodies (Figures S2A and S2B).

Long-Term Time-Lapse Experiments Revealed Two Modes of Astrocytic Reprogramming to Neurons

In order to investigate whether cell division is required for cell fate conversion to occur or astrocytes trans-differentiate directly to post-mitotic neurons following forced expression of CEND1 or NEUROG2, we performed continuous live-cell imaging for 1 week combined with Timm's Tracking Tool (TTT) analysis (Costa et al., 2011; Eilken et al., 2009) to track transduced cells' lineage trees (Figure 3; Figure S3). Results derived by tracking β III-TUBULIN⁺ neurons back in time revealed that the majority of CEND1-overexpressing astrocytes passed through one to two divisions, before neuronal trans-differentiation (Figures 3A–3G; Movie S1). Approximately 80% of divisions were asymmetric, giving rise only to one neuron (Figures



3J–3L), and 20% were symmetric, leading to the formation of two β III-tubulin⁺ neurons (Figures 3H and 3I). By contrast, as already reported using live-cell imaging for 5 days (Heinrich et al., 2010), newborn neurons derived from NEUROG2-transduced astrocytes were rarely produced in a proliferative manner (Figure S3). Analysis of β III-TUBULIN⁺ neurons 1 week after NEUROG2 overexpression revealed that up to 82% of NEUROG2-overexpressing astrocytes directly trans-differentiated into β III-TUBULIN⁺ neurons (Figure S3H; Movie S2) without passing through a proliferative stage. Additionally, whereas during the first days astrocytes were very motile, as neuronal trans-differentiation occurred their motility became more limited, and by the time they acquired neuronal identity, their cell bodies stopped moving and

only their processes headed toward different directions scavenging the environment (Movies S1 and S2).

Multipotent Neurospheres Formed in CEND1 and NEUROG2 Double-Transduced Astrocytic Cultures

Unexpectedly, only in double-transduced cultures, colonies of small round cells that formed highly proliferative three-dimensional spheres attached to the plate were detected 48 hr after transduction (Figure 4I). These clones, which amounted to approximately 20% of the whole-cell population (Figure 1L), were a transient population that could not survive for more than 72 hr when cultured in astrocyte medium and composed of cells with a mean diameter of 10–15 μ m, similar to the size of neurosphere cells derived from the subventricular zone (SVZ)

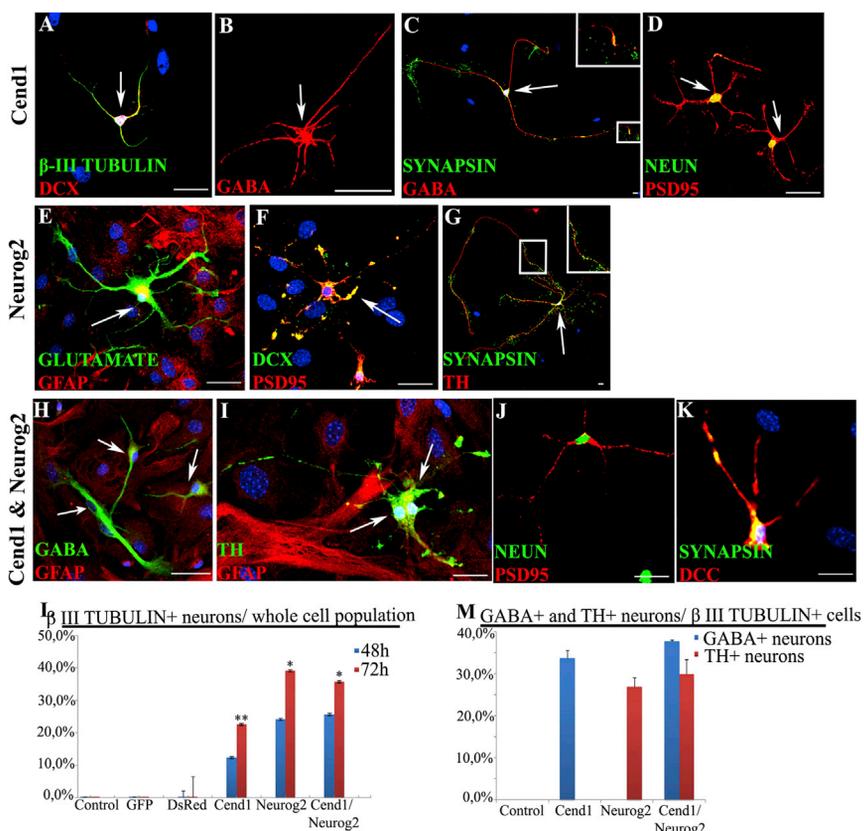


Figure 2. Distinct Subtype-Specific Neuronal Identity Acquired by Reprogrammed Astrocytes following CEND1 and/or NEUROG2 Forced Expression

(A–K) Differentiated neuronal morphology, subtype-specific neuronal phenotype, and synaptic marker expression in neurons derived from reprogrammed astrocytes after 20 days in culture, following CEND1 (A–D), NEUROG2 (E–G), and CEND1 and NEUROG2 (H–K) overexpression. CEND1 overexpression directed reprogrammed neurons (A) toward acquisition of GABA⁺ identity (B), whereas NEUROG2 overexpression directed reprogrammed neurons toward TH⁺ (G) and glut⁺ (E) identity. Both GABA⁺ and TH⁺ cells were present upon forced expression of both molecules (H–I). Functional markers PSD95, indicating post-synaptic activity (D, F, and J, arrows), and SYNAPSIN, indicating pre-synaptic activity (C, G, and K, arrows), were present in the synaptic membranes of reprogrammed NEUN⁺ and DOUBLECORTIN⁺ neurons.

(L) β III-TUBULIN⁺ cells appeared in astrocytic cultures 48 hr after CEND1 and/or NEUROG2 overexpression, and their percentage significantly increased after 72 hr. (M) Percentages of GABA⁺ and TH⁺ neurons

in the β III-TUBULIN⁺ neuronal population 72 hr after CEND1 and/or NEUROG2 forced expression.

DCX, DOUBLECORTIN. Nuclei were stained by TOPRO-3 (blue). Data were collected from at least three independent experiments. Scale bars, 40 μ m. See also Figure S2.

(Figure 4I). Live-cell imaging starting 12 hr after transduction combined with lineage tracing enabled us to visualize the timing of the first divisions resulting in sphere formations that took place during the first 21 hr following CEND1 and NEUROG2 double transduction (Figures 4A–4H; Movie S3). To investigate the potential of this cell population, we isolated spheres, either adherent or floating, and cultured them in NPC culture medium. This protocol resulted in the generation of double-transduced floating spheres resembling neurospheres (Figures 4J–4L), which we named “astrospheres” because of their cellular origin. Astrospheres could be propagated for more than 20 passages and exhibited proliferation and differentiation characteristics similar to NPCs derived from P5 mouse SVZ (Figures 4M–4Q). In particular, in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), astrospheres expressed NESTIN and exhibited similar proliferation characteristics as neurospheres (Figures 4M and 4N), whereas 3 days after growth factor withdrawal, they gave rise to β III-TUBULIN⁺ neurons, GFAP⁺ astrocytes, and O4⁺ oligodendrocytes (Figures 4O–4Q), in similar percentages with

those derived upon SVZ-derived neurosphere differentiation (Figures 4R–4U).

