REPROGRAMMING OF DISTINCT ASTROGLIAL POPULATIONS INTO SPECIFIC NEURONAL SUBTYPES IN VITRO AND IN VIVO
"REPROGRAMMING OF DISTINCT ASTROGLIAL POPULATIONS INTO SPECIFIC NEURONAL SUBTYPES IN VITRO AND IN VIVO"

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SUMMARY

Recently, the field of cellular reprogramming has been revolutionized by works showing the potential to directly lineage-reprogram somatic cells into neurons upon overexpression of specific transcription factors. This technique offers a promising strategy to study the molecular mechanisms of neuronal specification, identify potential therapeutic targets for neurological diseases and eventually repair the central nervous system damaged by neurological conditions. Notably, studies with cortical astroglia revealed the high potential of these cells to reprogram into neurons using a single neuronal transcription factor. However, it remains unknown whether astroglia isolated from different regions of the central nervous system have the same neurogenic potential and if they will generate the same phenotype of induced neurons. In this study we investigate the potential to reprogram astroglial cells isolated from the postnatal cerebellum into functional neurons using the proneural transcription factors Neurogenin-2 (Neurog2) and Achaete scute homolog-1 (Ascl1). We also evaluated the capacity of astroglia isolated from the cerebral cortex or cerebellum and reprogrammed into induced neurons to integrate into a neurogenic (subventricular zone – SVZ) or non-neurogenic (cerebral cortex) milieu in vivo. We observed that cortical astroglia underwent a full process of neuronal reprogramming in the brain, independently of the region of transplant and the transcription factor used. However, induced neurons behaved differently when transplanted in the SVZ or cerebral cortex. While astroglia overexpressing Ascl1 or Neurog2 reprogrammed into induced neurons migrated through the rostral migratory stream and integrated in the olfactory bulb (OB), astroglia overexpressing Neurog2 and transplanted in the cerebral cortex converted into spiny pyramidal neurons. Collectively, our results indicate that astroglial cells isolated from different regions undergo a full process of lineage reprogramming into induced neurons after transplantation in vivo. In vitro
and in vivo results suggest that the astroglia origin and the region of transplant play instructive roles in the phenotypic specification of induced neurons.
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INTRODUCTION

From cell discovery to the notion of genetic equivalence: a brief historical perspective

“There exists a general principle of construction for all organic products, and this principle of construction is cell formation”

(Theodor Schwann, 1839)

Cell discovery: the fundamental unit of life

The smallest and most basic form of life was observed for the first time under the light microscope of Robert Hooke in 1665. In his famous work *Micrographia* originally published in the same year, as illustrating thin slices of cork tissue, he observed several tiny pores and termed them “cells” which were boxlike cavities that reminded him of the cell of a monastery (Hooke, 1665). Observation of plant cells was then only the beginning of many important discoveries. Just after, Theodor Schwann extended cell observation to animals in 1839 while observing animal cartilage. In his book *Microscopical researches into the accordance in the structure and growth of animals and plants* (Schwann, 1847) he defined the cell as having three essential elements which are a nucleus, a fluid content and a wall. Subsequently, he stated the theory that cell is the basic unit of structure for all organisms. In other words, plants and animals consist of combinations of these basic units, which are arranged in accordance with definite rules. Despite the fact that cell theory enjoyed a general consensus, the question of how cells were formed was still highly controversial. Faithfull to the long dating theory of spontaneous generation, Schwann suggested that cells were formed by crystallization of inanimate material inside the cell. However, soon, Robert Remak put this theory to the question after his work on red blood cells. The Polish/German embryologist and neurologist stated in his own words “the extracellular
creation as postulated by Schwann cannot be proved... The cells of which the animal germ consists, multiply by continuous division, which starts as the nucleus as I had observed it” (Remak, 1855). Despite his brilliant statement, Remak’s theory required support from more popular scientists to be accepted. Luckily, the very famous German scientist Rudolf Virchow backed his statement and added a new doctrine to the cell theory “Omnis cellula e cellula”; or all cells develop only from existing cells. Since then, cell theory became widely accepted as an explanation of the relation between cells and living things: cells are the basic unit of life building up all organisms and old cells dividing into two create new cells.

**Birth of experimental embryology**

Subsequent to the cell discovery, many questions arise on how cells, such microscopical tiny things, organize themselves to develop into an entire and complex organism. These questions opened a new field in biology: development. Until the 1880’s, observation and histology were the only available techniques limiting studies to be mainly descriptive. Even though a lot can be learned by these simple techniques, many rising questions could not be answered. By the end of the XIX century, a group of scientists came out with the idea to directly manipulate vertebrate embryos in order to understand the rules that govern normal development, introducing by this way for the first time the field of experimental embryology. Subsequently, control of cellular differentiation became one of the most fundamental question in developmental biology. When do cells become unequal during development? Do they get permanent heritable alteration as they differentiate and adopt a specific fate? Does it happen sometime during cell division? Or is it determined at the very beginning in the substance of the undivided egg? Many pioneer scientific groups started to respond these questions by using amphibians at first stages of development as model systems. Wilhelm Roux, one of the early proponent of direct embryo
manipulation, published in 1888 results showing that killing one cell of the two cell stage amphibian embryo led to the development of only half of the animal. This work corroborated with the hypothesis of Weismann stating that development worked by “qualitative division” among daughter cells (Weismann et al., 1893). Indeed, some scientists started to believe that after division, each cell receives different subsets of heritable material to specify their unique traits. On the other hand, other biologists, amongst whom Hans Driesch, who was performing similar experiments on sea urchins embryos, observed different outcomes. Instead of killing one of the cells, he was able to separate the two cells of an embryo and keep them both alive. In 1882, he related that after separation of sea urchin embryos at the two cells stage, both cells were capable of forming a complete but half sized embryo. These conflicting results raised more questions than they answered. However, at that time, many scientists considered Driesch’s experiment, stating that Roux’s outcomes may result from killing one of the two cells. Driesch’s experiments suggested that each cell in the early embryo was capable of generating all the organs and tissues of the fully formed organism. It proposed that at least during embryonic development, each cell retains the same and the entire genetic material. Indeed, this statement enhanced the notion of nuclear equivalence at least at the two-cell stage.

**Nuclear transfer: the “fantastic experiment”**

Soon after Driesch’s observations, many scientists started to investigate the role of genetic material in cellular differentiation. Indeed, the question was whether development and differentiation were achieved by loss of information and/or irreversible changes contained within the genes. Hans Spemann, Nobel laureate for his work on the organizer and embryonic induction, was one of the first scientists interested in nuclear equivalence. After his retirement, he published an important book of experiments, *Embryonic development and induction* (1938) where he
proposed the “fantastical experiment”: to transfer the nuclei from advanced developmental stages back to the zygote from which the genetic material have been removed (Sutovsky, 2007). However, unfortunately, this procedure was not feasible at his time and was only completed after his death.

**Nuclear transfer: initial steps towards cell reprogramming**

Later on, thanks to technical advances allowing better nuclear cell transfers, many scientist could obtain enlightening responses regarding the overriding question of whether the process of development and cell differentiation requires a loss or stable change in the genetic constitution of cells (Gurdon and Byrne, 2003). The immense contribution to our actual understanding of nuclear equivalence dates from the second half of the XX century, thanks to the seminal work by Gurdon with nuclear transfer in Xenopus and other important researchers in the field (Gurdon and Byrne, 2003). Gurdon’s key contribution to cellular biology was the remarkable experiment showing that normal development to adulthood could be achieved by transferring a fully differentiated nucleus from an intestinal cell of feeding-stage larvae into an irradiated egg (Gurdon 1962; Gurdon and 1966). These results led to the assumption that some highly differentiated cells still retain all the important hereditary information as the zygote. Hence, it seems probable that neither discard of nuclear material nor irreversible genetic alteration take place during cell differentiation. In other words, nuclear content of undifferentiated and fully differentiated cells are equivalent. This principle of nuclear equivalence was further extended to adult mammalian somatic cells by the very famous work of Campbell and Wilmut that could successfully generate the adult sheep Dolly from the nuclear transfer of an adult ewe mammary cell (Campbell et al., 1996). From then on, the scientific community acknowledged what Gurdon called as the “remarkable
reprogramming activity of egg and oocyte cytoplasm” and endeavored to find out the mechanisms responsible for such nuclear reprogramming (Byrne 2003).

**Cellular reprogramming**

**Artificial transcriptional regulation**

In 2012, Gurdon’s work on nuclear transfer was awarded with the Nobel Prize in Medicine. The prize was shared with Yamanaka, a much younger scientist, but not less important, in the field of stem cell biology. Originally, Yamanaka’s group and many others were working on the isolation of key transcriptional regulators responsible for the maintenance of pluripotency in human embryonic stem cells (Mitsui et al., 2003; Chen and Daley, 2008). Yamanaka came up then with the brilliant and simple idea to test whether an adult somatic cell could be reprogrammed by artificially expressing transcriptional regulators instead of proceeding to nuclear transfer. His group investigated therefore the possibility to induce pluripotency in mouse fibroblasts by overexpression of some TFs instructing pluripotency. Surprisingly, his group found out that four transcription factors, namely Oct4, Klf4, Sox2, and cMyc, were capable of reprogramming murine adult and embryonic fibroblasts into pluripotent stem cells. In fact, reprogrammed cells expressed many of embryonic stem cells markers and showed the ability to differentiate into cells from all three germ layers. Subsequently, these cells were referred as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). One year later, generation of iPSCs was extended to human somatic cells (Takahashi et al., 2007; Yu et al., 2007; Huangfu et al., 2008; Park et al., 2008).

**Direct cell reprogramming**

The possibility to convert adult somatic cells into iPSCs by expression of few transcription factors opened up an important era in cellular reprogramming. Many laboratories
set to generate different cell types from iPSCs such as adipocytes, cardiomyocytes, primitive hematopoietic cells, pancreatic beta-cells, and several different neuronal cell types (review Amabile and Meissner, 2009). However, one important question remained unanswered: could an adult somatic cell be lineage reprogrammed into another cell type without passing through a pluripotent state? Several laboratories independently addressed this question and could show evidences for direct reprogramming of somatic cells into cardiomyocytes (Ieda et al., 2010), insulin producing cells B cells (Zhou et al., 2008) and even neurons (Vierbuchen et al., 2010) by overexpressing of TFs. These works provided direct evidence that cellular fates are not irreversible. Therefore, adult somatic cell can be directly lineage converted following expression of transcription factors. Direct cell reprogramming became with no doubt a trendy field concerning many cellular types. Recently, it gained ground also in neuroscience, with a growing number of groups focusing on the generation of neurons from somatic cells.

