HELLENIC REPUBLIC

National and Kapodistrian
University of Athens

Department of Biology

Athens International
Master's Programme
in Neurosciences

Neural Stem Cells and Neuroimaging Lab, Department of Neurobiology,
Hellenic Pasteur Institute

RESEARCH THESIS PROJECT

Mechanisms of Direct Reprogramming of Human Astrocytes To Induced-Neurons

Seyyedeh Vejdani

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Summary

Trans-differentiation of cells into induced-neurons is a promising strategy for CNS repair after acute injury or neurodegenerative diseases. Astrocytes are an ideal cell source for direct neuronal conversion. The purpose of the present study is the investigation of the neuronal reprogramming capacity of Cend1 and/or Neurog2, upon their overexpression on primary human adult cortical astrocytes. The newborn induced-neurons were studied for their morphology and molecular phenotype at specific time points after transgene overexpression. The astrocytes are reprogrammed by either CEND1 or NEUROG2 directly to cells with differentiated neuronal morphology, exhibiting long neurites and branched processes. Induced neurons exhibit either GABAergic (CEND1 overexpression) or Glutamatergic/dopaminergic (NEUROG2 overexpression) molecular phenotype. Exploration of gene expression dynamics along the entire conversion process revealed that neuronal genes are significantly up-regulated while astrocytic genes are down-regulated. On the other hand, in double-trasnsduced cultures, the levels of relative expression of neural progenitor/stem genes are high and this comes in accordance with the appearance of highly proliferative spheres with neural progenitor cell (NPC) properties in culture.

Highlights

- Human primary cortical Astrocytes differentiate towards subtype-specific neurons upon Cend1 or Neurog2 forced expression.
- In double-transduced cultures, highly proliferating free floating three-dimensional colonies are formed. These colonies can survive for many passages, and share the same morphology, size, proliferation and differentiation properties with human NPCs.

Keywords

Astrocytes, induced-neurons, astrospheres, Cend1, Neurog2, trans-differentiation

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Introduction

The irreversible loss of neurons is an important pathological feature of CNS injury and disease, resulting in persistent neurological disability. Subsequently, neuron regeneration in adult mammalian brain is important for alleviation of brain injuries or neurodegenerative diseases. However, the regenerative capacity of the mammalian CNS is not only largely restricted to the two areas of the subgranular and the subventricular zones; but also, strictly limited due to the disability of the neurons for self-renewal (Goldman, 2016; Hashemian et al., 2015). Since the generation of induced pluripotent stem cells by Takahashi and Yamanaka (2006), cell replacement therapy using exogenous cells has attracted attention as a potential therapeutic strategy for CNS injury and disease. However, potential risks, such as tumorigenesis and the difficulty of transplanting cells into the brain, are obstacles partially limiting their wide application (Hu et al., 2015; Li et al., 2015; Zhang et al., 2016).

Following nerve injury, glial cells including astrocytes, NG2 cells, and microglia proliferate and become reactive glial cells forming the glial scar in order to protect the neighboring CNS tissue from further damage (Burda and Sofroniew, 2014). Definitely, any injury transforms astrocytes into their activated status with great self-renewal capacity and neural stem cell characteristics, which makes them more potent to reprogramming. Astrocytes developmentally originate from the same precursor cells as neurons, are capable of proliferating in response to brain damage, and therefore are considered as ideal starting cells to regenerate neurons in situ (Amamoto and Arlotta, 2014; Chouchane and Costa, 2012). Additionally, reactive astrocytes share many characteristics with NSCs capable of generating neurons, astrocytes, and oligodendrocytes in the adult brain (Huang and Tan, 2015). Thus, astrocytes are likely to be ideal starting cells for neuronal conversion.

Previous reports have shown that, astrocytes have been successfully reprogrammed into different types of functional mature neurons using defined transcription factors, such as NEUROG2, ASCL1, and SOX2, in vitro (Berninger et al., 2007; Heinrich et al., 2010; Torper et al., 2015). Importantly, more recent studies have proven that the in vivo resident astrocytes can be also converted directly into functional neurons (Guo et al., 2014; Niu et al., 2013; Su et al., 2014). In a number of other studies use of a single transcription factor, such as SOX2, NEUROD1 or ASCL1, in adult mammal brain and/ or spinal cord was sufficient to convert astrocytes into mature neurons (Guo et al., 2014; Niu et al., 2013; Su et al., 2014). Furthermore, it has been shown that small molecules are capable of reprogramming mouse astrocytes and human fetal astrocytes into neuronal cells (Cheng et al., 2015; Zhang et al., 2015). However, up to now only in one study, human astrocytes were specifically trans-differentiated into induced glutamatergic neurons using a complicated combination of 9 small molecules (Zhang et al., 2016). Protocols of direct chemical reprogramming, which are still at an early stage of their implementation, are of particular direct translational interest, as

(a) they produce new born neurons fast, (b) they avoid use of viral vectors for transgene(s) expression and (c) they provide the possibility of therapeutic use of the endogenous astrocytes of the injured brain after their in vivo modification with neurogenic molecules.

Our previous study has shown that forced expression of the neurogenic protein Cend1 or the proneural transcription factor NEUROG2 in both mouse cortical astrocytes and embryonic fibroblasts resulted in acquisition of induced-neuronal cells expressing glutamatergic, dopaminergic and GABAergic markers, while Cend1/NEUROG2 double-transduced mouse astrocytes and fibroblasts formed free-floating spheres exhibiting neural stem cell properties. These spheres are highly proliferative and in the absence of growth factors, they spontaneously differentiate into neurons, astrocytes and oligodendrocytes, suggesting that they have the same multipotent potential as neural stem cells of the SVZ. Thus, the population of endogenous activated astrocytes, which significantly increase in number and size after injury, keep the epigenetic memory of their origin, and appear to be a cell source that can be used to produce new neurons at the site of a brain lesion. Also, RT-PCR arrays analysis revealed that components of the beta-Catenin/ Wnt-signaling pathway to be up-regulated in Cend1/Neurog2-double transduced cultures, most possibly being responsible for the formation of astrospheres exhibiting NSCs characteristics. Additionally, we have shown that Cend1 acts downstream and gets regulated by NEUROG2, while at the same time it enhances the expression of Neurog2 in reprogrammed astrocytes, indicating the existence of a cross-activating feedback loop between the two molecules (Aravantinou-Fatorou et al., 2015).

Despite the development of protocols for reprogramming astrocytes and other types of mouse glial cells and the creation of transgenic animal models that resemble human diseases or brain damage, it is still necessary and imperative to study reprogramming and differentiation of human astrocytes, as critical differences exist between human and rodent astrocytes. Human astrocytes in the neocortex are 27-fold larger and have 10 times more processes, which are 2.6-fold longer compared to those in rodents (Robertson, 2014) Additionally astrocytes of rodents cover 20.000-120.000 synapses; while, the human astrocytes cover 270.000-200.0000 synapses (Oberheim et al., 2012). Functionally, human astrocytes outcompete their rodent counterparts as presented in an elegant study where human glia progenitors were transplanted into mouse forebrains. The human glia progenitor cells migrated, integrated, and matured to astrocytes resulting in a mouse with improved long-term potentiation (LTP) and learning compared to mice in which murine progenitors were grafted (Han et al., 2013). Thus, morphological and functional variations present in human astrocytes make it essential to study their properties and their ability to reprogram them to neurons.

Therefore, the aim of this study is to investigate whether the two neurogenic molecules, CEND1 and NEUROG2, are capable of also reprogramming human

astrocytes to neural progenitor cells and neurons, in particular of GABAergic, dopaminergic and glutamatergic subtypes as they do in mice. This question, which was the main purpose of this research work, brings this line of research one step closer to clinical approaches through future translational exploitation of our experimental results.