CEND1 and NEUROG2 Trans-differentiate Mouse Embryonic Fibroblasts to NPCs and Differentiated Neurons

Next we sought to investigate whether the two molecules possess a broader neurogenic potential and can also convert more lineage-distant cell types toward a neuronal identity. On the basis of supporting evidence from the literature that fibroblasts can be directly reprogrammed to neurons if provided with combinations of transcription factors including those of the bHLH family, such as NEUROG2 (Vierbuchen et al., 2010), we tested the direct reprogramming efficiency of CEND1 and/or NEUROG2 upon their forced expression in mouse embryonic fibroblasts (MEFs). To this end, we developed a multistep culture protocol summarized in Figure 5. MEFs were transduced and cultured in reprogramming media until day 14, when reprogrammed cells were re-plated and cultured in neuronal differentiation medium. By day 14, transduced MEFs overexpressing either CEND1 or CEND1 and NEUROG2

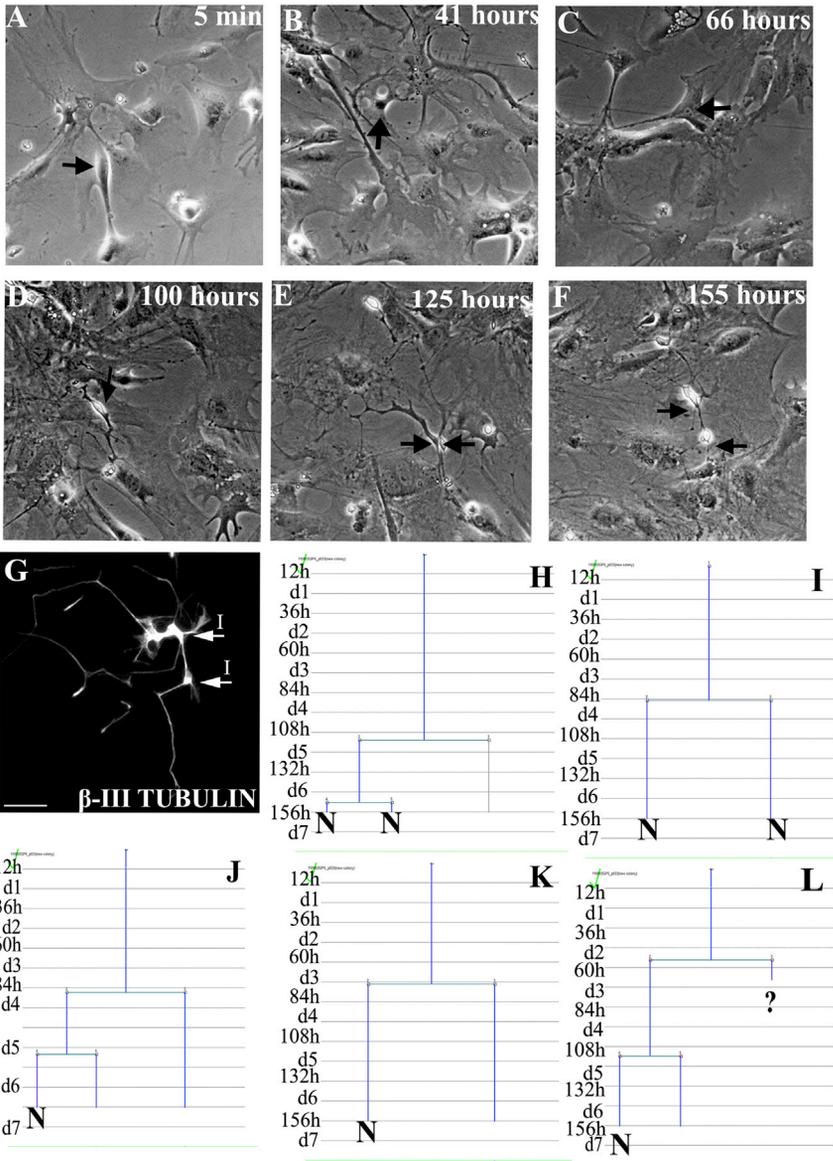


Figure 3. Long-Term Live-Cell Imaging upon CEND1 Overexpression

(A–L) Astrocytic cultures transduced with the CEND1-IRES-GFP retrovirus trans-differentiated, giving rise to β III-TUBULIN⁺ neurons (G) after passing through one or two divisions (lineage trees H to L). Snapshots of the following time points during 1 week live-cell imaging: 5 min (A), 41 hr (B), 66 hr (C), 100 hr (D), 125 hr (E), and 155 hr (F). Cell tracking and lineage tree drawings were correlated with β III-TUBULIN⁺ neurons produced (G). 80% of the divisions were asymmetric, giving rise to one neuron and one or more astrocytes and/or intermediate precursor cells (lineage trees J, K, and L), and 20% were symmetric, leading to the formation of two neurons (lineage trees H and I). The movie that accompanies this figure (Movie S1) concerns tracking of lineage tree (I). ?, cell lost from field of view. Scale bars, 40 μ m. See also Figure S3 and Movies S1 and S2.

(Figures 5A, 5D, and 5G) acquired a different morphology from control cultures (Figures 5A–5I). Upon CEND1 forced expression small spheres started appearing (Figure 5F), and the spheres were larger and better formed, spontaneously detaching from the culture dish, with combined CEND1 and NEUROG2 forced expression (Figure 5I). In cultures transduced with NEUROG2 only, small spontaneously floating spheres were also formed but could not be maintained for the long term, in contrast to the spheres derived by CEND1 or CEND1 and NEUROG2, which could be maintained for several passages and yielded viable cells after freezing and thawing.

Molecular phenotype analysis at different time points (Figures 5 and 6A–6D) revealed that a significant number

of NESTIN⁺ cells were already present in control cultures not exposed to either neurogenic molecule when these were maintained in MEF reprogramming medium (Figures 5J and 5K). However, upon CEND1 and/or NEUROG2 overexpression, the percentage of NESTIN⁺ and/or SOX2⁺ (Figures 5L and 5O; Figure 6A) cells was much higher as compared with controls, especially during the first 14 days in reprogramming medium (Figure 6A). As soon as cells were transferred to differentiation media, the population of NESTIN⁺ neural precursors in CEND1-transduced cultures remained similar to non-transduced controls, while it decreased most pronouncedly in NEUROG2 single-transduced, but also in double-transduced, cells (Figure 6A). At the same time, a significant percentage of