**Direct generation of neurons from somatic cells**

Direct reprogramming of fibroblasts into neurons has been widely demonstrated by several laboratories (Vierbuchen et al., 2010; Ambasudhan et al., 2011; Son et al., 2011; Pang et al., 2011; Liu et al., 2012, 2013; Victor et al., 2014; Lau et al., 2014; Zhou et al., 2014; Aravantinou-Fatorou et al., 2015; Hu et al., 2015; Li et al., 2015; Zhou et al., 2015; Blanchard et al., 2014). In a pioneer work, Vierbuchen and colleagues showed that viral-mediated delivery of neural-lineage specific transcription factors Ascl1, Brn2 and Myt11, is sufficient to reprogram mouse embryonic and adult fibroblasts into functional neurons (Vierbuchen et al., 2010). However, the neural conversion efficiency of these cells is still considered low (19.5%) (Vierbuchen et al., 2010). In order to ameliorate the reprogramming rates, additional molecules have to be used (Liu et al., 2013). Other studies focused on the generation of specific neuronal
subtypes such as striatal (Victor et al., 2014), dopaminergic (Liu et al., 2013), cholinergic (Liu et al., 2013) and even sensory neurons from fibroblasts (Blanchard et al., 2014). Nevertheless, it seems that a great genetic barrier has to be overcome in order to convert fibroblasts into specific neural subtypes given that it is necessary to use a complex combination of reprogramming factors such as several TFs, small molecules or microRNAs. Albeit the advantage of being easily isolated and the possibility to use patient fibroblasts for future cell therapies, these cells are still far from being the appropriate cell type to generate neurons. For those reasons, resident central nervous system glial cells become extremely attractive, as we shall discuss in the following section.

**Direct lineage reprogramming of astrocytes into neurons**

**Astrocytes and neurons: a common ancestor**

In the 19th century, Santiago Ramón y Cajal discovered that the brain and the rest of the nervous system consisted not of one mass of tissue, but of various distinct cells. At that time, drawings made by His provided scientists with the first evidence of the extraordinary cell diversity in the brain. Since then, scientists have been identifying and classifying brain cells for more than a hundred years. A very basic classification of cell type in the brain is the distinction between neurons and glia. For a long time scientists highly regarded the idea that both types of cells originate from distinct progenitor pools. Indeed the main claim was that neurons derive from specialized progenitors called neuroblasts whereas glial cells originate from spongioblasts (see historical facts in (Pease, 1971)). The different origin of neurons and glial cells became since then broadly approved and it is relatively recent that this concept has been demonstrated to be incorrect. Nowadays spongioblasts correspond to what we call radial glia cells (RGCs). This specialized cellular population has been identified in most regions of the vertebrate brain during
restricted developmental periods and has been shown to be a common progenitor for both glia and neurons in the CNS (Costa et al., 2010). Important studies showed that virtually all cortical astrocytes are derived from progenitors that contribute to the generation of neurons at early developmental stage (Costa et al., 2009; Magavi et al., 2012). The major advance in defining the nature and function of these cells came with the introduction of new methods in developmental biology such as electron microscopy, 3H-thymidine autoradiography and immunocytochemistry which provided higher resolution of cellular mechanisms and allowed more reliable identification of cell classes. Furthermore the use of cell culture, transgenic technology and retroviral gene transfer methods allowed a more accurate analysis of cell lineage relationship as well as their function in the developing mammalian brain (Rakic, 2003).

Nowadays it is well established that RGCs differentiate from neuroepithelial cells of the neural tube, and acquire typical astroglial features, characterized by the presence of glycogen storage granules and the expression of astroglial-specific proteins, such as the astrocyte-specific glutamate and aspartate transporter (GLAST), brain lipid-binding protein (BLBP) and tenascin-C (Rakic, 2003; Pinto and Götz, 2007). Although RGCs were first associated with neuronal radial migration (Rakic, 2003), there is now a general consensus that they comprise a specialized population of neural stem cells (NSCs) during cerebral cortex development (Kriegstein and Alvarez-Buylla, 2009). Many evidences showing that RGCs are multipotent cells generating both neuronal and glial progeny, and are capable of self-renewing by cell divisions generating either two new RGCs (symmetric division) or one RGC and a fate-restricted progenitor (asymmetric division) (Miyata et al., 2001; Noctor et al., 2004) indicate that RGCs exhibit defining stem cell hallmarks and are thus often considered as embryonic NSCs. Despite the fact that RGCs give rise to most neurons of the cerebral cortex during embryonic development
(Malatesta et al., 2000, 2003; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001), at the end of this period, the remaining radial glia lose their apical and basal processes and transform into parenchymal astrocytes (Voigt, 1989; Alves et al., 2002) which do not generate neurons in the adult brain under physiological conditions. These astrocytes will constitute a supportive glial cell population that has a heterogeneous morphology extending numerous processes surrounding neurons and blood vessels and containing intermediate filaments (Wang and Bordey, 2008). Astrocytes are essential for maintaining a viable nervous system environment for neurons. Among their well-established functions are: buffering excess of potassium and neurotransmitters, providing nutrients, structural support around synapses, and contributing to the integrity of the blood–brain barrier (Wang and Bordey, 2008). Interestingly, the disappearance of radial glia cells in the cerebral cortex has been suggested to account for the incapacity to generate new neuron (Kriegstein and Alvarez-Buylla, 2009). According to this view, the high regenerative capacity in the brain of some non-vertebrates is tightly linked to the persistence of immature RGCs that retain many developmental features (Pinto and Götz, 2007). Noteworthy, RGCs do not only give rise to parenchymal astrocytes, but in restricted zones of the adult central nervous system transform into astroglial neural stem cells (Merkle et al., 2004). These cells reside within two neurogenic niches, namely subventricular zone (SVZ) and subgranular zone (SGZ), and maintain the potential to generate new neurons throughout life. Interestingly, these adult NSCs display features of both RGCs and mature cortical astrocytes (Doetsch et al., 1999; Seri et al., 2001). Curiously, when astroglial cells from the early postnatal cerebral cortex are grown in vitro in the absence of serum factors but in the presence growth factors, these cells can still give rise to self-renewing and multipotent neurospheres (Laywell et al., 2000). However, the potential of this astroglia to give rise to neurospheres declines reaching
the second postnatal week. This transformation appears to be a gradual process during which the neurogenic potential of the astroglial cells becomes progressively diminished. Some mechanisms involving silencing of genes encoding the transcription of neurogenic fate determinants are thought to be involved (Hirabayashi et al., 2009). Interestingly, studies from Vaccarino’s lab have shown that early postnatal astroglia in the cerebral cortex may retain some capacity of neurogenesis in vivo. They showed by fate-mapping technique (which permits the follow up of astroglial cells using Cre-recombinase activity driven by the hGFAP promoter), that a very small percentage of the fate-mapped cells gave rise to neurons one month after recombination (Ganat et al., 2006). This potential is highly evoked in the early postnatal brain by damage such as caused by hypoxia (Fagel et al., 2006, 2009), indicating that early postnatal astroglia can respond to stimuli generating new neurons.