Methods

Induction of neuronal cells from human adult astrocytes

For induction of neuronal cells, human adult astrocytes were seeded on 0.015mM poly-L-lysine (sigma) pre-treated coverslips in human astrocyte growth medium (DMEM high glucose/F12, N2, B27, NEAA, FGF2, 10%FBS, Ascorbic Acid,Glutamax). The cells were seeded at a density of 50,000 cells per square centimeter. The following day, viral transduction was preformed using the retroviruses RV-IRES-GFP, RV-IRES-DSRED, RV-CEND1-IRES-GFP, RV-NEUROG2-IRES-DSRED. Two days after the seeding, the cells should be over 90% confluent. At this time point, the growth medium was replaced by the reprogramming medium, which was DMEM high glucose/Neurobasal (Gibco) supplemented with 1x B27 (Gibco), 1x N2(Gibco), 20 ng/ml BDNF (Peprotech), 20 ng/ml IGF (Peprotech), 0.2 µM ascorbic acid (sigma), 100 µM dibutyryl-cAMP (sigma), and the following small molecules: 0.5 mM VPA (Calbiochem) and 10 µM forskolin (Cayman). The reprogramming medium containing small molecules was changed every two days. At week 2, the culture medium was replaced by the differentiation medium, containing Neurobasal medium, 1x B27, 1x N2, 20 ng/ml BDNF, 20 ng/ml GDNF, 20 ng/ml IGF, 0.2 µM ascorbic acid, 100 µM cAMP and 1 µg/ml laminin.

Neurosphere assay

Neurosphere formation was achieved after the double transduction with the retroviruses RV-CEND1-IRES-GFP and RV-NEUROG2-IRES-DSRED on human adult astrocytes. The culture medium for neurosphere assay was DMEM/F12 supplemented with 1x B27 (Gibco), 20 ng/ml EGF, and 20 ng/ml bFGF. The NSC medium was changed every other day.

Immunofluorescence staining

Immunostaining of cells was performed as previously reported (Cheng et al., 2015). Briefly, after cells cultured on coverslips were washed with 1x PBS for 3 times, 4% PFA was used to fix the cells for 20 minutes at room temperature. After washed with 1x PBS for 3 times, blocking buffer (1% BSA and 0.5% Triton X-100 in PBS) was used for 1 hour at room temperature. Then, the cells were incubated with primary antibodies at 4 °C overnight and finally, with fluorescent probe-conjugated secondary antibodies for 1hour at room temperature. DAPI (Beyotime) or TopRoIII (Thermo Scientific) were used to stain Nuclei at room temperature at the same time with the secondary antibodies.

The primary antibodies were used as: DCX (1:200, Santa Cruz, Cat. #sc- 8066), b-III TUBULIN (1:500, Covance, Cat. #MMS435P), MAP2 (1:500, Millipore, Cat. #AB5622, #MAB3418), NEUN (1:500, Millipore, Cat. #ABN78, #MAB377), SYN1 (1:500, Millipore,

Cat. #AB1543), VGLUT1 (1:500, Synaptic system, Cat. #135302), TH (1:200, Chemicon, AB152), NESTIN (1:1000, Millipore, Cat. #MAB5326), SOX2 (1:50, R&D, Cat. #AF2018), GFAP (1:1000, DAKO, Cat. #Z033401), PSD95, clone K28/43 (Millipore). The following secondary antibodies were used at 1:1000 dilution: Donkey-anti-goat-Alexa 488 (Molecular Probes, Cat. #A11055), Donkey-anti-mouse-Alexa 488 (Molecular Probes, Cat. #A21202), Donkey-anti-Rabbit-Alexa 488 (Molecular Probes, Cat. #A21206), Donkey-anti-goat-cy3 (Jackson ImmunoResearch, Cat. #705-165-147), Donkey-anti-rabbit-cy3 (Jackson ImmunoResearch, Cat. #711-165-152), Donkey-anti-mouse-cy3 (Jackson ImmunoResearch, Cat. #715-165-150), Donkey-anti-rabbit-Alexa 647 (Molecular Probes, Cat. #A31573), Donkey anti- mouse- Alexa 647 (Molecular Probes, Cat. #A31571).

Conversion efficiency and neuronal purity

Briefly, 10-20 view fields were randomly selected for each sample by Confocal SP8 Leica microscope at each time point. The number of neuronal cells was counted on DCX-positive cells with typical neuronal morphology. The conversion efficiency was calculated by the ratio of the number of neuronal cells to that of initial cells seeded in each field. Neuronal purity was calculated by the ratio of the number of neuronal cells to the total cell number indicated by DAPI. The percentage indicated in characterizing human adult astrocytes or neuronal subtype was calculated similarly. Quantitative data were represented as average ± SEM of three independent experiments.

Time-Lapse Microscopy

Time-lapse microscopy (Costa et al., 2011; Eilken et al., 2009; Ortega et al., 2013) was performed at 370 C and 7% CO2. Phase contrast images were acquired every 5 min for 5 days.

Quantitative real-time PCR

The total RNA of indicated cell samples were isolated with Trizol (Sigma) following manufacturer’s instructions. Isolated RNA was used for reverse transcription with random hexamers and Superscript II transcriptase (Invitrogen) according to manufacturer’s instructions. Quantitative real-time PCR was conducted with primers and SYBR Green qPCR Master Mix (4472908, Invitrogen) in LightCycler® 480 PCR machine. The relative expression levels were normalized to the internal control (GAPDH). Primers used were listed in the Table 1.

Oligo Name	Sense/Antisense	cDNA	Sequence (5'-3')
GFAP F	Sense	Genomic	GGTTGAGAGGGACAATCTGG
GFAP R	antisense	Genomic	GGGTGGCTTCATCTGCTTC
TUJ1 F	Sense	genomic	CATTCTGGTGGACCTGGAAC
TUJ1 R	antisense	genomic	CCTCCGTGTAGTGACCCTTG
MAP2 F	Sense	genomic	GAGAATGGGATCAACGGAGA
MAP2 R	antisense	genomic	CTGCTACAGCCTCAGCAGTG
MASH1 F	Sense	genomic	AAGAGCAACTGGGACCTGAGTCAA
MASH1 R	antisense	genomic	AGCAAGAACTTTCAGCTGTGCGTG

Table 1. List of primer sequence.

Statistical Analysis

All quantified data were statistically analyzed and presented as mean ± SEM. Two-tailed Student's t tests were used to calculate statistical significance with p values. A p value < 0.05 was considered statistically significant.

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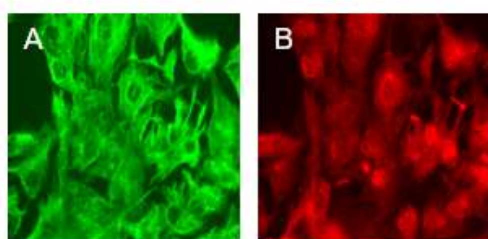
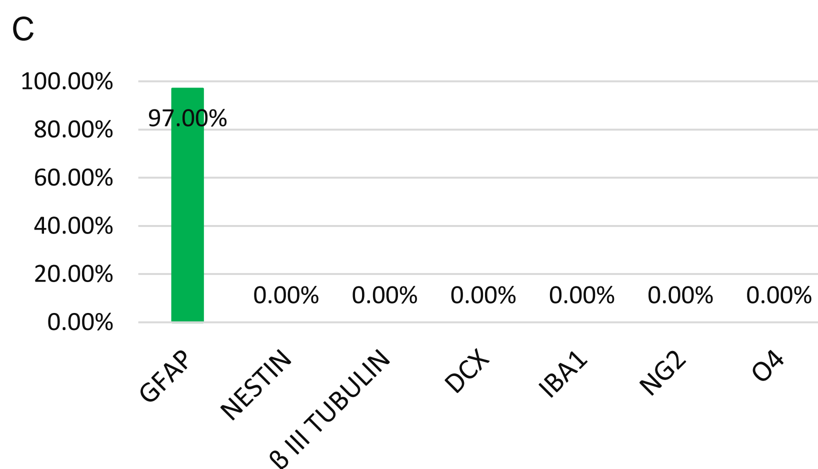
Results