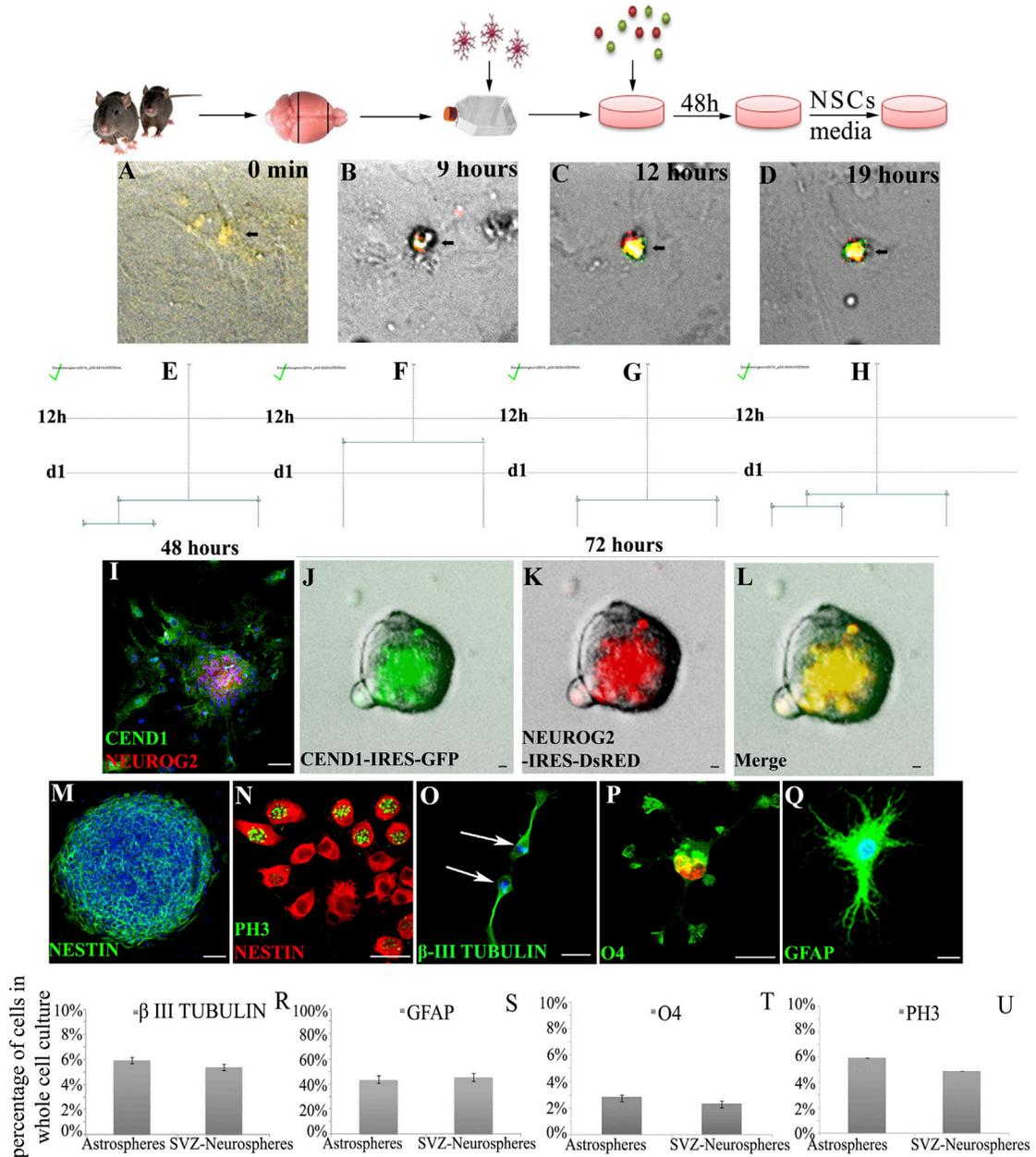


Figure 4. Formation of Three-Dimensional Spheres following Double CEND1 and NEUROG2 Forced Expression

(Top) Protocol schematically representing all stages of astrosphere formation.

(A–D) Snapshots of the following time points during 19 hr of live-cell imaging show the formation of GFP⁺ and dsRed⁺ clones: 0 hr (A), 9 hr (B), 12 hr (C), and 19 hr (D).

(E–H) Lineage tree drawings indicate the creation of clones in early stages with the first division to happen between day 1 and 2 in all samples shown here: 21 hr (E), 14 hr (F), 19 hr (G), and 18 hr (H).

(I) Three-dimensional colonies attached to the culture dish were present 48 hr after CEND1 and NEUROG2 double overexpression.

(J–L) Live spheres isolated from the astrocytic culture and cultured in NSC medium (L) expressed both GFP (J) and dsRed (K) after 72 hr.

(M and N) High NESTIN expression (M) and proliferative activity (N) in 10th passage astrospheres.

(O–Q) Upon growth factors removal, cells gave rise to βIII-TUBULIN⁺ neurons (O), O4⁺ oligodendrocytes (P), and GFAP⁺ astrocytes (Q).

(R–U) Quantification and statistical analysis of immunostaining data indicated that the differentiation process toward neural progenitors (R), astrocytes (S), oligodendrocytes (T), and the proliferation capacity of astrospheres (U) was not statistically significant different from that of P5 subventricular zone-derived neurospheres.

Nuclei were stained by TOPRO-3 (blue). Data were collected from at least three independent experiments. Scale bars, 40 μm. See also [Movie S3](#).