**Proneural transcription factors: explaining cell diversity in the CNS**

Nowadays, it is a general consensus that RGCs are common progenitors for neurons and astrocytes in the developing brain (Kriegstein and Alvarez-Buylla, 2009). Yet, it remains a matter of intense debate how these cells generate the huge cell diversity characteristic of the mammalian nervous system. Telencephalon is one of the best-studied system. It harbors a broad variety of progenitors that give rise to neurons and glial cells to the cerebral cortex and basal ganglia (striatum and pallidum). These regions are composed of subdivisions that have specific patterns of gene expression, reflecting the differential environmental influences which progenitors are exposed to. This early developed regional identity has been shown to contribute for the generation of the huge cell diversity in the cerebral cortex (Guillemot, 2007). In accordance with these observations, RGCs located at distinct regions of the telencephalon express different sets of transcription factors, which play a key role in cell fate choice. Amongst
these TFs, some belonging to the bHLH family (basic helix-loop-helix) have been assigned an important intrinsic role in the neuronal differentiation of progenitor cells (Bertrand et al., 2002). These proteins form a large superfamily of TFs that are found in almost all eukaryotes and have been assigned an important role in embryonic development. Due to the heterogeneity of their DNA sequences and dimer formation, bHLH proteins are involved in very diverse functions. Some of these proteins are widely expressed in different tissues and cells, however others are more cell specific. These latter are involved in cell fate determination in many cell lineages and have been shown to act on processes such as neurogenesis, cardiogenesis and hematopoiesis. bHLH proteins involved in neurogenesis include Ascl1 and Neurog2, belonging to the achaete-scute and neurogenin subfamilies respectively (For review see Jones, 2004). Several studies have shown that mice mutant for Ascl1 and Neurog2 show severe defects including a reduction of neurogenesis and premature excessive generation of astrocytic precursors (Guillemot et al., 1993; Fode et al., 1998; Horton et al., 1999; Ma et al., 1999). It has also been shown that both TFs dimerize with E-proteins forming by this way protein-protein interaction through their HLH domains, two α-helices connected by a loop. Following dimerization, these TFs use their DNA binding domains to bind E-box specific consensus sequences in the promoter or enhancers of their target genes (Murre et al., 1989; Johnson et al., 1992; Bertrand et al., 2002; Ross et al., 2003). E-box consensus sequences are frequent throughout the genome, nevertheless it is their clustering that promote strong bHLH binding. Interestingly, it has been shown that sequences between these consensus sites are also important given that they can influence target gene specificity, partly explained by the cooperative binding of additional TFs (Bertrand et al., 2002; Castro et al., 2006; Seo et al., 2007).
Proneural factors also affect the balance between proliferation and differentiation. Following cell division, newly generated cells can either re-enter the cell cycle continuing to proliferate or exit the cell cycle, differentiating. Consistent with their role in promoting neuronal differentiation, proneural TFs play an important role in the control of cell cycle exit. Indeed, Neurog2 has been shown to promote cell cycle exit in cortical progenitors and other neural cell populations (Farah et al., 2000; Mizuguchi et al., 2001; Mattar et al., 2008). On the other hand, Ascl1 role in this cellular event is not as sharp. It has been shown that it can induce either proliferation or differentiation of progenitor cells (Castro et al., 2011). Beside their generic function, Ascl1 and Neurog2 have been also shown to display context-dependent effects contributing to the differentiation of specific neuronal subtypes (Bertrand et al., 2002; Powell and Jarman, 2008). It is nowadays established that these genes are expressed in non-overlapping patterns in the murine central and peripheral nervous system where they drive the production of different neuronal populations (Fode et al., 2000; Parras et al., 2002; Osório et al., 2010; Brzezinski et al., 2011). For instance, several works showed that Ascl1’s implication in the generation of neurons is highly time and space dependent (Mizuguchi et al., 2006; Jo et al., 2007; Peltopuro et al., 2010). Ascl1 is also involved in the development of several neuronal subtypes such as hindbrain serotonergic neurons (Pattyn et al., 2004), central and peripheral noradrenergic neurons (Goridis and Rohrer, 2002), mesencephalic dopaminergic neurons (Park et al., 2008). RGCs in the ventral telencephalon express Nkx2.1, which acts upstream of Ascl1 and Dlx2 (Xu et al., 2004). Ascl1 has been shown to induce the expression of Dlx2 genes and direct the differentiation of GABAergic phenotype in the developing telencephalon (Fode et al., 2000). In a similar way, Neurog2 proneural functions are also temporally and spatially regulated (Mattar et al., 2008; Li et al., 2012). Corroborating with a tight temporal dynamic control of
these TFs regulation, it has been shown that continued expression of Neurog2 in post mitotic neurons leads to cell death or neuronal degeneration (Cai et al., 2000; Guichet et al., 2013). Cortical dorsal radial glia express the transcription factor paired box gene 6 (Pax6), which is required for their proper development (Götz et al., 1998). Indeed, it has been shown that Pax6 acts upstream of neurogenin 2 (Neurog2), which is specifically expressed in the dorsal telencephalon and have the dual role of repressing ventral identity and activating downstream transcription factors that control neuronal differentiation (Götz et al., 1998; Heins et al., 2002). Neurons generated from Pax6-expressing cells are distinguished by the early expression of T-box brain 1 gene (Tbr1), coding for a transcription factor involved in the specification of the glutamatergic lineage in the telencephalon (Hevner et al., 2006). Only a handful of Neurog2-regulated cortical genes have been validated until now as direct target among which are NeuroD1, NeuroD4, Tbr2 (Mattar et al., 2004; Schuurmans et al., 2004; Seo et al., 2007; Gohlke et al., 2008). An interesting study showed that Neurog2 activity can also be modulated by phosphorylation events. It has been shown that phosphorylation of Neurog2 by GSK inhibits Neurog2 transcriptional activity by preventing the formation of homodimers and instead promoting the formation of another complex Neurog2-E47 heterodimer which have a reduced ability to transactivate target gene promoter (Li et al., 2012). It is therefore not a coincidence that GSK is progressively activated in later stage cortical progenitors, but not early stage cortical progenitors where Neurog2 is most active (Li et al., 2012).

Briefly, these data suggest that cell diversity in the telencephalon is established early in development and is sustained, partly, by different expression pattern of distinct TFs in RGCs. Nevertheless, mechanisms by which these TFs function seem to be very far from simple and controlled at different levels.
Astrocytes reprogramming into neurons: development inspired reprogramming

A gene transcriptional regulation is nowadays considered as the guiding path of cellular differentiation. Developmental TFs, such as the ones belonging to the bHLH family have a considerable role in the determination of neural fate and subtype specification (Nieto et al., 2001). Amongst the member of this important family Pax6, Neurog2, Dlx2 and Ascl1 have been tested as potential neuronal reprogramming factors in astrocytes. Indeed, an interesting study showed that retrovirus mediated expression of Pax6 in cultured astroglia isolated from the cerebral cortex resulted in a rapid down-regulation of astroglial-specific genes such as GFAP and the up-regulation of neuronal genes such as βIII-tubulin (Heins et al., 2002). However, this study did not evaluate the functional maturation of these cells. A following study showed that not only forced expression of Pax6, but also of the other neurogenic transcription factors, such as Neurog2 and Ascl1, reprogrammed astroglial cells into fully functional neurons that could fire repetitive action potentials (Berninger et al., 2007). Strikingly, only Neurog2 expression induced the expression of Tbr1 in reprogrammed neurons, indicating that these neurons were following a program towards a glutamatergic fate (Berninger et al., 2007), similar to what has been described in the developing dorsal telencephalon. Likewise, forced expression of Ascl1, a transcription factor involved in the genesis of GABAergic neurons during embryonic development and adult neurogenesis (Petryniak et al., 2007), showed that the same cortical astroglia can be directed towards the genesis of GABAergic neurons. These data suggest that early postnatal astroglia can be reprogrammed towards different neuronal lineages even if they are restricted to glial identity under physiological conditions. However this first study failed to demonstrate that reprogrammed astrocytes could establish functional presynaptic complexes. This failure is thought to be a consequence of the low levels of gene expression and tendency to be silenced of
the retroviral vector (pMXIG) used in the study (Berninger et al., 2007). To overcome this technical limitation, another study of the same group used a different type of retroviral vector driving the neurogenic gene expression under the control of a chicken beta actin promoter pCAG optimized for long-term expression over months in the adult mouse brain (Zhao et al., 2006). In fact, this allowed a more complete reprogramming towards a fully mature, synapse forming neuronal phenotype (Heinrich et al., 2010). Reprogrammed neurons showed not only the ability to receive synaptic inputs but also to form functional presynaptic outputs onto other astroglia-derived neurons (Heinrich et al., 2010). It has been shown in another publication of the same group that reprogrammed astrocytes could even generate networks of spontaneously active neurons (Blum et al., 2011). A subsequent study showed that by only adding two other neurogenic TFs, such as Nurr1 and Brn2 to Ascl1, it is sufficient to convert cortical astrocytes into dopaminergic neurons (Addis et al., 2011). All these data are suggesting that astrocytes may be a suitable cell population for neuron generation. Not only they can be reprogrammed via a minimum number of TFs but also they can generate different subtypes of induced neurons (iNs) depending on the TFs used.

**Astrocytes induced neurons: alternative for cell therapy?**

The dream of therapeutic organ replacement in man returns to antiquity. Legends back far from the Roman and Greek civilizations recited magical substitution of lost tissues, such as restoration of limbs or eyes, or even replacement of decapitated heads. In the course of the last four centuries, humanity witnessed from the first attempts at blood transfusion (Lower et al, 1667) to the first successful full face transplant in 2010 (Barret et al, 2011). However, organ transplant presents many real limiting problems such as immunological rejection, side effects of long term treatment with immunosuppressive, infrequent availability of the organs and ethical
difficulties concerning who should be treated and from whom the organs should be taken. A promising and more simplistic alternative to partially sidestep all these problems is stem cell therapy. Many studies on animal models observed neuronal replacement and partial reconstruction of affected neuronal circuitry following stem cells grafts. Also, clinical trials with cell replacement on diseased human brain revealed the possibility of symptomatic relief. However, many basic issues remain unresolved such as the control of undesired growth and genetic alterations, increasing the risk for tumor formation (Amariglio et al, 2009). In addition, efficacy of functional integration and differentiation into the appropriate cell type still need to be ameliorated.

Sustainability of stem-cell tissue replacement may become less reliable as the cellular complexity of the concerned organ turns higher. A relevant example is the central nervous system. Moreover, it is generally known that susceptibility to neuronal conditions is strongly neuronal class specific. Therefore, generation of specific neuronal subtypes for the purpose of cell replacement therapy or in vitro disease modeling becomes important. Concerning all the studies related previously in this text, astrocytes reprogramming into neurons may be an interesting potential for cell therapy. Their abundancy and omnipresence make them even more reachable than any other cell type in the brain. However, we are still a way far from any clinical trials and things are not very promising if we refer to studies that tried in vivo reprogramming of astrocytes (Heinrich et al., 2015). More studies should be done in order to understand the mechanisms by which astrocytes differentiate into neurons, what could help us to find out a way to produce specific neuronal subtypes.
GENERAL QUESTIONS AND OBJECTIVES

Our work introduces many relevant questions concerning astroglia reprogramming and subtype specification of induced neurons (iNs). One of our main concerns is to discuss and propose some experiments that could help to understand how finely we can tune the fate of an iN. We believe that the final phenotype of a reprogrammed cell is not restrained by a simple factor. Instead, we think that cell reprogramming is a complex mechanism that can be controlled by harmonizing several distinct elements. Transcription factor, cell origin and environmental context are amongst the elements that could potentially influence the fate of an iN.

Here, we present a perspective about the possible application of lineage-reprogrammed astroglia iN for the treatment of neurological disorders, focusing on brain ischemia (manuscript 1). Next, we present our results using two different neurogenic TFs, Ascl1 and Neurog2, to convert different types of astroglial populations into iNs and assessed the phenotypes of these cells in vitro (manuscript 2). To the best of our knowledge, this is the first description of the neurogenic potential of astroglial population isolated from the hindbrain. This work contribute to answer (1) whether neurogenic potential can be extended to other astroglial cell types and (2) if the origin of the source cell is important in the phenotypic determination of the iN. Finally, we transplanted lineage-reprogrammed astroglia in different brain regions of the postnatal and adult mouse (manuscript 2). This allowed us to investigate whether origin of astroglial cell, TF used for reprogramming, region of integration and age of animal affect the integration of iN in the host animal. Altogether, our work brings new insights on the potential of astroglial cells to generate neuronal diversity and contributes to discuss some of the challenges in the field of cell lineage reprogramming for the use of such strategy in cell-based therapies to treat neurological disorders.
MATERIAL AND METHODS

Animals

In this work, we used C57BL/6 and GFP mice (Okabe et al., 1997) from the animal facility of the Brain Institute (UFRN, Natal). All animal procedures were done in accordance with national and international laws and were approved by the local ethical committee (CEUA/UFRN, license # 008/2014).