Characterization of Cultured Human Adult Astrocytes

The human cerebral cortex astrocytes (HA#1800) are purchased from ScienCell (San Diego, CA). To check the possibility of presence of neural progenitor cells (NPCs) in the astrocytic culture, we have been culturing the cells in the presence of 10% fetal bovine serum (FBS). After more than 1-month of culture in basic astrocytic medium, the cells are unable to form neurospheres or any neuronal cells and about 97% of the cells express the astrocytic markers GFAP and S100B (Figure 1A and 1B). In addition, the cultured cells are negative for the neuronal markers βIII-tubulin, DCX, radial glial marker Nestin, microglial marker IBA1, glial marker NG2, or oligodendrocyte marker O4 (Figures 1C). These results collectively suggest that the cultured cells are pure astrocytes without detectable contamination of NPCs, neuronal cells, or other glial cells. Besides, the qRT-PCR results for GFAP mRNA expression levels confirm that we have a homogeneous population of primary astrocytic cells with stable functional properties as the basis for our cell reprogramming experiments (Figure 1D). More specifically qRT-PCR analysis revealed that the mRNA levels of S100b are higher as compared to GFAP, in accordance with in accordance with previous reports showing higher expression of S100b in grey matter astrocytes and GFAP in white matter astrocytes, respectively (Ben Haim and Rowitch., 2017; Rusnakova et al., 2013).





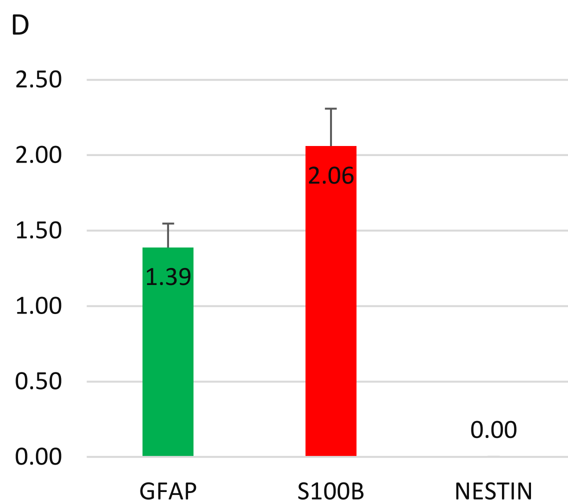
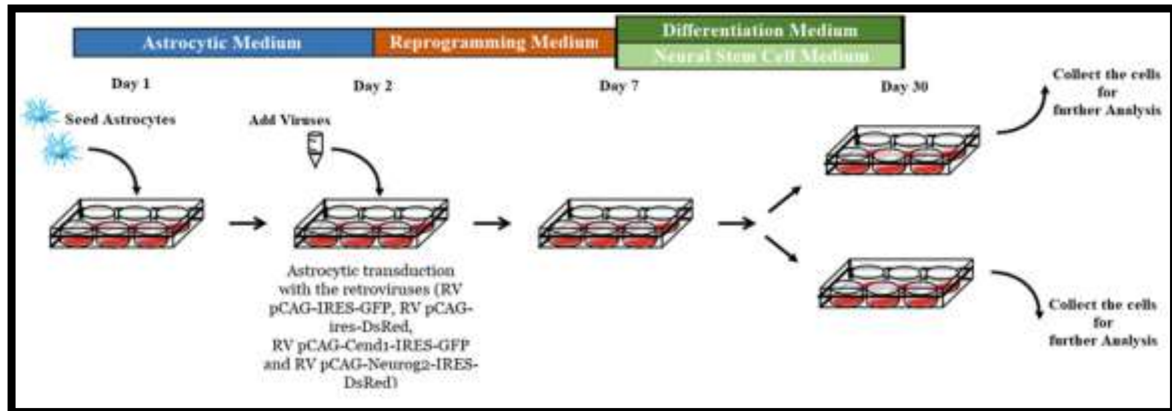


Figure 1. Characterization of Human Adult Astrocytes in vitro

(A and B) Human astrocytes stained for GFAP marker (green) and S100β marker (red). (C) The cultured astrocytes are negative for the markers βIII-tubulin, DCX, IBA1, NG2, and O4. (D) Real-time PCR validation of the expression of representative astrocyte-enriched genes. (mean ± SEM, n = 3 independent experiments).

Reprogramming of Human Cortical Astrocytes by CEND1's and NEUROG2's overexpression to Induced Neurons with Subtype-Specific Identity

The aim of the study is the investigation of the neuronal reprogramming capacity of the two neurogenic molecules, CEND1 and NEUROG2, used either alone or in combination, upon their overexpression on human adult primary cortical astrocytes. CEND1 and NEUROG2 are expressed under the control of chicken b-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 (Aravantinou-Fatorou et al., 2015). The small molecules, Forskolin and Valproic acid, both frequently used in direct neuronal reprogramming or differentiation protocols (Hu et, 2015; Hou et al, 2013; Liu et al 2013; Jung et al 2008) have been also applied in the reprogramming cocktail. To induce direct reprogramming of human astrocytes, we have developed a multistep culture protocol summarized in Figure 2A. Briefly, astrocytes are transduced and cultured under reprogramming media until day 7, then reprogrammed cells are re-plated and cultured in neuronal differentiation medium or neural stem cell medium. From the starting point, the transduction efficiency, is very high as shown in Figures 2B-2E.



RV-IRES-GFP	Cend1	Neurog2

B: GFP; GFAP; GFAP GFP

C: Cend1; GFAP; TopRoIII; Cend1 GFAP TopRoIII

D: GFAP; TopRoIII; Ngn2; GFAP Ngn2 TopRoIII

E

	Control	Cend1	Neurog2
	95.52%	88.97%	88.73%

Axis: 0.00%, 10.00%, 20.00%, 30.00%, 40.00%, 50.00%, 60.00%, 70.00%, 80.00%, 90.00%, 100.00%

Figure 2. Astrocytes Reprogramming upon Forced Expression of CEND1 and/or NEUROG2

(A) Schematic drawing of the protocol used for astrocytes reprogramming. (B-D) Confocal images showing the high retroviral transduction efficiency in control (B), CEND1 (C) and (D) NEUROG2-transduced cultures. (E) Graph showing the average transduction efficiency of more than 5 reprogramming experiments.

72 hours after transduction, the vast majority of the cells in control GFP-virus-transduced cultures are positive for glial fibrillary acidic protein (GFAP) (figure 3A). Overexpression of CEND1, NEUROG2, or both results in a decrease in the number of GFAP$^+$ astrocytes (figures 3B-3D). After 7 days in reprogramming medium, molecular phenotype analysis at different time points reveals that a significant number of NESTIN$^+$ cells are present in control cultures, which are not exposed to either neurogenic molecule (Figures 3E and Figure 4B). Additionally, few of them form 3D spheres attached to the plate and differentiate into βIIITubulin$^+$ spheres that never mature any further. Upon CEND1 overexpression, the percentage of NESTIN$^+$ cells is much higher compared to the control culture (Figures 3F,4D); while, as soon as cells are transferred to differentiation media, the population of NESTIN$^+$ neural precursors in CEND1-transduced cultures decrease and βIII-tubulin$^+$ neurons appear in culture (Figure 3J, 4E-F). This reduction of NESTIN$^+$ and subsequent increase of more mature NeuN$^+$ neurons is also obvious in NEUROG2 single-transduced cells (Figure 3G-K and 4H-I). In double-transduced cultures, a significant percentage of NESTIN$^+$ progenitors appear (Figure 4H), which reaches 87.94% t of the cell population, indicating that combined CEND1 and NEUROG2 forced expression induce a cell fate choice toward proliferating multipotent neural precursors, whereas higher expression of these molecules in single-transduced cells drives neuronal differentiation. At the same time, in NEUROG2 cultures, newborn neurons, besides their higher maturation level are also expressing subtype-specific neuronal markers, such as tyrosine hydroxylase (TH) for the first time (data is not shown). The early neuronal marker DCX is never detectable in the control group, (Figure 3I and Figure 4B) however the induction medium itself can activate a low level of reprogramming to β-III TUBULIN positive cells in the population of control human astrocytes. Further analysis, with multiple neuronal markers as well as morphological features should be examined when defining induced-neurons identity.