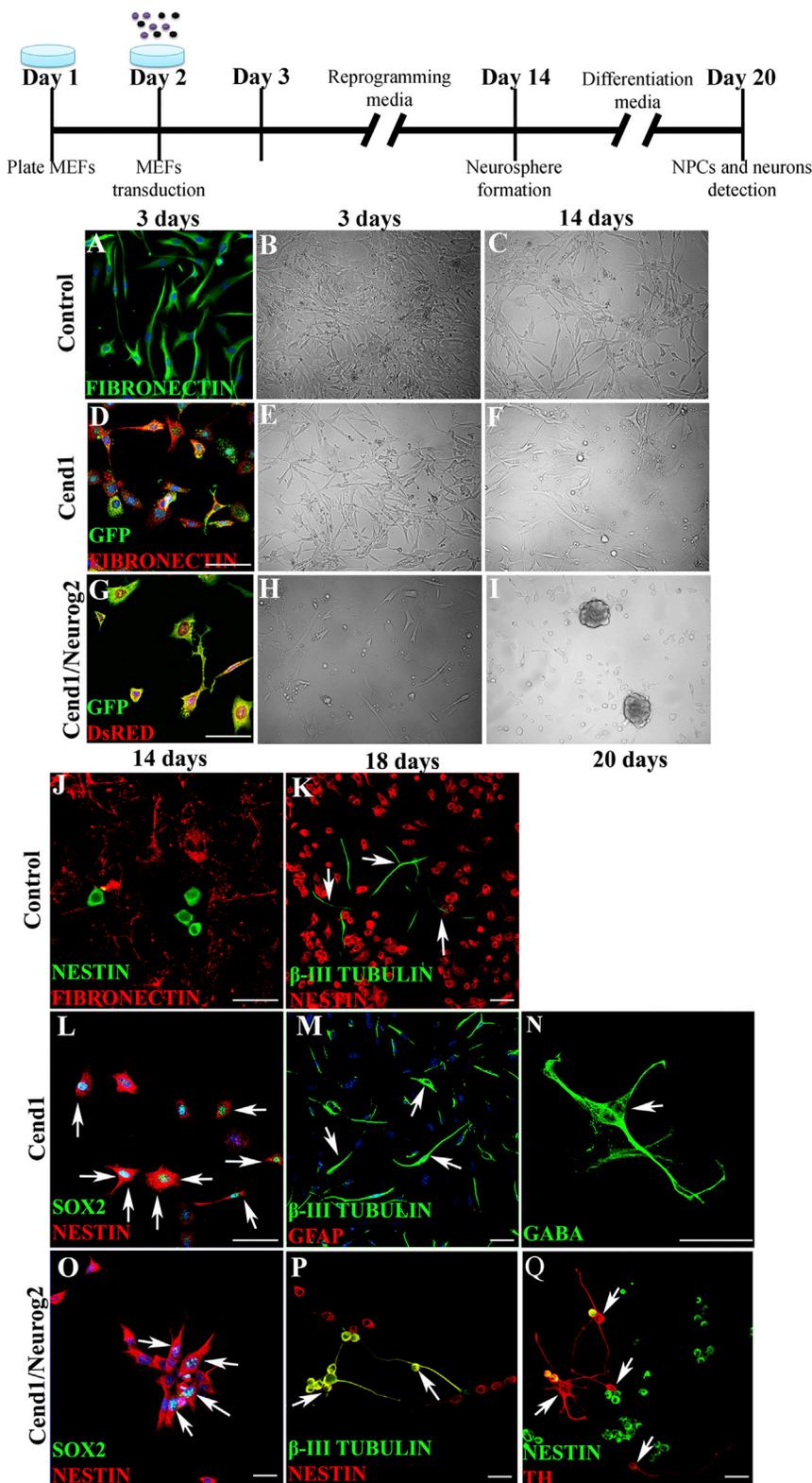


Figure 5. MEF Reprogramming upon Forced Expression of CEND1 and/or NEUROG2

(Top) Schematic drawing of the protocol used for MEF reprogramming.

(A, D, and G) 3 days after viral transduction, cells transduced with both *GFP*- and *dsRed*-*cre* viruses exhibited high FIBRONECTIN expression (A), which became diminished following CEND1 overexpression (D). (G) High transduction efficiency was observed in MEFs co-transduced with CEND1 and NEUROG2.

(B, C, E, F, H, and I) In day 3 an alteration on the phenotype of the cell cultured can be noticed with CEND1- and NEUROG2-transduced cultures to have the biggest change (H), while CEND1⁺ cultures (E) have changed too compared to the control cultures (B). By day 14, cultures transduced with either CEND1 or CEND1 and NEUROG2 formed smaller (F) or bigger diameter spheres (I), depending on the molecule(s) being force expressed. No such cell types appeared in control cultures at the same time point (C).

(J, L, and O) Cells expressing the NPC markers NESTIN and Sox2 in naive cultures (J) and following CEND1 (L) and CEND1 and NEUROG2 (O) transduction by the end of reprogramming period (day 14).

(K, M, and P) A small percentage of β III-TUBULIN⁺ neurons appeared in *ctrl* cultures by day 18 (K), while their number was significantly higher in CEND1-transduced cultures (M) and in particular CEND1- and NEUROG2-transduced cultures (P).

(N and Q) After 20 days, a subpopulation of β III-TUBULIN⁺ cells was expressing the neuronal subtype-specific markers, GABA (N) and TH (Q) in CEND1 and CEND1- and NEUROG2-transduced cultures, respectively.

Nuclei were stained by TOPRO-3 (blue). Scale bars, 40 μ m.

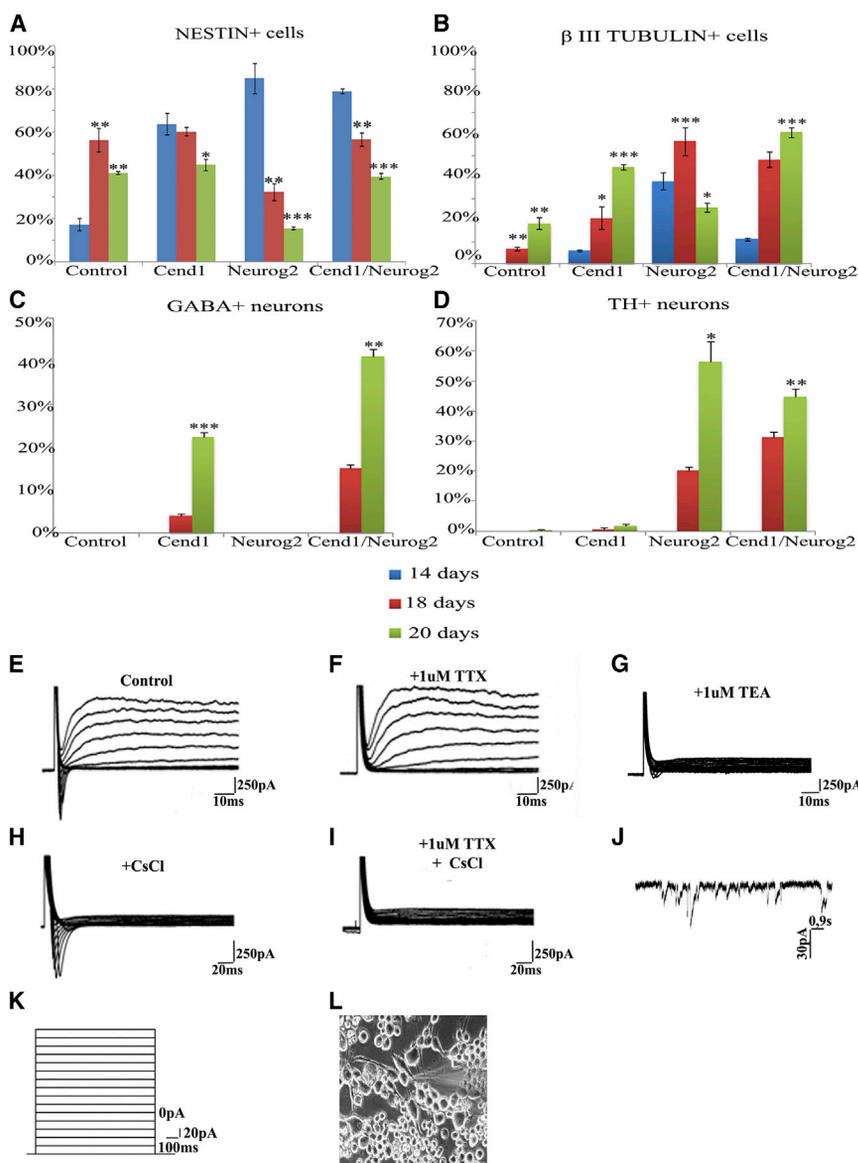


Figure 6. MEF Culture Shift to Induced Neural Precursor Cell Culture and Neuronal Cells Shown via Immunohistochemical and Electrophysiological Analysis

(A and B) Graphs indicating the percentages of NESTIN⁺ NPCs (A) and βIII-TUBULIN⁺ neurons (B) in the whole-cell population during reprogramming and neuronal differentiation stages both in naive cultures and upon CEND1 and/or NEUROG2 forced expression.