Astroglia culture

Postnatal cerebellum and cortical astroglial cells were isolated from postnatal day (P) 5-7 mice, as previously described (Berninger et al., 2007). Briefly, animals were killed by decapitation and had both brain and cerebellum removed from the skull and immersed in PBS. Using a stereoscopic microscope, meninges were removed and the cerebral cortex gray matter and entire cerebellum were removed and maintained separately. Next, tissues were mechanically dissociated to obtain small pieces of 2-4 mm. Cerebellum and cortical tissues were then directly plated in T75 culture flasks containing Astromedium - DMEM/F12 (Gibco), 3.5 mM glucose (Sigma), 10% fetal bovine serum (Gibco), 5% horse serum (Gibco), penicillin/streptomycin (Gibco), and supplemented with 2 % B27 (Gibco), 10 ng/mL epidermal growth factor (EGF, ) and 10ng/mL fibroblast growth factor 2 (FGF2). Cultures were incubated at 5% CO₂ and 37°C without moving. After 3-4 days, cultures were washed vigorously with PBS in order to remove unattached and weakly attached cells and medium was replaced with fresh Astromedium. Purified astrocytes reached confluency after 7-9 days. Then, cells were chemically detached using trypsin/EDTA (Gibco) 0, 5% for 10 minutes, transfected with plasmids of interest and plated in differentiation medium (below).
Astroglia transfection

Transfection of cells was proceeded via nucleofection. Astroglial cells were transfected using the 4D nucleofector device and the nucleofection solution kit P3 (Lonza). Briefly, 10^6 cells were suspended in 20µl of P3 solution containing 1-2 µg of plasmid DNA. Cell/DNA suspension was dropped in the nucleofection well to receive an electrical shock with the program EM110 for mammalian glial cells (Lonza). Astroglial cells were nucleofected with either pCAG-Neurog2-IRES-DsRed, pCAG-Ascl1-IRES-DsRed or the control plasmid pCAG-IRES-DsRed. Next, cells were plated at a density of 70,000 to 100,000 cells/well in poly-D-lysine coated 24-well tissue plates containing a medium composed of DMEM/F12 (Gibco), 3.5 mM glucose (Sigma), 10% fetal bovine serum (Gibco), 5% horse serum (Gibco), penicillin/streptomycin (Gibco), and supplemented with 2 % B27 (Gibco). 24 hours after nucleofection, medium was replaced with a serum free differentiation medium composed of DMEM/F12, 3.5 mM glucose, penicillin/streptomycin and 2% B27. Brain-derived neurotrophic factor (BDNF, Sigma) was added at 20ng/mL every fifth day of the differentiation process.

Observations: It is important to prevent bubble formation while loading the cell/DNA suspension in the wells. This will prevent more cell death. We also observed that cell clusters are more resistant to nucleofection than totally dissociated cells. It is preferable to plate cells in an already heated and equilibrated medium. Although using the same protocol, cell nucleofection efficiency and cell death vary a lot from one experiment to the other. Therefore, we always took into account experiments with higher efficiency in our analysis.
Co-culture of iNs with hippocampal neurons

Due to the decreasing survival rate of iNs starting from 20 days post transfection, we co-cultured transfected cells 5 days after nucleofection with hippocampal neurons isolated from P0 pups. Cortical and hippocampal tissues were dissected and dissociated in trypsin/EDTA for 15 min. Cells were then centrifuged (1000 rpm, 4°C) and suspended in a serum containing medium to stop trypsin activity. Next, cells were centrifuged again and suspended in a serum free medium to be subsequently added at a density of 50,000 to 70,000 cells/well.

Transplantation of transfected astroglia

Cerebellar and cortical astroglia were isolated from postnatal GFP animals (Okabe et al., 1997) and cultured as mentioned in a previous section. At confluence (70-80%), cells were nucleofected with either pCAG-Neurog2-IRES-DsRed, pCAG-Ascl1-IRES-DsRed or the control plasmid pCAG-IRES-DsRed. Cells were then resuspended and mechanically dissociated in DMEM-F12. Next we kept cells at a density of 50,000 cells/µl in ice until transplantation procedure.

Observations: Cells can survive up to 2 hours in ice. However, it is preferable to keep them less than one hour before transplantation. It is very important that the cell suspension is well dissociated to a single cell suspension otherwise this can block the glass capillary.

P0-2 C57BL/6 mice were anesthetized by hypothermia for 10 min and positioned under a light source. Make sure to see where the midline and bregma are. Injection was targeted to approximately 1mm to the right of the midline and 1mm anterior to bregma. Glass capillaries and manual injector were used to inject the cells. Glass capillary was manually and very slowly lowered into the SVZ (1mm to the pial surface). Injection in the cortex was done at 0.5 mm to
the pial surface with an angle of 45°. Approximately 1 to 2 µl of cell suspension was injected for transplantation. Glass capillary must be slowly withdrawn. After the procedure, animals were revived on a heat pad and returned to their mothers. Transplantation in adults were proceeded on P30 C57BL/6 mice under isoflurane anesthesia. Cells were stereotaxically injected using a nanoinjector (NANOLITER 2010, WPI) coupled to a glass capillary in the following coordinates: SVZ (in mm) (AP: 1.58, ML: 3.44, DV: 1.55) and cortex (AP: 1.58, ML: 3.44, DV: 1.40). 1µl of cell suspension was injected for each transplant at a speed of 1µl/min. After the procedure, animals received the essential care and returned to the animal facility.

Observations: Glass capillaries were prepared using a heater at 62°C. Before transplantation capillaries were cut (1mm) in order to push up the cells.

Tissue preparation and histology

Cell cultures were fixed with 4% PFA for 10 minutes at room temperature and stored in PBS. For anti-glutamate staining, we also added 0.3% glutaraldehyde to the fixative solution. Primary antibodies were prepared in a solution of 0.5% Triton X-100, 10% normal goat serum (NGS) and 2% bovine serum albumin (BSA). Samples were incubated with primary antibody solution at 4°C overnight. The following primary antibodies were used: polyclonal anti-green fluorescent protein (GFP, chicken, 1:500, Aves Labs, GFP-1020), polyclonal anti-Glial Fibrillary Acidic Protein (GFAP, rabbit, 1:4000, DakoCytomation, Z0334), polyclonal anti-Red Fluorescent Protein (RFP, rabbit, Rockland, 1:1000, 600-401-379), monoclonal anti-Microtubule Associated Protein 2 (MAP2, mouse IgG1, 1:200, Sigma-Aldrich, M4403), monoclonal anti-NeuN (mouse, 1:500, Millipore, MAB377), monoclonal anti-synapsin 1 (mouse IgG2, 1:2000, Synaptic Systems, 106001), polyclonal anti-Tbr1 (rabbit, 1:500, Abcam, ab51502), monoclonal anti-bIII tubulin (mouse IgG2b, 1:500, Sigma, T5076), monoclonal anti-calbindin 28K (mouse
IgG1, 1:2000, Swant), monoclonal anti-parvalbumin (mouse IgG1, 1:2000, Sigma, p3088), monoclonal anti-Cux1 (mouse IgG1, 1:500, ABCAM), monoclonal anti-glutamate (mouse, 1:1000, Sigma, g9282), monoclonal anti-GABA (mouse, 1:2000, Swant). For some nuclear staining, TO-PRO-3 (1:2000, Invitrogen) was incubated together with secondary antibody solution. After washing with PBS cells were incubated with appropriate species secondary AlexaFluor (Invitrogen) antibodies for 2 hours at room temperature. After 3 washes in PBS, cell nuclei were stained with DAPI and coverslips were mounted on glass slides with an anti-fading mounting medium (Aqua Poly/Mount). Coverslips were analyzed using fluorescence (AxioImager, Zeiss) and confocal microscopy (LSM 710, Zeiss). Images were acquired using the software ZEN 2 blue edition (Zeiss).

Tissue fixation was performed 20 to 30 days post transplantation. For that, animals were deeply anesthetized with THIOPENTAX (Cristalia) and transcardially perfused using a ventricular catheter with 0.9% saline solution for 15 min and 4% phosphate-buffered saline-buffered paraformaldehyde (PFA) for another 15 min. Brains were removed and kept in phosphate-buffered saline (PBS) overnight. The next day, brains were kept in 30% sucrose solution for cryoprotection before freezing procedure. Brains were cut in slices going from 40 to 50µm of thickness using a cryostat (Leica). Subsequently, slices were mounted on gelatin-coated slides and stored at -20ºC until immunohistochemistry.

Quantifications and statistical analysis

For the in vitro study, cells were quantified for colocalization of DsRed and BIII-tubulin immunoreactivity at 7 days post nucleofection (dpn). Colocalization of DsRed, Map-2 and NeuN was done 15 dpn. Calbindin and parvalbumin expression were analyzed at 20 dpn whereas expression of GABA, Glutamate and synapsin was analyzed later, at 30 dpn. Induced neurons
(iNs) or DsRed+ neurons terms refer to DsRed positive cells that have a clear neuronal morphology. Morphological analysis was performed using the plugin “Sholl Analysis” (version 3.4.5 June 2015) in ImageJ version 1.49 (NIH) at 15 dpn. For the in vivo experiments, we studied GFP+ cells for their morphology, hodology and neurochemical markers such as NeuN and Cux1. For each transplant, cells were quantified through the entire brain 20 or 30 days post transplantation.

Student t-test, One-way ANOVA with Tukey’s post test, or Two-way ANOVA with Bonferoni´s post test were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego California USA, www.graphpad.com). Confidence interval is 95%.
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Cell therapy for stroke: use of local astrocytes

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Abstract

Stroke refers to a variety of conditions caused by the occlusion or hemorrhage of blood vessels supplying the brain, which is one of the main causes of death and the leading cause of disability worldwide. In the last years, cell-based therapies have been proposed as a new approach to ameliorate post stroke deficits. However, the most appropriate type of cell to be used in such therapies, as well as their sources, remains a matter of intense research. A good candidate cell should, in principle, display high plasticity to generate diverse types of neurons and, at the same time, low risk to cause undesired outcomes, such as malignant transformation. Recently, a new approach grounded on the reprogramming of endogenous astrocytes towards neuronal fates emerged as an alternative to restore neurological functions in several central nervous system diseases. In this perspective, we review data about the potential of astrocytes to become functional neurons following expression of neurogenic genes and discuss the potential benefits and risks of reprogramming astrocytes in the glial scar to replace neurons lost after stroke.