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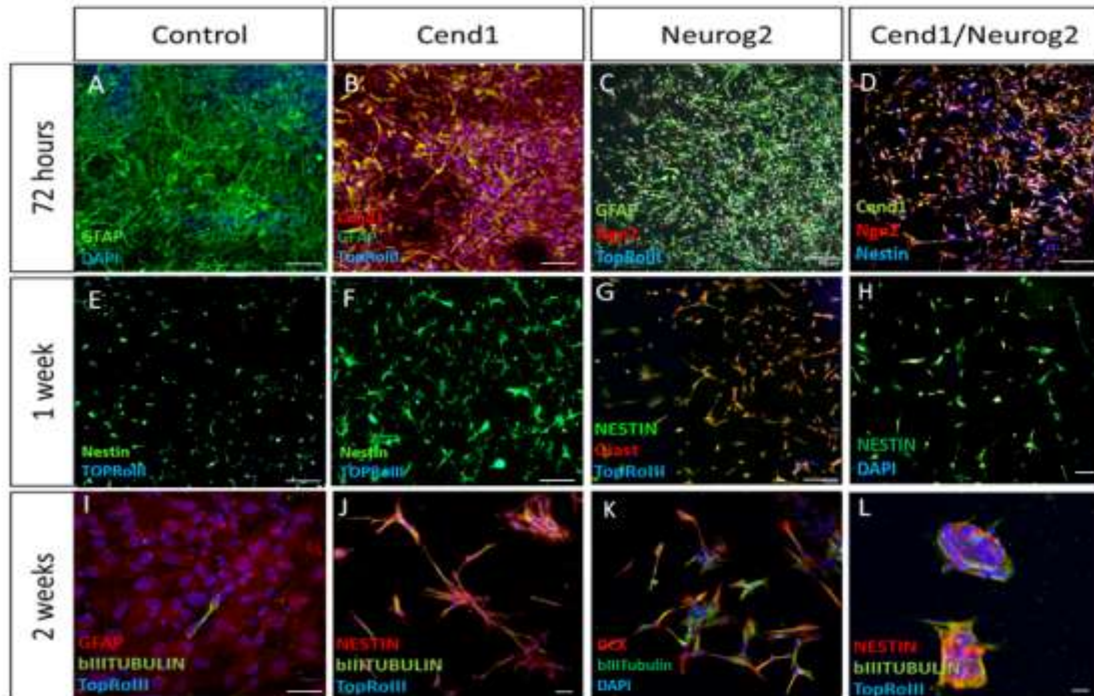

Figure 3. CEND1 and/or NEUROG2 Overexpression Drives Astrocytes Toward Radial Glia Phenotype

(A-D) GFAP expression pattern starts to be different among the different groups even 72 hours following viral transduction. E-H) 1 week after transduction NESTIN and Glast markers are expressed by the neural progenitor cells that emerge, while some cells are already positive for bIII-TUBULIN (data not shown). (I-K) The reduction in expression of NESTIN+ cells and increase in appearance of βIII-TUBULIN^{+} neuronal cells with elongated axonal morphology is now more obvious. L) In double transduced cultures, most of the cells are included in spheres, known as human astrospheres, positive for NESTIN and βIII-TUBULIN.

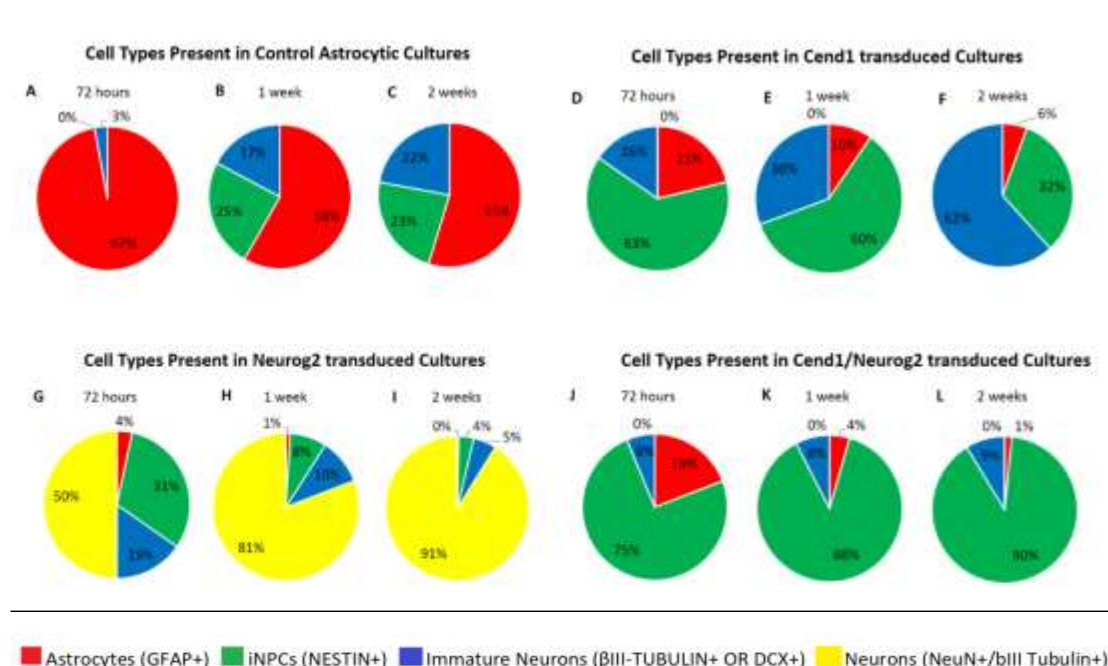

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Figure 4. Appearance of different cell types in different conditions

(A–H) Quantification of at least three independent experiments show different cell types present in control astrocytic cultures (A & B), CEND1 (C & D), NEUROG2 (E &F), and double-transduced (G & H). The most profound changes occur in double transduced cultures, where 87.94% of the cells become NESTIN^{+} neural progenitor cells (green). In the contrary, in NEUROG2 transduced cultures the vast majority of the reprogrammed cells are NeuN+ neurons (yellow) even from 72 hours post-transduction. Finally, in the Cend1 transduced cultures, most of the cells are either NESTIN^{+} neural stem cells (green) or β-III TUBULIN^{+} immature neurons (blue). GFAP fluorescence appears in red color.