(C and D) Percentages of GABA⁺ (C) and TH⁺ (D) neurons in the βIII-TUBULIN⁺ neurons population in naive cultures and upon CEND1 and NEUROG2 forced expression. All statistical data were collected from at least three independent experiments.

(E and F) Representative whole-cell current response of CEND1- and/or NEUROG2-induced neurons. Step depolarizing pulses evoked fast activating and rapidly inactivating inward currents followed by outward non-inactivating ones. The inward component of the current response was abrogated by the selective sodium channel blocker TTX.

(G) The outward component of the current response was blocked by the selective potassium channel blocker TEA.

(H) Representative traces of whole-cell patch clamp recordings with CsCl in the recording pipette. When K⁺ ions were substituted by Cs⁺ ones in the patch pipette, depolarizing steps elicited only the fast inactivating inward current response but suppressed the outward ones.

(I) The inward currents were also totally blocked by the presence of 1 μM of the selective voltage-gated sodium channel blocker TTX.

(J) Representative recording of spontaneous current events of various amplitudes from a cell held at -60 mV.

(K) The voltage protocol used to elicit whole-cell current responses. The holding potential was at -60 mV. The membrane was stepped at -120 before and after each depolarizing voltage step.

(L) Whole-cell patch-clamp experiments were performed on neurons present in cell cultures for up to 42 days.

βIII-TUBULIN⁺ neurons appeared (Figure 5M), which reached $61 \pm 0.8\%$ out of all cells upon combined CEND1 and NEUROG2 forced expression (Figures 5P and 6B), indicating the need of both molecules for more efficient neuronal differentiation. Although NEUROG2-derived cultures could be differentiated to βIII-TUBULIN⁺ neurons (Figure 6B), NEUROG2 was found to be less potent in driving and sustaining MEFs neuronal trans-differentiation, unless its downstream effector CEND1 was also force expressed (Figure 6B). This is in agreement with recent data

indicating that the downstream neuronal differentiation factors MYTL1 and BRN2 are required for NEUROG2 to reprogram MEFs to induced neurons (Chanda et al., 2013).

Cells maintained for up to 46 days in culture, according to the protocol in Figure 5, further differentiated expressing the neuronal subtype-specific markers GABA (Figure 5N) and TH (Figure 5Q). In accordance with our astrocytic reprogramming data, the subpopulation of GABA⁺ neurons appeared only in CEND1 or CEND1 and NEUROG2 transduced cultures, with the latter reaching $41.2\% \pm 3\%$ of



β III-TUBULIN⁺ neurons (Figure 6C). On the other hand, TH⁺ neurons appeared in either NEUROG2 or CEND1 and NEUROG2 double-transduced cultures (Figure 6D). Such subtype-specific neurons were never seen in control cultures. Within the time period tested and the culture media used, which favor neuronal differentiation, only neuronal, but not glial, lineage markers were expressed by reprogrammed MEFs (Figure 5M), unlike astrospheres, which were cultured in neurosphere medium that retained the potential to differentiate to neurons, astrocytes, and oligodendrocytes.

Induced neurons derived from reprogrammed fibroblasts by CEND1 or CEND1 and NEUROG2 overexpression exhibited neuronal-like electrical characteristics, such as voltage-gated sodium and potassium currents, while a number of cells were able to fire action potentials (Figures 6E–6K). Data were collected from 43 out of 60 cells at different days in culture (22 to 43 days). Cells were held at –60 mV and currents were recorded in response to 10-mV step depolarizations up to +50 mV (Figure 6K). Step depolarizations were preceded by a 50-ms hyperpolarizing pulse to –120 mV in order to remove residual current inactivation. All neurons displayed fast activating and rapidly inactivating inward currents followed by outward ones (Figure 6E), while in the majority of the cells injection of depolarizing currents evoked single action potentials. Application of 1 μ M tetrodotoxin (TTX) abrogated the inward current in the majority of the cells (Figures 6F and 6I). Few cells exhibited a small but significant inward current at –50 mV that was blocked by 1 mM NiCl₂, suggesting the presence of T-type calcium channels. The outward currents were blocked by 10 mM tetraethylammonium (TEA), suggesting the presence of voltage-gated potassium channels (Figure 6G). This was further supported by the fact that no outward currents were elicited when KCl was substituted by CsCl (Figure 6H). In general, the amplitude of voltage-gated currents was higher in cells that stayed longer in culture, while the width of the fired action potentials decreased. Furthermore, few neurons overexpressing both molecules presented spontaneous post-synaptic current activity at –60-mV holding potential (Figure 6J).

CEND1 and NEUROG2 Cross-Activate Each Other during Astrocytic Reprogramming

NEUROG2 is a bHLH factor exhibiting a well-characterized mechanism of action in inducing neuronal reprogramming in vitro by binding to the promoter of its downstream neuronal target genes. However, the mode of the reprogramming action of CEND1, which is not a transcription factor, as well as its synergistic action with NEUROG2 is less obvious. Our previous results indicate that NEUROG2 activates CEND1 through its binding to the E box present at the CEND1 promoter and that this effect is abolished

upon E box mutation (Katsimpardi et al., 2008). To investigate the causal relationship between the two molecules in inducing astrocytic reprogramming, we measured the mRNA levels of each one of them in astrocytic cultures overexpressing CEND1, NEUROG2, or both. Real-time qRT-PCR data verified overexpression of *Cend1* and *Neurog2* 48 and 72 hr following transduction (Figures 7A and 7B). As expected (Katsimpardi et al., 2008), forced expression of NEUROG2 resulted in increased levels of *Cend1* (Figures 7A and 7B). Interestingly, CEND1 overexpression also resulted in elevated levels of endogenous *Neurog2* expression (Figures 7A and 7B), while immunocytochemical observation of reprogrammed astrocytes verified the induction of endogenous CEND1 upon NEUROG2 overexpression (Figure 7C) and vice versa (Figure 7D). It thus seems that a reciprocal activating feedback loop exists between the two molecules, where expression of each one is activated by overexpression of the other.