Background

Ischemic insults result in a severe loss of neural cells in the core of the lesion and variable effects in the surrounding area, commonly described as ischemic penumbra. While cell death occurs during the first hours after interruption of blood supply within the core of the ischemic lesion, tissue damage in the surrounding regions is a delayed process, involving several physiopathological events, such as inflammation and immune response (for a review see: Doyle et al., 2008). Treatments aiming to increase neuronal survival after stroke are usually limited to the area of penumbra and their success in promoting functional recovery is largely dependent on
the extension of the core ischemic area (Goldstein, 2007). Currently, the only treatment available to reduce the size of the ischemic area is the use of recombinant tissue plasminogen activator (t-PA)(1995), which is approved to be administered within 3 hours after the onset of ischemia (Goldstein, 2007). This narrow time window as well as a number of contraindications for t-PA therapy makes such treatment accessible to an extremely low number of stroke victims (Katzan et al., 2000), boosting the necessity to develop new strategies to treat stroke patients.

In the last years, cell-based therapies have been proposed as a new approach to ameliorate post stroke deficits. Different from t-PA, cell-based therapies could, in principle, be administered at any time following the ischemic event and contribute to replace neurons lost after ischemia and presumably restore neurological functions (Lindvall and Kokaia, 2011). Chiefly, two main types of intervention have been proposed: i) transplantation of exogenous cells (Benchoua and Onteniente, 2011; Lindvall and Kokaia, 2011); and ii) mobilization of endogenous stem or progenitor cells (Leker et al., 2009; Lindvall and Kokaia, 2011). Both strategies have been shown to promote some degree of improvement in animal models of stroke (Lindvall and Kokaia, 2004; 2006). Yet, important limitations regarding the number of neurons replaced, specification of these neurons into the appropriate neurochemical subtypes, integration to the preexisting circuitry and potential side effects hamper the translation into clinical practice of such therapies.

Amongst the potential side effects, the most feared is the generation of tumors. In fact, it has been shown that both stem cell transplantation and stimulation of endogenous neural stem cell proliferation can lead to tumor formation in rodents and humans (Doetsch et al., 2002; Erdo et al., 2003; Amargilio et al., 2009). Therefore, the development of new strategies to replace neurons following stroke circumventing the limitations and risks discussed above is imperative.
to move cell-based therapies into clinic. In this scenario, we put in perspective the potential of a new approach grounded on the reprogramming of local astrocytes into neurons.

**Astroglial cells in the adult brain possess neurogenic potential**

Contrary to the previous notion that neurons were not generated in the mammalian brain after birth, in the last two decades two neurogenic regions in the adult mammalian brain have been uncovered: the subependymal zone (SEZ), located along the lateral walls of the lateral ventricles, which holds a population of astroglial neural stem cells (ANSC) that constantly supply the olfactory bulb with interneurons (Kriegstein and Alvarez-Buylla, 2009); and the subgranular zone (SGZ) of the hippocampus, which also contains a population of ANSC capable of generating neurons to the dentate gyrus throughout life (Gage, 2000). Besides the significance of these findings to our understanding of brain physiology (Lledo et al., 2006), they also opened a new and promising avenue to brain repair after damage (Lindvall and Kokaia, 2006).

In fact, it has been shown that global and focal ischemic injuries in rodents lead to a significant increase in the number of neurons generate from ANSC both in the SGZ and the SEZ (Liu et al., 1998; Kee et al., 2001; Arvidsson et al., 2002; Parent et al., 2002). Some of these newly generate neurons change their route of migration and roam to lesioned areas after focal ischemia, where they acquire some characteristics of local neurons (Arvidsson et al., 2002; Parent et al., 2002). However, survival of these neurons in the lesioned areas is extremely poor, suggesting that some survival signal might be missing in re-routed neurons, leading to their premature death. As mentioned before, treatments with growth factors, used to increase the generation and survival of newborn neurons from endogenous neural stem cells (Leker et al., 2009), have also been related with glioma formation (Doetsch et al., 2002), making such approaches unsafe. Moreover, it remains to be shown whether such approach could be of any use in humans, given
the great distance that newly generated neurons would need to transverse to reach the lesioned cortical tissue as compared to the rodent brain. Last but not least, the functionality of therapies aiming to recruit neurons from endogenous neurogenic niches relies on the occurrence of neurogenesis in the adult human brain, what has not been observed under physiological conditions (Sanai et al., 2004; Sanai et al., 2011).

More recently, astrocytes of the cortical parenchyma, another population of astroglial cells, were suggested as an alternative source for neuronal replacement in neurological diseases (Robel et al., 2011). Compared to ANSC residing in neurogenic compartments, cortical astrocytes would have four main advantages: i) they are located within the lesioned site, eliminating the need of relocation; ii) their amount is significantly increased after stroke (Buffo et al., 2008), generating a large amount of exploitable cells; iii) they can be efficiently reprogrammed into neurons using simple molecular manipulations (Berninger et al., 2007; Heinrich et al., 2010; Blum et al., 2011); and iv) they are involved in the formation of the glial scar, which contributes to generate an anti-neurogenic environment (Pekny and Nilsson, 2005). Therefore, astrocyte reprogramming could provide at once a source of new neurons in large numbers to replace the circuitry lost after stroke and reduce some negative effects of the glial scar (see below).

Efficient reprogramming of astrocytes into glutamatergic and GABAergic neurons

Astrocytes isolated from rodent postnatal brain are highly susceptible to neuronal reprogramming following forced expression of a single neurogenic fate determinant, such as Neurogenin 2 (NEUROG2), Distal-less homeobox 2 (DLX2) or Achaete-scute homolog 1 (ASCL1, also known as MASH1) (Berninger et al., 2007; Heinrich et al., 2010; Blum et al., 2011). Interestingly, expression of NEUROG2 induces a glutamatergic phenotype whereas the
expression of MASH1 and DLX2 induces a GABAergic phenotype, resembling the roles of those transcription factors (TFs) in the developing forebrain (Guillemot, 2005). Astrocytes can not only be reprogrammed into neurons of specific subtypes but also acquire electrical properties compatible with a mature neuronal phenotype, such as intrinsic excitability and the ability to generate action potentials and synaptic contacts (Berninger et al., 2007; Heinrich et al., 2010).

Reprogramming of postnatal astrocytes using neurogenic TFs is a highly efficient process. Approximately, 70% of NEUROG2-transduced astrocytes differentiated into βIII tubulin-positive neurons after 7-10 days (Berninger et al., 2007; Heinrich et al., 2010). By 2-3 weeks post-transduction, reprogrammed neurons acquire MAP2 immunoreactivity, indicative for dendritic maturation, and express the vesicular glutamate transporter 1 (VGLUT1), present in synaptic vesicles within presynaptic terminals of glutamatergic neurons (Heinrich et al., 2010). Thus, astrocytes reprogrammed by forced expression of a single TF (NEUROG2) adopt a full neuronal glutamatergic phenotype forming presynaptic specializations. Indeed, electrophysiological recordings of neurons reprogrammed from astrocytes with NEUROG2 demonstrated both autaptic and synaptic currents that were blocked by CNQX (an AMPA/kainate glutamate receptor antagonist), further confirming the glutamatergic nature of the reprogrammed neurons. Amongst all NEUROG2-transduced astrocyte-derived neurons recorded, ~60% exhibited either glutamatergic autaptic connections onto themselves or glutamatergic synapses onto nearby neurons (Heinrich et al., 2010). Calcium-imaging experiments demonstrated that cultures of astrocytes reprogrammed with NEUROG2 are even capable of generating networks of spontaneously active neurons (Heinrich et al., 2010; Blum et al., 2011).

The efficiency of reprogramming astrocytes into GABAergic neurons using MASH1 or DLX2 (~35%) is lower than into glutamatergic neurons using NEUROG2 (Berninger et al.,
Nevertheless, neurons reprogrammed from astrocytes using DLX2 express GAD67 immunoreactivity, display autaptic responses with slow decay time kinetics which are abolished by the GABAA receptor antagonist and show spontaneous synaptic currents exhibiting a slow decay time, characteristic of GABAergic current (Heinrich et al., 2010), indicating that astrocytes are converted to functional GABAergic neurons. Co-expression of MASH1 and DLX2 in postnatal astrocytes increased the rate of neuronal conversion up to 90% (Heinrich et al., 2010), showing that the efficiency to reprogram astrocytes into GABAergic neurons can be drastically improved by combining two TFs. Taken together, these data clearly indicate that astrocytes can be efficiently converted to functional glutamatergic or GABAergic neurons through simple molecular manipulations.

**Reprogramming of postnatal astrocytes into dopaminergic neurons**

Postnatal cortical astrocytes can also be reprogrammed into dopaminergic neurons, although this requires a more complex set of TFs (Addis et al., 2011). Astrocytes transduced with a polycistronic lentiviral vector encoding for MASH1, LIM homeobox transcription factor 1 (LMX1) and nuclear receptor related 1 protein (NURR1) differentiate into neurons expressing biochemical and electrophysiological characteristics analogous with midbrain dopaminergic neurons. However, the efficiency of astrocyte conversion to dopaminergic neurons (~18%) is much lower than that described previously for glutamatergic and GABAergic neurons (Heinrich et al., 2010; Addis et al., 2011). Yet, the finding that astrocytes isolated from a region that normally do not generate dopaminergic neurons can be reprogrammed into these types of neurons using few TFs reveals the great plasticity of astrocytes and supports the notion that these cells are good candidates to replace distinct types of neurons in damaged brain areas.
Astrocytes account for up to one-fifth of the dividing cells in the first 7 days following traumatic or ischemic brain injury (Buffo et al., 2005) and at least part of these cells are mature astrocytes that resume proliferation after lesion (Buffo et al., 2008). These reactive, proliferating astrocytes acquire some neural stem cell-like properties after injury (Buffo et al., 2008; Robel et al., 2011) and are suitable to reprogramming into functional neurons (Heinrich et al., 2010).