Reprogrammed astrocytes cultured for 1 month exhibit a highly differentiated neuronal morphology with long neurites and multiple and complex processes emanating from their small cell somas. In addition to their differentiated branched morphology and β-III TUBULIN expression, reprogrammed astrocytes start to express the neuronal subtype-specific markers GABA (Figure 5B), Tyrosine Hydroxylase (TH) (Figure 5G), and GLUTAMATE (Figure 5H), depending on the neurogenic factor being forced expressed. In particular, 29% ± 3.4% of the β-III TUBULIN^{+} neurons are GABA^{+} upon CEND1 overexpression, while in NEUROG2- transduced cultures, 34% ± 5.1% of β-III TUBULIN^{+} neurons are TH^{+} and 18% ± 7.1% of β-III TUBULIN^{+} neurons are GLUTAMATE^{+}. Moreover, the pre- and post-synaptic markers SYNAPSIN and PSD95 appear (Figures 5C-5D and 5I-5J), indicating further maturation of the induced neurons. Quantification of the total neurite length in the three different neuronal subtypes produced (GABA^{+}, TH^{+}, GLUTAMATE^{+}) indicate that average total neurite length is 39 ± 0.4 um in the CEND1-overexpressing for GABA+ neurons and 53 ± 0.5 um in the NEUROG2-overexpressing for TH^{+} neurons, whereas NEUROG2-overexpressing glutamatergic neurons exhibit the highest neurite length of 110 ± 0.7 µm. By contrast, no significant morphological change is observed in the control group, where the viruses and the small molecules are not added (data not shown). However, in the control cultures, cultured in reprogramming medium, we observe a spontaneous neuronal differentiation phenomenon, where few immature β III-TUBULIN^{+} neuronal cells appear (Figure 6A). It is worth mentioning that the remaining non-converted astrocytes form a neuron-astrocyte co-culture condition, with the iNs migrating onto the surface of astrocytes, and the astrocytes serving as a feeder layer to promote neuronal survival and maturation (Figure 5E) (Tang et al., 2013).

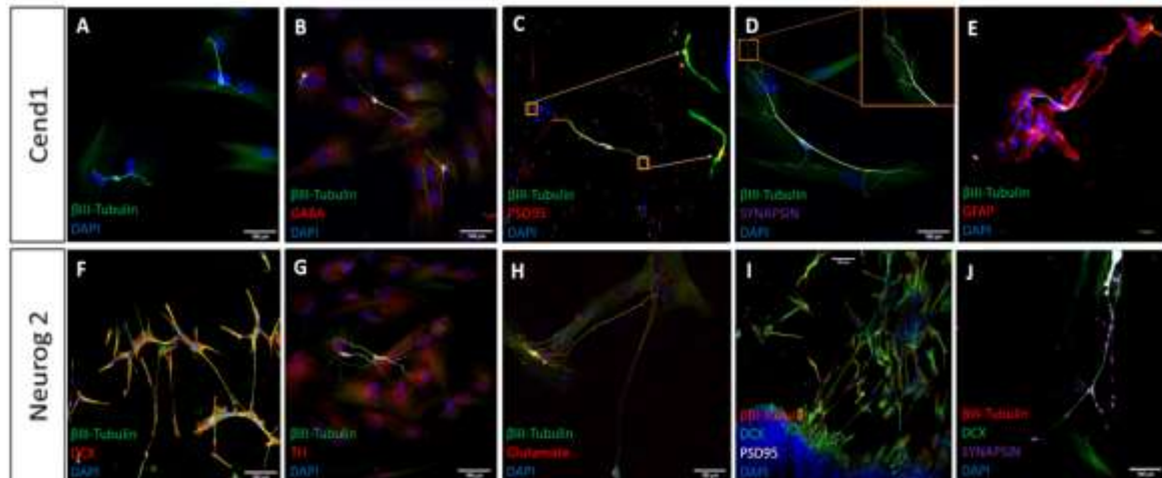


Figure 5. Reprogrammed astrocytes cultured for 1 month exhibit a highly differentiated neuronal morphology

(A-D) Mature, subtype-specific neuronal cells, exhibiting GABAergic phenotype and synaptic proteins expression are present in Cend1 transduced cultures. (E) A more mature morphology is observed mostly in neurons lying on top of the remaining astrocytes. (F-J) Upon Neurog2 overexpression, a significant population of cells are highly branched neurons expressing neuron-specific markers, such as βIII-TUBULIN, GLUTAMATE, TH, DCX, PSD95 and SYNAPSIN.

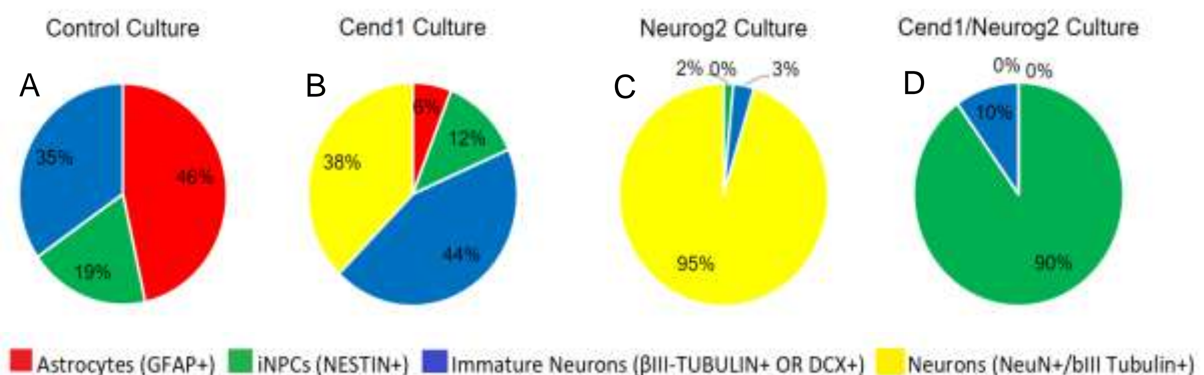


Figure 6. The final cellular populations, one month post-transduction and while the cultures are under the differentiation medium or neural stem cell medium.

(A) In control cultures, astrocytes (red), immature neurons (blue) and neural stem/precursor cells (green) are present at percentages of46.54%, 34.63% and 18.84% respectively. (B) At the same time point in Cend1 transduced cultures 37.58% of DCX+ neurons (yellow), 44% β-III TUBULIN+ neural progenitors (blue), 12.43% NESTIN+ neural stem cells (green) and 6% GFAP+ astrocytes (red) appear. (C) Amazingly, 95.3% of the cell cultures transduced with the Neuroge2 virus, are $NeuN^+$ neurons, followed by 3.21% of β- III $TUBULIN^+$ immature neurons, 1.47% of $Nestin^+$ NPCs and 0.03% $GFAP^+$ astrocytes. (D) The majority of cells in double transduced cultures contain a pure neural stem cell population (90.07% Nestin+, green).

To this end, neuronal purity and conversion efficiencies based on β-III TUBULIN expression and cell morphology 30 days post transduction, upon Cend1 and Neurog2 overexpression, were estimated to be 57.7% and 81.1% respectively (Figure 7).

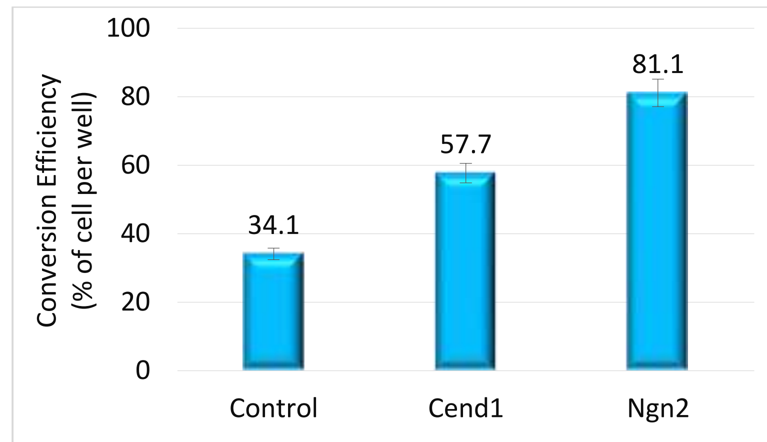


Figure 7. Conversional efficiency of astrocytes to neurons based on the expression of β-III TUBULIN 30 days following transgene over-expresssion.