CEND1 Is a Key Mediator of NEUROG2 Reprogramming Function

To address whether CEND1 is needed for NEUROG2 to exert its neurogenic action, we co-transduced cortical astrocytes with NEUROG2 in the presence of a sh-CEND1-GFP silencing lentiviral vector that efficiently knocks-down *Cend1* mRNA (Katsimpardi et al., 2008). As control we used a sh-*Luc*-GFP lentiviral vector targeting the *Luciferase* mRNA (Figures 7E–7G). Analysis of double-transduced cultures after 48 hr indicated that upon CEND1 silencing, the majority of NEUROG2-overexpressing astrocytes remained GFAP⁺ (Figures 7I and 7J, arrows) and were not reprogrammed to either GLAST⁺ (Figure 7H, arrow) and NESTIN⁺ radial glia or β III-TUBULIN⁺ neurons, as NEUROG2 single-transduced (Figure 7K, arrowheads) or control sh-*Luc* and NEUROG2 co-transduced astrocytes, expressing both GLAST and β III-TUBULIN (Figures 7F and 7G, arrows). More specifically, only 12.9% \pm 3% (versus 29.5% \pm 4% in sh-*Luc* and NEUROG2 controls) and 16% \pm 4% (versus 26.7% \pm 2% in sh-*Luc* and NEUROG2 controls) of sh-CEND1- and NEUROG2-transduced astrocytes gave rise to NESTIN⁺ and GLAST⁺ radial glia, respectively, while no β III-TUBULIN⁺ neurons were observed in CEND1-silenced and NEUROG2-overexpressing cells. This finding indicates that CEND1 not only influences NEUROG2 expression levels but is also a key mediator of NEUROG2-induced neurogenic reprogramming.

Mechanism of CEND1 and NEUROG2 Synergistic Action

To get insight into the mechanism through which CEND1 and/or NEUROG2 exert their reprogramming function in astrocytic cultures, we performed a real-time qRT-PCR array analysis on plates with 84 key neurogenesis factors and

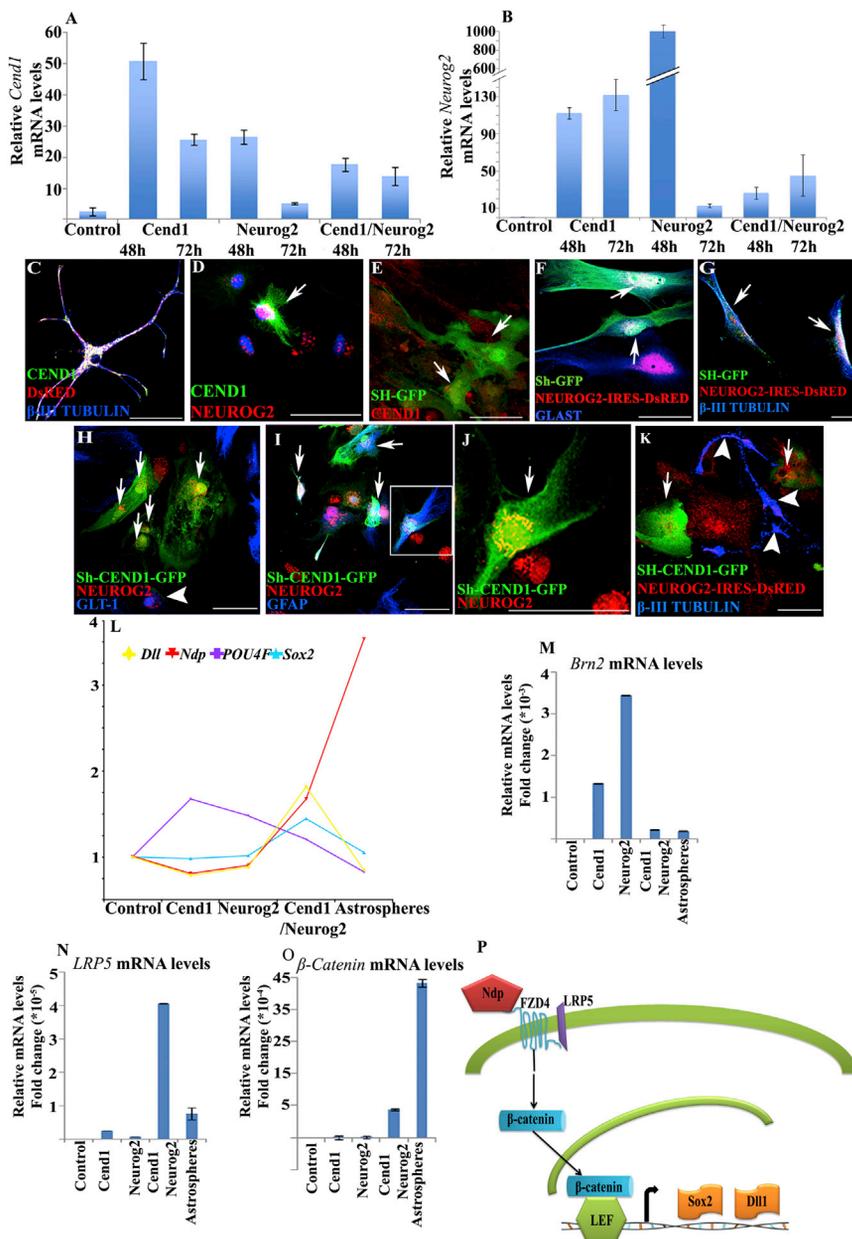


Figure 7. CEND1 and NEUROG2 Form a Positive Feedback Loop Inducing Neuronal Reprogramming

(A and B) mRNA levels of *Cend1* (A) and *Neurog2* (B) 48 and 72 hr following their overexpression in astrocytic cultures as revealed by real-time qRT-PCR.

(C) NEUROG2 overexpression resulted in elevated endogenous levels of CEND1.

(D) CEND1 overexpression also induced endogenous NEUROG2 expression 48 hr following transduction.

(E–K) Co-transduction of NEUROG2 together with a CEND1-silencing lentiviral vector expressing GFP (sh*Cend1*-GFP) (H–K) or the control lentivirus sh*Luc*-GFP (E–G). In contrast with sh-*Luc*- and NEUROG2-transduced control cultures (E–G), where astrocytes expressing low levels of CEND1 (E, arrows) gave rise to GLAST⁺ radial glia (F, arrows) and βIII-TUBULIN⁺ neurons (G, arrows), the majority of sh-CEND1- and NEUROG2-transduced cells (H–K) retained their astrocytic morphology and GFAP expression (I and J, arrows) and did not express GLAST (H, arrows) or βIII-Tubulin (K, arrow). (J) Magnification of the squared cell in (I) exhibiting high GFAP expression. In the same cultures (H–K), single-transduced NEUROG2 cells trans-differentiated to GLAST⁺ radial glia (H, arrowhead) and βIII-TUBULIN⁺ neurons (K, arrowheads). GLT1, GLAST. Scale bars, 40 μm.

(L) mRNA levels of four genes (*Dll1*, *Ndp*, *POU4f1*, *Sox2*) with differential mRNA levels in single CEND1- or NEUROG2-overexpressing astrocytic cultures 48 hr after transgene overexpression versus double-transduced or astrosphere cultures, as revealed by real-time qRT-PCR array analysis of 84 genes related to multipotency and neurogenesis.

(M) The *Brn2* mRNA levels of expression.

(N and O) The mRNA levels of receptor *Lrp-5* (N) and mRNA levels of *LRP5*'s downstream molecule β-catenin (O). The levels of *Ndp*, *Lrp-5*, and β-catenin are strongly upregulated in double-transduced and astrosphere cultures. By contrast, the mRNA levels of the neurogenic factor *Brn2* are highly upregulated only in single-transduced cultures.