Although astrocyte activation may play beneficial roles at early time-points after stroke, there is convincing evidence that astrocytes in the glial scar are detrimental for regeneration of the adult brain (Pekny and Nilsson, 2005; Robel et al., 2011). For instance, attenuation of reactive gliosis through genetic deletion of intermediate filaments (IFs) glial fibrillary acidic protein (GFAP) and vimentin in animals subjected to traumatic brain injury improved regeneration, with a positive effect on complete synaptic restoration (Wilhelmsson et al., 2004). In these same models, neuronal differentiation and dendritic growth of transplanted cells were enhanced after transplantation, indicating that reactive gliosis adversely affects integration of neuronal cells (Widestrand et al., 2007). In an opposite direction, increasing reactive gliosis worsens brain injuries, as demonstrated by the finding that overexpression of S100b, an astrocyte-derived protein, enlarged infarct size and impaired neurological outcome after ischemia (Mori et al., 2008). Therefore, reprogramming of astrocytes in the glial scar could per se improve neurological functions after stroke.

In an ideal scenario, we should be able to find a balance between diminishing the number of detrimental astrocytes in the glial scar through reprogramming of these cells into neurons and, at the same time, conserve non-reprogrammed astrocytes that could contribute to create an
appropriate environment for the development and functioning of new synaptic contacts between reprogrammed neurons and the pre-existing circuitry (Wang and Bordey, 2008). To this point, it is unclear whether reactive astrocytes acquiring stem cell-like properties after injury represent a subpopulation of astrocytes and what would be the role of such cells in the glial scar. Future studies should help to clarify this point and indicate methods to target specific subpopulations of astrocytes to reprogramming.

**Reprogramming of human astrocytes into neurons**

An important question towards translation of astrocyte reprogramming into clinic would be whether human astrocytes possess the same potential to be reprogrammed into neurons. A partial answer to this question has been recently published in a paper from Corti and colleagues (2012). By cultivating astrocytes from the human cerebral cortex and inducing the expression of transcription factors involved in pluripotency (Takahashi and Yamanaka, 2006; Wernig et al., 2007), the authors could show that astrocytes expressing OCT4, SOX2 or NANOG generated colonies of neural stem cells (Corti et al., 2012). These colonies could be expanded and differentiated into the three major neural cell types – neurons, astrocytes and oligodendrocytes (Corti et al., 2012). Neurons expressed typical neuronal proteins, such as MAP2, synapsin and GABA, suggesting that human astrocytes could be reprogrammed into neurons acquiring part of the machinery to establish synaptic contacts. Expression of MASH1 in NSCs derived from human astrocytes significantly increased the frequency of neuronal differentiation (Corti et al., 2012), further supporting the key role of neurogenic determinants to convert astrocytes into neurons.

Strikingly, human astrocytes transduced with NANOG and transplanted in the lateral ventricles of immunosuppressed mice survived and integrated into the host brains 2 months after
delivery. Some transplanted cells expressed MAP2 and displayed complex and long neuritic extensions, compatible with neuronal differentiation (Corti et al., 2012). Thus, human astrocytes can be efficiently reprogrammed into neurons both in vitro and in vivo. Noteworthy, neuronal conversion of human astrocytes occurred without regression to a pluripotent state, what could contribute to avoid some complications linked to that state, including the risk of malignancy.

**Reprogramming of astrocytes into subtype-specific neurons**

The adult human brain harbors a large variety of neuronal cell types, each exhibiting specific structural, molecular and functional features (DeFelipe, 1993; Douglas and Martin, 2004; Markram et al., 2004; Klausberger and Somogyi, 2008). Therefore, one important step towards the clinical translation of astrocyte reprogramming as a therapy for stroke would be to direct the specification of defined neuronal subtypes. It remains to be evaluated, for instance, whether astrocytes reprogrammed by forced expression of NEUROG2 will generate principal and local glutamatergic neurons of different cortical layers. Similarly, it is unclear whether GABAergic neurons generated from astrocytes reprogrammed with MASH1, DLX2 or combination of these two TFs will adopt distinct morphological and electrophysiological properties, contributing to generate distinct subtypes of GABAergic interneurons, such as basket and chandelier cells (Markram et al., 2004) (Figure 2). These questions can only be answered by experiments assessing the differentiation of neurons reprogrammed from astrocytes in vivo, either by expressing neurogenic transcription factors in astrocytes directly in the brain or transplanting astrocytes previously transduced with neurogenic TFs in vitro into the healthy or injured brain. Such experiments will allow the evaluation of neuronal morphology, connectivity and synaptic formation adopted by reprogrammed astrocytes exposed to the brain environment.
Nevertheless, data from studies unraveling the molecular machinery responsible for the generation of neuronal diversity during development may help to suggest strategies to reprogram astrocytes into specific subtypes of neurons. In the last decade, several works have contributed to identify the genetic machinery involved in the specification of distinct populations of cortical glutamatergic neurons (Arlotta et al., 2005; Molyneaux et al., 2007; Leone et al., 2008). For example, family zinc finger 2 (FEZF2) is necessary for the specification of subcerebral projection neurons (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005), whereas SATB homeobox 2 (SATB2) is required for proper specification of callosal projection neurons (Alcamo et al., 2008). It is tempting to speculate that co-expression of NEUROG2 and FEZF2 or SATB2 in astrocytes would drive reprogrammed neurons into subcerebral and callosal projection neurons, respectively (Figure 2). In accordance with this possibility, expression of FEZF2 in striatal progenitors during development is sufficient to generate cortifugal neurons (Rouaux and Arlotta, 2011).

Similarly, subtypes of cortical GABAergic interneurons originate from separate progenitor domains characterized by expression of distinct sets of TFs (Wonders and Anderson, 2006; Hernandez-Miranda et al., 2010). For instance, parvalbumine-expressing basket cells originate from progenitors in the medial ganglionic eminence that express the TFs NK2 homeobox 1 (NKX2.1) and LIM homeobox 6 (LHX6), whereas calretinin-expressing interneurons originate from the caudal ganglionic eminence areas that do not express NKX2.1 (Wonders and Anderson, 2006; Hernandez-Miranda et al., 2010). Therefore, it is also feasible that distinct subsets of cortical GABAergic neurons could be generated from astrocytes through the expression of specific combinations of TFs (Figure 2).

**Targeting astrocytes for reprogramming in vivo**
Finally, for future clinical approaches, two additional points remain to be elucidated. The first is how to target specifically astrocytes within the glial scar, avoiding reprogramming of other astrocytic populations. The second point is how to deliver reprogramming factors to astrocytes in vivo. As we discussed above, techniques to convert astrocytes into neurons rely on genetic manipulations of the cells, which may cause unexpected genetic modifications (Yamanaka, 2007). One possible way to circumvent the need to introduce exogenous genes in astrocytes could be the use of recombinant proteins. This has been done to convert fibroblasts into induced pluripotent stem cells (Zhou et al., 2009) and it is likely to work in astrocytes. Another possibility would be the use of molecules capable of modifying extracellular signals involved in cell specification. Kondo and Raff (2000), for instance, have shown that oligodendrocytes precursor cells isolated from the optic nerve and cultured sequentially in platelet derived growth factor (PDGF), fetal calf serum (FCS) and basic fibroblast growth factor (bFGF), could revert to a multipotent neural stem cell state and differentiate into neurons (Kondo and Raff, 2000). It is tempting to speculate that pharmacological manipulations of the extracellular milieu or use of recombinant proteins could substitute DNA elements needed for reprogramming of astrocytes in the glial scar and, therefore, represent a safe strategy to replace neurons in human patients after stroke.

Conclusions

Reprogramming of astrocytes in the glial scar into neurons is a promising approach towards regeneration of nervous tissue after stroke. In contrast to stem cell transplantation and recruitment of neural stem cells from neurogenic regions in the adult brain, parenchymal astrocytes possess the advantage of being present in large amounts around the lesion. Moreover, conversion of reactive astrocytes into neurons would not only contribute to replace neuronal
populations lost but also help to create an environment more suitable for neuronal growth and synaptic integration.

Future studies should clarify the potential of reprogrammed astrocytes to generate different subtypes of neurons \textit{in vivo}, as well as identify the subpopulations of astrocytes more suitable to reprogramming after stroke. Simultaneously, TFs networks capable of reprogramming astrocytes into specific subtypes of neurons should be identified, contributing to design more sophisticated approaches to selectively replace neuronal populations lost after stroke.
Figure 1. Direct conversion of astrocytes into neurons *in vitro*. A-D, culture of astrocytes isolated from the postnatal cerebral cortex of transgenic mice expressing green fluorescent protein (GFP) under the control of the astrocyte-specific promoter hGFAP (Nolte et al., 2001). Astrocytes were transduced with NEUROG2 2h after plating and processed for immunocytochemistry after 7 days using antibodies against GFP (green, A and D), NEUROG2 (white, C and D) and the neuronal marker TUJ1 (red, B and D). Observe that astrocytes
transduced with NEUROG2 (three of these cells are highlighted with arrows) adopted a neuronal phenotype, but still express residual levels of GFP, indicating their astrocytic origin.