Long-Term Time-Lapse Experiments Reveal Two Modes of Astrocytic Reprogramming to induced-neurons

In order to investigate whether cell division is required for cell fate conversion to occur or astrocytes directly trans-differentiate to post-mitotic neurons following forced expression of CEND1 or NEUROG2, we have performed continuous live-cell imaging for up to 5 days, using an Olympus IX81 time-lapse imaging system and Cell Profiler software to track transduced cells' lineage trees. Analysis reveals that the majority of CEND1-overexpressing astrocytes passed through one to two asymmetrical divisions, before neuronal trans-differentiation (Figures 8A-8B and 8I), just as previously described (Aravantinou-Fatorou K. et al., 2015). By contrast, as already reported, using live-cell imaging for 5 days (Heinrich et al., 2010; Aravantinou-Fatorou K. et al., 2015) during mouse strocytic reprogramming, newborn neurons derived from NEUROG2-transduced astrocytes are rarely produced in a proliferative manner (Figures 8E-8H and 8J). Furthermore, during the first days astrocytes are very motile, while as soon as neuronal trans-differentiation is occurring, their motility becomes more limited, and by the time they acquire neuronal identity, their cell bodies stop moving and only their processes head towards different directions scavenging the environment.

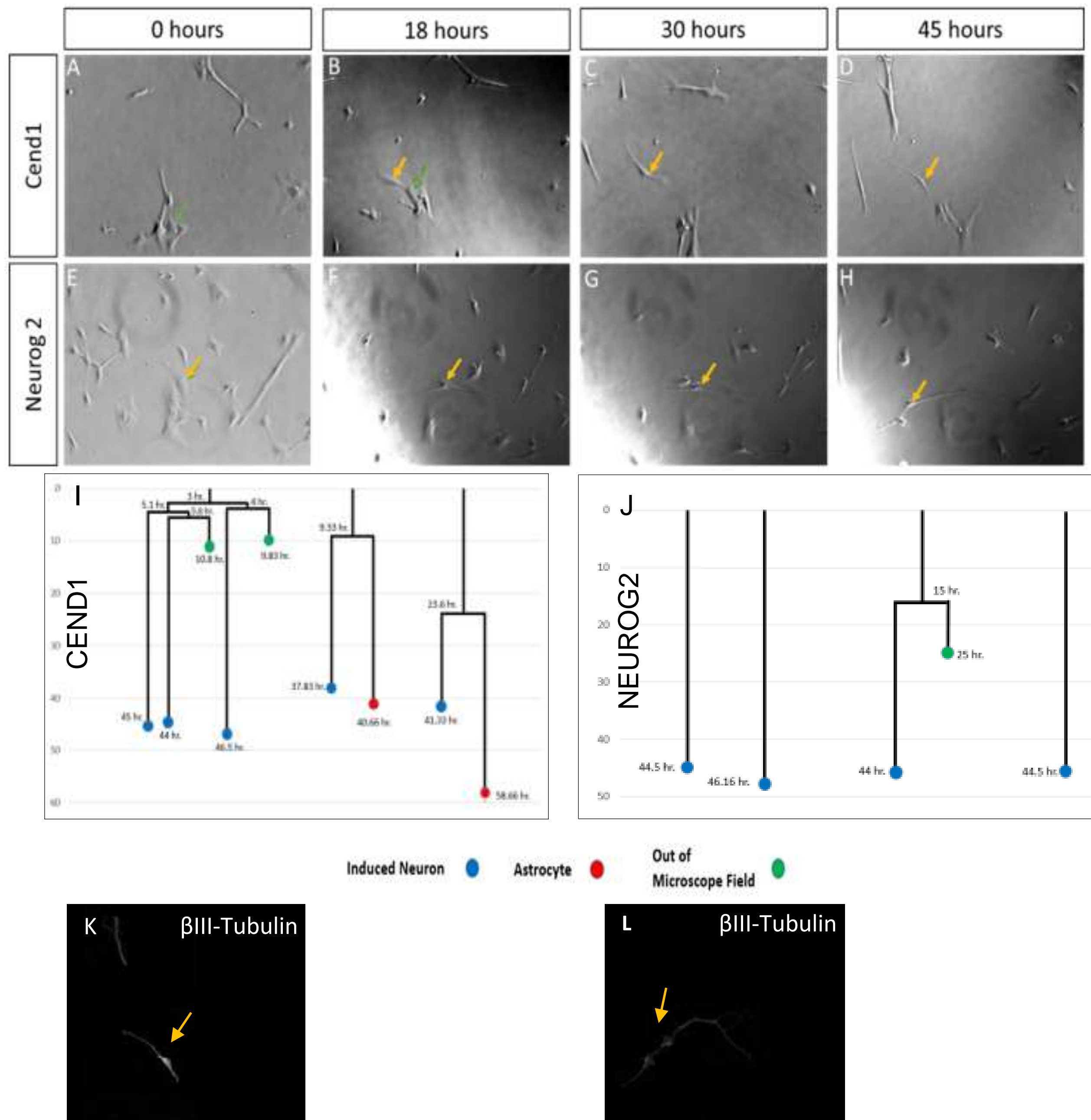


Figure 8. Long-Term Live-Cell Imaging upon CEND1 or NEUROG2 Overexpression

Astrocytic cultures transduced with the CEND1-IRES-GFP (A-D and I) or Neurog2- IRES-DsRed (E-H and J) retroviruses trans-differentiat, giving rise to neurons, after passing either through one or two divisions (I) or directly (J). Snapshots of the following time points during 5 days live cell imaging: 0 hr (A, E), 18 hr (B, F), 30 hr (C, G), 45 hr (D, H). 80% of the divisions in Cend1 transduced cultures are asymmetric, giving rise to one neuron and one astrocyte (lineage trees I), while in Neurog2 transduced cultures the vast majority of astrocytes directly form neurons at a very short time interval (lineage trees J, video 2). Blue circles refer to neurons, red circles refer to astrocytes, and green circles refer to the cells lost from field of view. Scale bars, 40 mm. See also Movies S1 and S2. (K-L) Cell tracking and lineage tree drawings are correlated with bIII-TUBULIN+ neurons produced.

CEND1 and NEUROG2 double transduced astrocytic cultures form multipotent astrospheres

In double-transduced cultures, colonies of small round cells form highly proliferative three-dimensional spheres attached to the plate, detected around 60 hours after transduction as revealed by live cell imaging experiments (Figure 9I). These clones, which amount to approximately 90% of the whole cell population (Figure 6D), are a transient population that cannot survive for more than 72 hours when cultured in astrocyte medium and is composed of cells with a mean diameter of 22.1-24.8 µm.

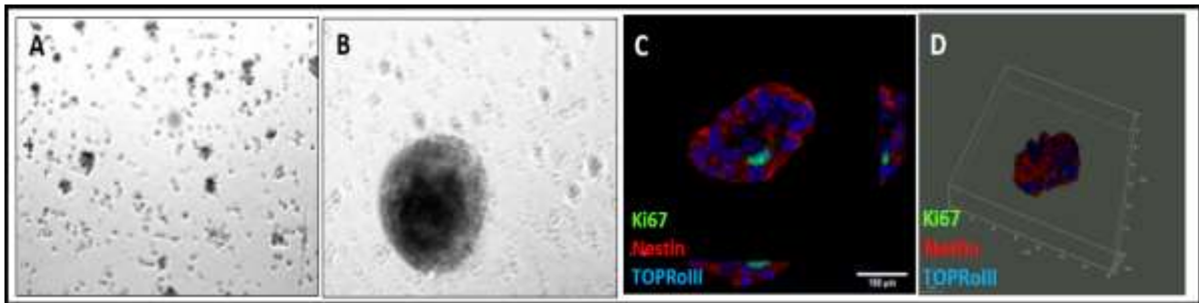
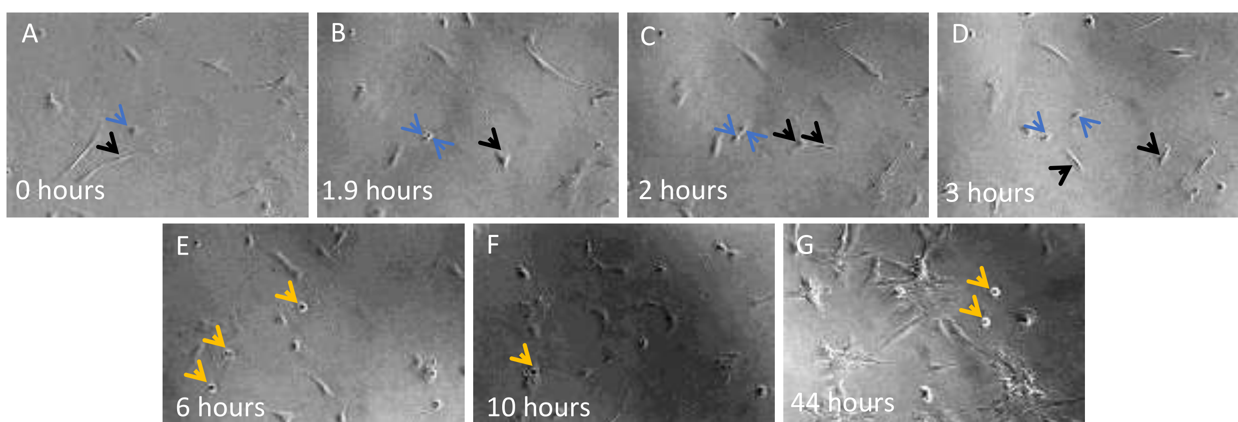