(P) Schematic diagram of the canonical Wnt/β-catenin pathway, leading to subsequent activation of multipotency and/or proliferation genes.

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(P) Schematic diagram of the canonical Wnt/β-catenin pathway, leading to subsequent activation of multipotency and/or proliferation genes.

See also Figures S4 and S5. mRNA level quantification by qRT-PCR is based on three biological replicates.

compared the mRNA profiles of control, single-induced, and double-transduced cultures 48 hr after transgene overexpression (Figure S4). Comparison of the mRNA profiles of CEND1—versus NEUROG2—overexpressing cultures by scatterplot analysis indicated that approximately 90% of tested genes in single-transduced cultures exhibited similar expression levels (Figure S5). Among these, 28 genes related

to neuronal differentiation, axonogenesis, and synaptic transmission were upregulated upon CEND1 or NEUROG2 overexpression as compared with control astrocytic cultures. These included *Pou4f1* (Figure 7L), *Map2*, *BDNF*, *NrCAM*, *S100a6*, and *Rtn4*. Additionally, the mRNA levels of *Brn2*, not included in the PCR array, were also elevated as revealed by real-time qRT-PCR for individual genes of interest



(Figure 7M). Importantly, comparison of differentially expressed genes in single- versus double-transduced cultures revealed a significant upregulation of genes associated with multipotency only in double-transduced and astrosphere cultures, including the transcription factor *Sox2* and the Notch ligand *Dll1* (Figure 7L). Interestingly, *Ndp*, encoding for the secreted protein Norrin that activates β -catenin leading to neural stem cell proliferation (McNeill et al., 2013), was significantly upregulated in double-transduced cultures with even higher levels detected in astrospheres (Figure 7L). To address whether downstream components of the Norrin signaling pathway are also activated upon double CEND1 and NEUROG2 overexpression, we performed real-time qRT-PCR for the Norrin receptor *Lrp-5* and its effector *β -catenin*. Our data indicate a robust increase in the mRNA levels of both molecules in double-transduced and astrosphere cultures (Figures 7N and 7O), implying activation of the canonical Wnt/ β -catenin pathway upon CEND1 and NEUROG2 forced expression, resulting in upregulation of multipotency genes, such as *Sox2* and *Dll1* (Figure 7P).

DISCUSSION

In this study we addressed the question of whether lineage-distant somatic cell types residing inside and outside the CNS can be turned into NPCs and neurons upon forced expression of factors that induce neurogenesis during development. We demonstrated that (1) the neurogenic molecule CEND1 can reprogram mouse astrocytes toward GABA⁺ neurons and acts synergistically with NEUROG2 to trans-differentiate them toward proliferating NPCs and subtype-specific neurons, (2) CEND1 and NEUROG2 possess a broader neurogenic potential, inducing neuronal reprogramming of MEFs, (3) CEND1 is a key downstream player in NEUROG2-induced astrocytic reprogramming participating in a positive feedback loop leading to neurogenesis, and (3) double overexpression of CEND1 and NEUROG2 results in activation of the Wnt/ β -catenin signaling pathway driving astrocytes to acquire an NSC multipotent character. Taken together, our results demonstrate that CEND1 is not only a potent inducer of neuronal differentiation in neural precursor cells as previously shown (Katsimpardi et al., 2008; Politis et al., 2007), but it also has a neurogenic capacity in different cell contexts.

Molecular characterization of neurodegenerative pathologies designates that reactive astrogliosis involves a number of changes in morphology, gene expression, and function of astrocytes, which in that state share hallmarks with NPCs (Robel et al., 2011). Astrocytes constitute the most abundant glial cell type, and they endogenously possess the tendency to proliferate following brain trauma and are easily reprogrammable to neural precursors and

functional neurons (Magnusson et al., 2014; Torper et al., 2013). They thus constitute a target cell source for neuronal replacement strategies following brain injury. In the present study, we show that the two neurogenic factors CEND1 and NEUROG2 can synergistically trans-differentiate postnatal cortical astrocytes in vitro to multipotent, expandable NPCs and differentiated neurons possessing GABA⁺, TH⁺, or LUT⁺ subtype specificity. Moreover, long-term live-cell imaging proved that astrocytic trans-differentiation to neurons is not always direct, but depending on the reprogramming factor, cells may undergo a limited number of divisions before giving rise to post-mitotic neurons. This is the case for CEND1, which unlike NEUROG2 allows a couple of asymmetric divisions prior to neuronal reprogramming. An intriguing and rather unexpected finding is the de-differentiation of astrocytes to a NPC state upon overexpression of both molecules. It is noteworthy that in this situation the mRNA levels of the two transgenes are relatively lower than in single-transduced astrocytes. Recent findings highlight an oscillatory mode of expression of bHLH factors in neural precursors that underlines their action during normal development and in cell reprogramming (Imayoshi and Kageyama, 2014). Depending on expression dynamics, bHLH factors seem to have opposing functions in promoting NPC proliferation and cell-cycle exit and differentiation, with the multipotent state correlating with lower levels and oscillatory expression and the differentiation state correlating with sustained expression of a single bHLH factor (Imayoshi et al., 2013). Our results concerning the reprogramming function of CEND1 and NEUROG2 may thus be explained in light of these findings. We postulate that lower levels of NEUROG2 and CEND1, as observed upon their combined forced expression, induce a cell fate choice toward proliferating multipotent neural precursors, whereas higher levels as in single-transduced cells drive neuronal differentiation. It is true that CEND1 has been repeatedly shown before to drive neural stem cells out of the cell cycle toward neuronal differentiation (Katsimpardi et al., 2008; Makri et al., 2010). However, its function had not been tested in astrocytes before, which apparently undergo the above-described proliferative switch when exposed dually to CEND1 and NEUROG2. It is not uncommon that the mechanism of action of molecules participating in neurogenic pathways is in many cases dependent upon the cell context and developmental stage. For example, NEUROG2, which exhibits a well-characterized neurogenesis-promoting action during forebrain development (Schurmans et al., 2014) and leads to direct neuronal reprogramming of astrocytes as shown by Heinrich et al. (2010) and confirmed in this study, has been also shown by lineage tracing studies to regulate cell-cycle progression of neuronal progenitors in the developing cerebellum (Florio and Barbieri, 2012).



Therefore, our present data on astrosphere formation, although unexpected, are not contradictory, but rather are complementary to our previous observations. Interestingly, the proliferative switch observed in double-transduced cultures seems to be linked to activation of the Wnt/ β -catenin signaling pathway via a notable upregulation of *Norrin* (Zerlin et al., 2008), as revealed by real-time qRT-PCR array analysis. Binding of the secreted protein Norrin to its receptor LRP5 leads to activation of β -catenin and its translocation to the nucleus where it is known to promote cell proliferation (McNeill et al., 2013).