**Figure 2.**

**Figure 2.** Direct reprogramming of astrocytes into subtype specific neurons. Astrocytes can be converted into glutamatergic neurons by forced expression of NEUROG2 and into GABAergic neurons following expression of DLX2 and MASH1 (filled arrows). Up to now, it is unknown which subtype of glutamatergic and GABAergic will be generated in vivo. We suggest that co-expression of additional TFs, such as FEZF2, SATB2 or NKX2.1/LHX6, could contribute to generate more specific subtypes of neurons such as subcerebral projection neurons, callosal projection neurons or basket cells, respectively (dashed arrows).
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Lineage reprogramming of astroglial cells from different origins into distinct neuronal subtypes

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SUMMARY

Astroglial cells isolated from the rodent postnatal cerebral cortex are particularly susceptible to lineage reprogramming into neurons using a single transcription factor (TF). However, it remains unknown whether other astroglial populations retain the same potential. Likewise, little is known about the fate that iNs could adopt in vivo. In this study we addressed these questions using astroglia isolated from the postnatal cerebellum and the proneural TFs Neurogenin-2 (Neurog2) or Achaete scute homolog-1 (Ascl1). We show that cerebellum astroglia can be reprogrammed into iNs with distinctive neurochemical and morphological properties in vitro. Yet, iNs efficiently migrate and differentiate into olfactory bulb neurons following transplantation in the postnatal and adult mouse subventricular zone. In contrast, iNs differentiate at low efficiencies after transplantation in the postnatal cerebral cortex. Altogether, our data indicate that the origin of the astroglial population and TF used for reprogramming, as well as the region of integration, affect the fate of iNs.
INTRODUCTION

Direct lineage-reprogramming of somatic cells into induced neurons (iNs) is a promising strategy to study the molecular mechanisms of neuronal specification, identify potential therapeutic targets for neurological diseases and eventually repair the central nervous system (CNS) after acute or neurodegenerative injury (Arlotta and Berninger, 2014). Brain resident cells such as forebrain astrocytes present some great advantages when we consider the easiness and efficiency of conversion into iNs both in vitro (Berninger et al., 2007; Heinrich et al., 2015) and in vivo (Niu et al., 2013, 2015; Guo et al., 2014; Hu et al., 2015) compared to other cell types (Vierbuchen et al., 2010; Ambasudhan et al., 2011; Son et al., 2011; Pang et al., 2011; Marro et al., 2011; Liu et al., 2012, Karow et al., 2012, 2013; Victor et al., 2014; Lau et al., 2014; Zhou et al., 2014; Aravantinou-Fatorou et al., 2015; Zhou et al., 2015; Blanchard et al., 2014).

However, still little is known about the role of the environment in the specification of iNs fates during astroglia lineage-reprogramming in vivo (Heinrich et al., 2015). Also, it remains poorly understood what are the subtypes of iNs generated by lineage-reprogramming and how they could be controlled. Following retroviral-mediated expression of Achaete-scute homolog 1 (Ascl1) or Neurogenin-2 (Neurog2) in vitro, forebrain astrocytes are reprogrammed mostly into GABAergic or glutamatergic neurons, respectively (Heinrich et al., 2010). These observations led to the suggestion that Ascl1 and Neurog2 would be instructing neuronal phenotypes reminiscent of their reported role in cortical development (Schuurmans and Guillemot, 2002). However, the same TFs are also expressed by non-overlapping progenitor populations in the developing cerebellum, contributing to the generation of GABAergic neurons in cerebellar nuclei, Purkinje cells and inhibitory interneurons of the cerebellar cortex (Zordan et al., 2008). Thus, it is possible that astroglial cells isolated from cerebral cortex or cerebellum and lineage-
reprogrammed with *Neurog2* or *Ascl1* could retain a "molecular memory" of their origin and generate iNs with distinctive phenotypes.

To test this possibility, we first investigated the potential to reprogram astroglial cells isolated from the postnatal cerebellum (CerebAstro) into iNs. Next, we compared the phenotypes of iNs derived from lineage-reprogrammed CerebAstro and cortical astroglia (CtxAstro), both in vitro and in vivo, following transplantation in the mouse cerebral cortex or subventricular zone (SVZ). Our results show that either *Ascl1* or *Neurog2* is sufficient to convert CerebAstro into iNs adopting mostly a GABAergic phenotype. Following transplantation in the postnatal cerebral cortex, only iNs derived from CtxAstro reprogrammed with Neurog2 adopted fates reminiscent of cortical pyramidal neurons. However, after transplantation in the postnatal subventricular zone, both types of astroglial cells generate iNs that migrate to the olfactory bulb and integrate as granular or periglomerular neurons, albeit at different ratios depending on the reprogrammed astroglial population. Collectively, our results suggest that both the origin of the astroglial population used for reprogramming and the region of grafting in the brain affect the phenotype of iNs.
MATERIALS AND METHODS

Animals

We used C57BL/6 and GFP mice (Okabe et al., 1997). All procedures were done in accordance with national and international laws and were approved by the local ethical committee (CEUA/UFRN, license # 008/2014).

Astroglia culture and nucleofection

Postnatal CerebAstro and CtxAstro were isolated from postnatal day (P) 5-7 mice. Cerebral cortex gray matter and entire cerebellum were removed and mechanically dissociated. Tissues from both regions were plated separately in culture flasks containing Astromedium (see supplemental data). After 3-4 days, medium was replaced with fresh Astromedium. After confluence, astroglial cells were nucleofected with either pCAG-Neurog2-IRES-DsRed, pCAG-Ascl1-IRES-DsRed or the control plasmid pCAG-IRES-DsRed using 4D nucleofector (LONZA) (see supplemental data). Next, cells were plated at densities from $7 \times 10^4$ to $1 \times 10^5$ cells/well on poly-D-lysine coated 24-well tissue plates containing serum free differentiation medium. For some experiments, primary cells isolated from the embryonic brain were co-cultured at 5 days post-nucleofection (See supplemental data).

Cell transplantation

CerebAstro and CtxAstro were isolated from postnatal GFP animals and cultured as described. After nucleofection with pCAG-Neurog2-IRES-DsRed, pCAG-Ascl1-IRES-DsRed or the control plasmid pCAG-IRES-DsRed, cells were counted, suspended in serum free DMEM-F12 (Gibco) and maintained on ice until transplantation procedure.
One microliter of cell suspension (3-5.10^5 cells/µl) was injected very gently using a pulled glass capillary coupled to a manual injector in the cerebral cortex or SVZ of postnatal day (P) 0-2 C57BL/6 mice anesthetized by hypothermia. Transplantation in early adults (P30) C57BL/6 mice were performed under isoflurane anesthesia. Cells were injected using a nanoinjector (NANOLITER 2010, WPI) coupled to a glass capillary using the following stereotactic coordinates (in mm): SVZ (AP: 0.6, ML: 1.2, DV: 1.8); cortex (AP: 1.58, ML: 3.44, DV: 1.40).

**Fixation, immunohistochemistry and microscopy**

Cell cultures were fixed at different time points with PFA 4% for 10 minutes at room temperature and stored in PBS for immunocytochemistry (See supplemental data). Grafted animals were deeply anesthetized with a lethal injection of Thiopental (Cristalia) and transcardially perfused with saline (15 min) and PFA 4% (15 min) 20-30 days after cell-transplantation. Entire brains were isolated and cryoprotected in TissueTek solution (Sakura Finetek). Coronal sections (50µm) were obtained using a cryostat (Leica) and stored at -20°C for immunohistochemistry (See supplemental data). Samples were analyzed using epi-fluorescence (AxioImager, Zeiss) and confocal microscopy (LSM 710, Zeiss). Images were acquired using the software ZEN 2 blue edition (Zeiss).

**Quantifications and statistical analysis**

Quantification of neuronal reprogramming and iNs phenotype in vitro was performed in at least three-independent cultures. For the transplantation studies, we analyzed at least 4 animals for each condition (type of astroglial cell and TF used, region of grafting and age). Total number of cells analyzed is described throughout the text (also see supplemental data). Neuronal morphology was quantitatively analyzed using Sholl analysis. Concentric circles were centered
at the centroid of the cell body with starting radius of 40 µm and increments of 40 µm. Fifteen to twenty neurons were randomly sorted and analyzed in each group. Neuronal polarity was quantified by counting the number of cell processes within 4 quadrants centered at the soma.

Statistical tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego California USA, www.graphpad.com). Confidence interval is 95%. Statistical significance is indicated as follows: *p<0.05; **p<0.01; ***p<0.001.
RESULTS

Expression of Ascl1 or Neurog2 efficiently reprograms cerebellum astroglia in iNs

Direct lineage-reprogramming of astroglial cells into induced neurons (iNs) following viral or chemical delivery of neurogenic transcription factors (TFs) is well established for astrocytes isolated from the cerebral cortex of postnatal mice (Berninger et al., 2007; Gascón et al., 2015; Heinrich et al., 2010; Masserdotti et al., 2015). However, it remains unknown whether astroglial cells from different regions of the central nervous system hold the same potential. To address this possibility, we set out to investigate whether the expression of single pro-neural TFs could lineage-reprogram CerebAstro into iNs. Towards this aim, we purified cerebellum astroglia and nucleofected the cells with plasmids carrying the genes encoding for NEUROG2 (Neurog2-DsRed), ASCL-1 (Ascl1-DsRed) or only the reporter protein DsRED (DsRed). To determine the cellular composition of cultures prior to nucleofection, cells were immunostained for the astrocytic marker glial fibrillary acidic protein (GFAP), the pan-neuronal marker class III β-tubulin (BIII-tubulin), the neural stem cell transcription factor Sox2 and the oligodendrocyte marker O4. We observed that the vast majority of cells were positive for GFAP, whereas only a very small percentage of cells were positive for BIII-tubulin (GFAP: 93 ± 1%; BIII-tubulin: 3±1% ; n=2254 cells) (Figure S1 A-B, E). We did not detect expression of O4 and Sox2 in the cultures (data not shown). One-day post-nucleofection (dpn), we observed that virtually all transduced cells expressed GFAP (Figure S 1F-H). However, 7-8dpn, most CerebAstro transduced with Ascl1 or Neurog2 adopted a neuronal-like morphology (Figure 1B-C) and expressed BIII-tubulin (Figure 1D-G, H; Ascl1: 73±2% BIII-tubulin+ cells, n=1320 DsRed+ cells; Neurog2: 54±2% BIII-tubulin+ cells, n=1234 DsRed+ cells). In sharp contrast, cells nucleofected with DsRed only kept an astrocytic morphology and did not express BIII-
tubulin (Figure 1A, S 1I-J). Similar rates were observed with cerebral cortex astroglia nucleofected with Neurog2-Dsred, Ascl1-DsRed or control plasmids (Figure S 1C-D, E, K-Q), indicating that both CerebAstro and CtxAstro are equally prone to lineage reprogramming into iNs after expression of NEUROG2 or ASCL1.
Figure 1. *Ascl1* and *Neurog2* convert cerebellum astroglia into induced neurons. (A-C) Representative pictures taken from CerebAstro cultures 8 days post nucleofection (dpn) with Control-DsRed (A), Ascl1-DsRed (B) or Neurog2-DsRed plasmid (C). Observe that nucleofected cells (RFP+) maintain astroglial morphologies in control conditions (A), but adopt neuronal-like morphologies after transduction with Ascl1 or Neurog2. (D-H) Representative pictures and quantification from CerebAstro culture 7 dpn with Ascl1-DsRed (D) or (F) Neurog2-DsRed.
Immunocytochemistry for RFP and the immature neuronal marker BIII-tubulin show RFP+/BIII-tubulin+ iNs only in cells transfected with Ascl1 (D, E) or Neurog2 (F, G). Cells transfected with control plasmid are BIII-tubulin negative (H, see also Figure S1). (I-V) Expression of mature neuronal markers in lineage-reprogrammed CerebAstro iNs 15 dpn. Example of RFP+ Ascl1 iNs (I - N) and Neurog2 iNs (P - U), stained for MAP2 (J-K, Q-R) or NeuN (M-N, T-U). (O, V) Quantification of MAP2+ and NeuN+ amongst RFP+ cells 15dpn with Ascl1 (O) or Neurog2 (V). N.F: not found. Nuclei are stained with DAPI (blue). (Student t-test is used in H, Mean±SD). Scale bars: 25µm. (See also Figure S1).