Figure 8. Astrospheres' formation upon CEND1/NEUROG2 Overexpression

(A) NSC-like colonies 60h after Cend1/Neurog2 double transduction. (B) A mature astrosphere grown after 1 week in culture. (C) IF of these cells shows that all cells express the neural precursors marker Nestin. (D) 3D demonstration of a sphere attached to the plate.

Live-cell imaging starting 12 hours after transduction combined with linage tracing enabled us to visualize the exact time of the first divisions resulting in sphere formations which take place during the first 20 hours following CEND1/NEUROG2 double transduction (Figures 4A–4H; Movie S3).



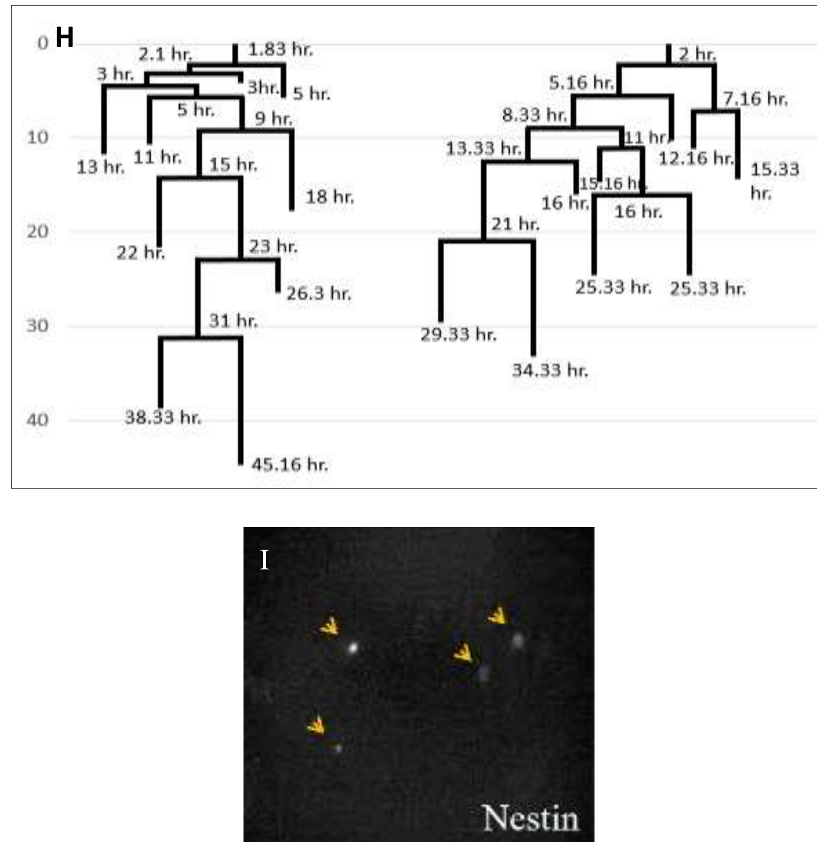


Figure 10. Long time live cell imaging experiments show the process of the formation of the free floating astrospheres.

(A-D) Astrocytes start proliferate fast. The arrows point to distinct divisions on this area. (E-G) As soon as astrospheres are formed, they detach from the bottom of the well and start to float. (H) The first divisions resulting in sphere formations take place during the first 20 hours following CEND1 and NEUROG2 double transduction. (I) Cell tracking and lineage tree drawings are correlated with Nestin+ spheres produced

RT-PCR analysis reveal the neuronal conversion of the astrocytic cultures over time

Generation of Neuronal Cells from Human Adult Astrocytes is achieved quite quickly during the reprogramming process. Exploration of gene expression dynamics along the entire conversion process reveals that neuronal genes (MAP2, and βIII-TUBULIN) are significantly up-regulated (Figures 9B-9C), while at the same time, the astrocytic gene, GFAP, is down-regulated (Figure 11A), indicating that the induced-neuronal cultures are proceeding to a neuronal state. On the other hand, in double-transduced cultures that trans-differentiate primarily to a NSC-like phenotype, the levels of the late neuronal differentiation gene MAP2 are low (Figure 11C), while the relative expression of bHLH transcription factor present in neural progrenitors, MASH1 is high (Figure 11D).