In accordance with recently published data, our approach involves use of target-specific neurogenic factors for direct conversion toward a neural fate. Findings of other groups have provided strong evidence of reprogramming of mouse and human fibroblasts to functional neurons following forced expression of a single transcription factor or a combination of transcription factors (Lujan et al., 2012; Ring et al., 2012; Vierbuchen et al., 2010). This conversion is either direct driving fibroblasts to post-mitotic neurons (Vierbuchen et al., 2010) or first drives them toward an NPC state (Han et al., 2012; Lujan et al., 2012; Ring et al., 2012). Here, we have used a reprogramming protocol that primes naive MEFs toward acquisition of *NESTIN*⁺ neuroepithelial cell identity. By forced expression of CEND1 and NEUROG2, these primed fibroblasts have acquired a neuronal precursor fate, and upon addition of bFGF and BDNF they differentiated to subtype-specific functional neurons capable of firing action potentials. NEUROG2 has been previously shown to possess reprogramming action, directly converting human fibroblasts to cholinergic neurons when overexpressed together with the transcription factor SOX11 (Liu et al., 2013) and dopaminergic neurons upon its overexpression together with MASH1, SOX2, NURR1, and PITX3 (Liu et al., 2012). However, as recently demonstrated, NEUROG2 alone is not capable of inducing neuronal reprogramming of MEFs unless downstream effectors are also co-expressed (Chanda et al., 2013). In support of this, our findings demonstrate that NEUROG2 alone possesses a poor neuronal reprogramming action in MEFs, unless co-expressed together with CEND1. The generation of neuronal precursors from fibroblasts with subsequent differentiation to neurons observed in our system exhibits the advantage that induced NPCs can self-renew and expand for many passages, an event facilitating applications where large cell numbers are needed, such as high-throughput drug screening or cell transplantation.

CEND1 is associated with the dynamics of neuron-generating divisions of embryonic and postnatal NPCs (Katsimpardi et al., 2008; Politis et al., 2007) and as shown here of primary astrocytes in vitro. Our previous data also indicate that CEND1 participates in bHLH proneural genes pathway(s) and is activated by bHLH factors, such as

NEUROG1/2 and MASH1 in neuronal precursors (Katsimpardi et al., 2008; Papadodima et al., 2005; Politis et al., 2007). Here we show that CEND1 can also enhance the endogenous expression levels of NEUROG2 in reprogramming astrocytes, indicating the existence of a cross-activating feedback loop and, most importantly, that CEND1 expression is a key downstream player in NEUROG2-driven astrocytic reprogramming. NEUROG2 protein has a short half-life in NPCs—less than 30 min (Ali et al., 2011)—while its expression is dynamically regulated at both the mRNA and protein level by a number of factors. NEUROG2 mRNA stabilization promotes neuronal differentiation (Knuckles et al., 2012), while NEUROG2 protein phosphorylation by Cyclin-dependent kinases diminishes it (Hindley et al., 2012). In particular, it has been demonstrated that the cell-cycle machinery negatively regulates neurogenesis by diminishing NEUROG2 protein stability and binding to the E box of its downstream effectors (Hindley et al., 2012). Our findings demonstrate that CEND1 also affects endogenous NEUROG2 expression and/or stability and that its presence is required for NEUROG2 to exert its neurogenic action. Of relevance, since proneural genes are readily downregulated in differentiated neurons, their ability to sustain neuronal differentiation not only during development but also during reprogramming relies on activation of downstream genes participating in cellular differentiation networks, one of which is CEND1 (Katsimpardi et al., 2008; Politis et al., 2007). Therefore, sustained expression of CEND1 in differentiated neurons above a certain threshold level (Katsimpardi et al., 2008) is crucial for astrocytic reprogramming to differentiated neurons by NEUROG2. This observation is in line with the findings of Chanda et al. (2013), who show that NEUROG2 cannot induce efficient neurogenic reprogramming of MEFs unless the neuronal differentiation mediator MYTL1 is co-expressed. Last, while NEUROG2 is a proneural factor linked to dorsal cell identity of cortical neurons, CEND1 has a wider neuronal distribution being highly enriched in GABAergic striatal neurons and motor neurons of the spinal cord. Indeed, our previous in vivo CEND1-overexpression studies in the early chick neural tube (Politis et al., 2007) indicated that CEND1 can give rise to both ventral and dorsal neuronal identities. Thus, unlike NEUROG2, CEND1 seems to exhibit a general—not cell-type-specific—potential in conferring neuronal identity, a fact that explains the different neuronal phenotypes present upon CEND1 or NEUROG2 overexpression.

Astroglia and fibroblast neurogenic reprogramming by the same factors is an exciting finding, indicating that the borders between mature cell phenotypes are not as rigid as originally thought and cells can trans-differentiate between different lineages upon challenging the levels of a certain small group of common genes that inactivate a somatic



cell program and activate a program associated with NPC proliferation and differentiation state. From a translational point of view, it is important that CEND1 and NEUROG2 share the potential to be used in gene therapy approaches to enhance the intrinsic neuronal reprogramming capacity of endogenous brain cells, such as astrocytes, or in cell therapy approaches involving transplantation of reprogrammed cells into the injured brain to enhance functional recovery.

EXPERIMENTAL PROCEDURES

Animals

All experiments were complied with the European Directive 2010/63/EU and the Greek National Law 161/91 for Use of Laboratory Animals, according to FELASA guidelines for euthanasia and the NIH Guide for Care and Use of Laboratory Animals. Our protocols were approved by the Institutional Animal Care, and the Use Committee of the Hellenic Pasteur Institute approved all protocols used.

Retroviral and Lentiviral Transduction

Retroviral transduction of astrocytes and MEFs was performed using vesicular stomatitis virus G pseudotyped retroviruses expressing *Cend1* and *Neurog2* under the control of chicken β -actin promoter and cytomegalovirus enhancer (pCAG), with GFP or dsRed, respectively, located behind an IRES site. For silencing CEND1, astrocytic cultures were transduced with pTrip.CMV-sh*Cend*-GFP lentiviral vector, allowing visualization of CEND1 silencing by GFP expression (Katsimpardi et al., 2008).

MEFs Direct Reprogramming

MEFs were transduced with retroviral vectors and cultured in neural reprogramming medium (DMEM and F12 and Neurobasal [1:1], 2 mM Glutamax, 0.11 mM β -mercaptoethanol, 0.05% BSA, 20 ng/ml EGF, 20 ng/ml bFGF, 1 \times N2, 1 \times B27) with gradually decreasing fetal bovine serum concentration. For neuronal differentiation, cells were cultured in DMEM and F12 and Neurobasal (1:1), with 3.5 mM glucose, 25 μ g/ μ l insulin, 10 ng bFGF, and 5 ng/ml BDNF.

Time-Lapse Microscopy

Time-lapse microscopy (Costa et al., 2011; Eilken et al., 2009; Ortega et al., 2013) was performed at 37°C and 7% CO₂. Phase contrast images were acquired every 5 min for 7–10 days. Single-cell tracking of BIII-TUBULIN neurons was performed using TTT software (Rieger et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three movies and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.07.012>.

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