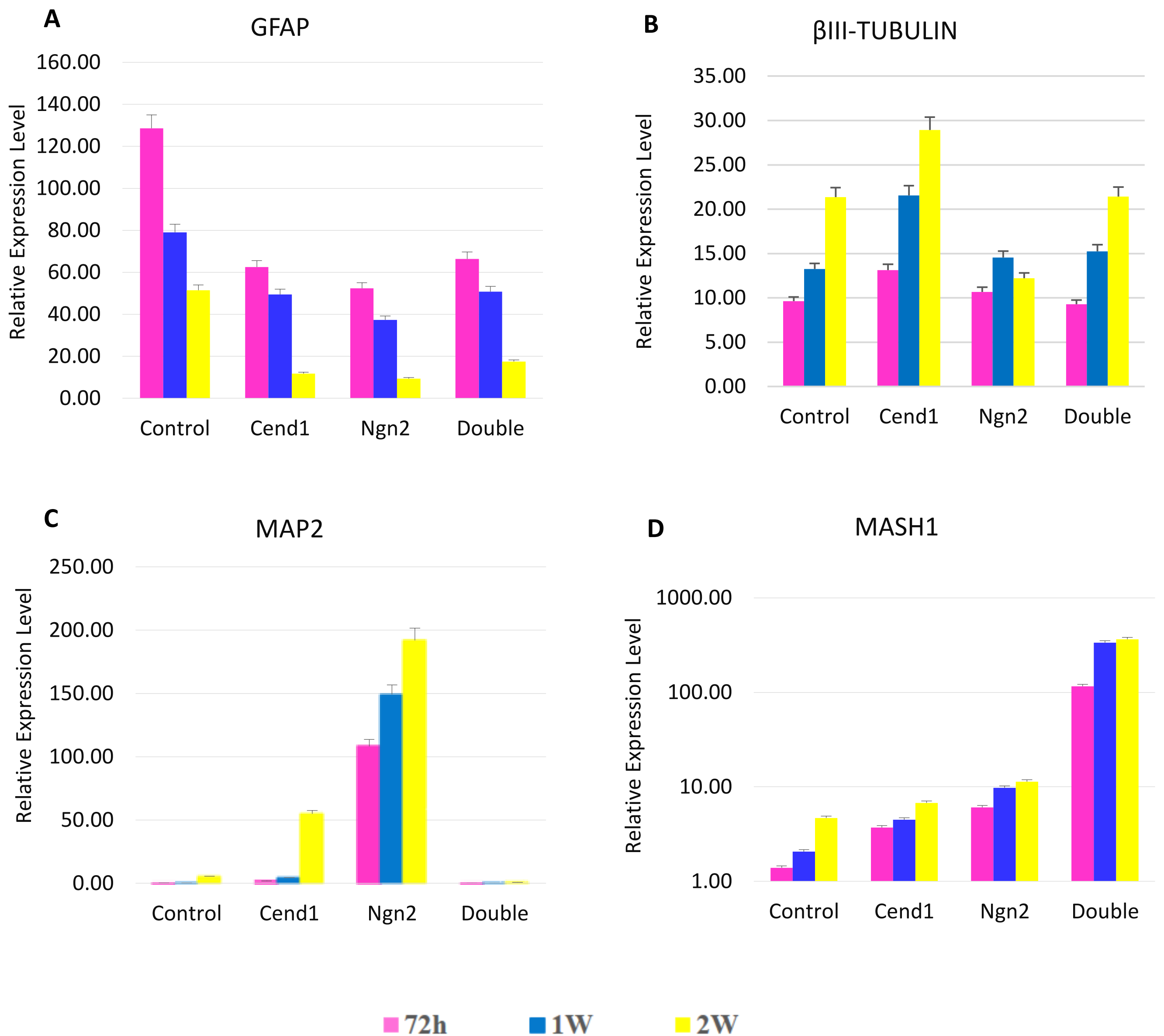


Figure 11. Real-time PCR Analysis of reprogrammed cells over the time

Real-time RT-PCR expression of representative astrocyte- (A), neuron- (B and C), and NSC- (D) enriched genes. 72 hours' time point is shown in pink, 1 week time point in blue, and 2 weeks' time point in yellow. (mean ± SEM, n = 3 independent experiments)

Discussion

Direct lineage reprogramming of astrocytes into induced neurons provides a valuable cell source for regenerative medicine, drug discovery and disease modeling. Because of the ubiquitous distribution of astrocytes in the CNS, the close lineage to neurons (Chandrasekaran et al., 2016; Zheng et al., 2018), and their endogenous tendency to proliferate following brain trauma and in some cases convert into neural precursors and functional neurons (Magnusson et al., 2014; Torper et al., 2013), astrocytes have been selected as an ideal candidate for neuronal reprogramming. In the present study, we show that two neurogenic factors, Cend1 and Neurogenin2, in combination with the small molecules Forskolin and VPA, can trans-differentiate primary adult cortical astrocytes into (a) differentiated neurons possessing GABA^{+}, TH^{+}, or GLUT^{+} subtype specificity and (b) multipotent NPCs. Use of the small molecule VPA has been selected to enhance reprogragramming, as VPA has been reported to promote neurogenesis and neuronal maturation (Hsieh et al., 2004; Niu et al., 2013). Also it is reported that VPA, as a histone deacetylase inhibitor, is able to increase the reprogramming efficiency and even reduce the number of factors required for reprogramming (Dhruba Biswas and Peng Jiang, 2016). The second chemical molecule used, forskolin, has been identified to reduce lipid peroxidation and promote neuronal conversion efficiency (Gascon et al., 2016; Liu et al., 2013), and it was also included into the reprogramming cocktail enhancing the morphological changes of the cells, as previously reported (Gao et al., 2017).

Currently, mouse astrocytes have been successfully reprogrammed into different types of functional mature neurons using defined transcription factors in vitro (Berninger et al., 2007; Heinrich et al., 2010), while the protocols for differentiating pluripotent stem cells (iPS) to mature, subtype-specific neurons are continuously improving (Lundin and Falk, 2018). Regarding astrocytes, the main goals are to simplify and shorten the process, standardize the culture conditions, and validate their functionality as reflecting that of their in vivo counterparts (Zheng et al., 2018). Transcription factors (TFs) are considered as the major determinants of specific cell lineages and lineage conversions (Mertens et al., 2016; Xu et al., 2015), and small molecules enable TFs to induce neuronal conversion more efficiently (Gasco´n et al., 2017; Ladewig et al., 2012; Liu et al., 2013). Our previous study (Aravantinou-Fatorou K. et al., 2015), in accordance with other reports further, supports that ectopic expression of neurogenic factors in some cases along with certain small molecules could activate key neuronal Transcription Factors in fibroblasts or astrocytes and induce their reprogramming to mature neuronal cells (Cheng et al., 2015; Hu et al., 2015; Li et al., 2015; Zhang et al., 2015).

CEND1 is a neuronal-lineage specific modulator involved in synchronization of cell cycle exit and differentiation of neuronal precursors. It is expressed all along the neuronal lineage, from neural stem/progenitor cells to mature neurons and is

associated with the dynamics of neuron-generating divisions of both embryonic and postnatal NPCs (Katsimpardi et al., 2008; Politis et al., 2007; Gaitanou et al., 2019) and as shown here of primary astrocytes in vitro. Achaetescute homolog 1 (Ascl1) and Neurogenin2 (Neurog2) are the prominent pro-neural factors in charge of the neuronal identity specification in the nervous system. Our previous data 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). While, Neurog2 is expressed in dorsal progenitors and instruct them to generate glutamatergic neurons (Colasante et al 2019), CEND1 has a wider neuronal distribution being highly enriched in GABAergic striatal neurons and motor neurons of the spinal cord. It is proven that in vivo CEND1-overexpresssion in the early chick neural tube (Politis et al., 2007) drives neural precursors towards 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.

Our new results using human adult astrocytes demonstrate that (1) the neurogenic molecule CEND1 can reprogram human astrocytes towards subtype-specific GABAergic neurons, while NEUROG2 drives them towards acquisition of glutamatergic and dopaminergic neuronal identity and (2) simultaneous overexpression of CEND1 and NEUROG2 results in activation of the multipotent characteristics of astrocytes transforming them to free floating Astrospheres of high proliferative capacity. Immunofluorescence results from single-transduced cultures also inducate that the induced-neurons are positive not only for neuronal markers, but also for synaptic proteins, supporting their functional maturation. On the other hand, astro-derived spheres are self-renewing, presenting specific NPCs-like morphology and molecular marker expression. and can be propagated for more than 10 passages exhibiting proliferation and differentiation characteristics similar to human neural progenitor cells. More specifically, withdrawal of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) from the astrosphere cultures, leads to production of βIIITUBULIN$^+$ neurons, GFAP$^+$ astrocytes, and O4$^+$ oligodendrocytes, proving their multipotency properties. Addtionally, our long term live-cell imaging experiments have proven that astrocytic trans-differentiation to neurons is not always direct, but depending on the reprogramming factor being forced-expressed, human astrocytes 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. In contrast, we observed a de-differentiation of astrocytes to an NPC state upon overexpression of both proteins simultaneously. Our outcomes on human cells are in accordance with our previous results on mouse primary astrocytes and embryonic fibroblasts (Aravantinou-Fatorou et. al, 2015).

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 astrocytes, or in cell therapy approaches involving transplantation of reprogrammed cells into the injured brain to enhance functional recovery. Therefore, the synergetic effect and underlying mechanism of action of the two molecules molecules in neuronal conversion need to be in depth investigated. Moreover, whether the small molecules used in this can also enhance the in vivo reprogramming capacity of resident astrocytes into neuronal cells in vivo and therefore help against neurological diseases or brain injuries still remains to be answered. Overall, the completion of this research work enriches our knowledge of direct neuronal differentiation mechanisms. Moreover, the production of functional neuronal subtypes by human astrocytes provides regenerative medicine with new approaches for the treatment of brain trauma and neurodegenerative diseases that can be used for therapeutic purposes in the future.

Title: Mechanisms of direct reprogramming of human astrocytes to induced-neurons

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