Next, we investigated the phenotypic maturation of lineage reprogrammed CerebAstro iNs. For this, nucleofected cultures were maintained for 2 weeks in differentiation medium and assessed for the expression of the neuronal microtubule associated protein 2 (MAP2) and the neuronal nuclei protein Foxb3 (NeuN). We observed that CerebAstro transfected with either Ascl1 or Neurog2 reprogrammed into iNs expressing Map2 (Ascl1: 83±7%, n=321 cells; Neurog2: 91±9%, n= 236 cells) and NeuN (Ascl1: 90±9, n=365 cells; Neurog2: 92±5%, n= 210 cells) (Figure 1I-V). To further stimulate the synaptic maturation of iNs, transfected CerebAstro were grown in the presence of co-cultured neurons (See supplemental data). Thirty days after nucleofection, we could observe expression of synapsin 1, a synaptic vesicle protein involved in the control of neurotransmitter release (Hvalby et al., 2006), in juxtaposition to DsRed+ processes (Figure 2A-H) suggesting that iNs could be establishing synaptic contacts with co-cultured neurons. Collectively, these observations suggest that Neurog2 or Ascl1 can efficiently reprogram cerebellum astroglia into mature iNs.

**Neurog2 and Ascl1 induces distinct morphological features in iNs**

Another important aspect of neuronal differentiation is the establishment of axial polarity and process growth (Kristin L. Whitford et al., 2002; BARNES and POLLEUX, 2009; Takano et al., 2015). Interestingly, we noted that iNs displayed different morphologies 15 days after expression
of \textit{Ascl1} or \textit{Neurog2} in CerebAstro (Figure 2I and J) or cortical astroglia (Figure S2E and F). To quantify these differences, we analyzed morphological features of iNs using Sholl analysis (Figure 2L and Figure S2A-C). We found that iNs derived from CerebAstro expressing \textit{Ascl1} displayed significantly longer processes, with an increased amount of secondary and tertiary branches as compared to iNs expressing \textit{Neurog2} (Figure 2L). Notably, we also found that iNs derived from CerebAstro expressing \textit{Ascl1} were more complex than those derived from CtxAstro expressing the same TF, whereas the opposite was observed with \textit{Neurog2} (Figure S2A, B).

Next, we set out to measure the axial distribution of cell processes (Figure 2M and Figure S2D). It has been shown that intrinsic programs control polarized growth of neuronal processes (Wu \textit{et al.}, 2015). Therefore, this quantification aims at elucidating whether \textit{Ascl1} and \textit{Neurog2} could trigger distinct polarization programs in reprogrammed cells. We observed that expression of \textit{Ascl1} in CerebAstro generate higher polarized iNs than \textit{Neurog2} (Figure 2M). The opposite result was observed when we compared iNs derived from CtxAstro (Figure S2D). Collectively, these morphological parameters indicate that both the TF and the type of reprogrammed astroglial cell may interfere with iNs phenotypes.
Figure 2. Cerebellum iNs express the presynaptic protein synapsin 1 and adopt distinct neuronal morphologies. (A-H) Lineage-reprogrammed CerebAstro iNs express synapsin 1 (green) 30dpn with Ascl1-DsRed or Neurog2-DsRed. Example of RFP+/synapsin+ iNs 30 dpn with Ascl1 (A-D) or Neurog2 (E-H). (D-H) Magnifications of dashed boxes in C and , respectively. (I, J) Examples of CerebAstro derived iNs 15dpn with Ascl1 (I) or Neurog2 (J). (K-M) Quantification of morphological parameters of iNs. (K) Example of an iN plotted on concentric circles for Sholl and polarization analyses. (L) Sholl analysis comparing the
arborization complexity of Ascl1 (red line) iNs and Neurog2 iNs (green line). Note the difference in neuronal complexity between the two iNs populations (p<0.0001, F=153.1, Two-way ANOVA, n=20 iNs for each condition, Mean ±SEM). (M) Cartesian plot representing the mean frequency of intersections per quadrant. Note the higher polarization of Ascl1 derived iNs (Two-way ANOVA followed by Bonferroni post test). Scale bars represent 25µm. (See also Figure S2).

Ascl1 and Neurog2 induce different neurotransmitter identity in astroglia of different origin

To further investigate possible phenotypic distinctiveness of iNs, we analyzed the expression of the neurotransmitters GABA and glutamate, as well as Tbr1, a transcription factor associated with glutamatergic neurons (Hevner et al., 2006), in iNs derived from CerebAstro or CtxAstro upon expression of Ascl1 or Neurog2 (Figure 3 and S3). We observed a higher percentage of GABA- than glutamate-expressing iNs following expression of both Ascl1 and Neurog2 in CerebAstro (Figure 3M; CerebAstro GABA+ iNs - Ascl1: 70±6% n=68 cells and Neurog2: 59±7% n=58 cells; CerebAstro Glut+ iNs - Ascl1: 38±6% n=52 cells and Neurog2: 48±2%, n=63). In contrast, these TFs induced different fates in CtxAstro. While iNs derived from CtxAstro expressing Neurog2 expressed mostly glutamate, Ascl1 induced more GABA expression (Figure 3M; CtxAstro GABA+ iNs -Ascl1: 64±15% n=60 cells and Neurog2: 19±7% n=50 cells; CtxAstro Glut+ iNs - Ascl1: 50±9% n=67cells and Neurog2: 81±4% n=54 cells). We also noted that Neurog2 induced Tbr1 expression only in CtxAstro (Figure S3A-F, 79±2% of iNs Tbr1+, n=30 cells).
Figure 3. Lineage-reprogrammed cortical and cerebellar iNs adopt different neurotransmitter phenotypes. (A-L) Example of CerebAstro derived iNs expressing GABA or
glutamate 20 dpn with either Ascl1 (A-C and G-I) or Neurog2 (D-F and J-LI). RFP+/Glutamate+ iNs (yellow arrowhead), RFP+/Glutamate- iNs (white arrowhead). Quantification of GABA+ and glutamate + cells amongst RFP+ iNs derived from CerebAstro nucleofected with Ascl1 (orange) or Neurog2 (yellow) and CtxAstro nucleofected with Ascl1 (dark green) or Neurog2 (light green) (M) (See also Figure S3) (Two-way ANOVA, followed by Bonferroni post test, Mean ±SD) Scale bars represent 25µm. (See also Figure S3).

**Ascl1 and Neurog2 generate iNs population expressing calcium binding proteins**

GABAergic neurons in the cerebral cortex and cerebellum are extremely diverse and express distinct calcium binding proteins (Brandão and Romcy-Pereira, 2015; Leto et al., 2009). To evaluate whether iNs derived from astroglia isolated from these regions adopt the phenotype of distinct GABAergic classes, we compared the expression of calbindin and parvalbumin in iNs after expression of Neurog2 or Ascl1 (Figures 4). We observed that Ascl1 expression in CerebAstro induced a higher ratio of calbindin+ iNs, whereas Neurog2 induced a higher ratio of parvalbumin+ iNs (Figure 4M; Ascl1: 72±11%, and Neurog2 40±10% calbindin+ iNs; n=170 and 118 cells respectively; Ascl1: 30±4% and Neurog2 65±7% parvalbumin+ iNs; n=120 and 143 cells respectively). Interestingly, more than half of those calbindin+ iNs expressed CTIP2 (Figure S4) a transcription factor present in Purkinje neurons (Leid et al., 2004). In contrast, Ascl1 expression in CtxAstro induced similar rates of calbindin+ and parvalbumin+ iNs (Figure 4M; Calbindin: 53±17% iNs; Parvalbumin: 55±6% iNs; n=138 and 127 cells respectively). Yet, Ascl1 induced a significantly higher fraction of parvalbumin+ iNs in CtxAstro as compared to CerebAstro (Figure 4M). Altogether, these results indicate that Ascl1 and Neurog2 instruct different subtypes of GABAergic iNs depending on the origin of the reprogrammed astroglial cell.
Figure 4. Expression of calcium binding proteins indicates that iNs derived from cortical and cerebellar astroglia adopt distinct GABAergic phenotypes. (A-L) Examples of CerebAstro derived iNs expressing calbindin or parvalbumin 30 dpn with either Ascl1 (A-C and
G-I) or Neurog2 (D-F and J-L). (M) Quantification of calbindin+ and parvalbumin+ cells amongst RFP+ iNs derived from both CerebAstro nucleofected with either Ascl1 (orange) or Neurog2 (yellow) and CtxAstro nucleofected with Ascl1 (green). (Two-way ANOVA, followed by Bonferroni post test, Mean ±SD). Scale bars represent 20µm. (See also Figure S4).

CtxAstro nucleofected with Neurog2 differentiate into pyramidal cell like neurons after transplantation in the postnatal mouse cortex.

The distinctive iNs phenotypes observed in vitro raised the question as to whether these cells would keep such hallmarks after integration in a pre-existing circuitry. To investigate this possibility, we transplanted cortical and cerebellum astroglia following nucleofection with Neurog2, Ascl1 or control plasmid in the postnatal mouse cerebral cortex and studied the phenotypes of grafted cells. In order to facilitate the identification of grafted cells, we transplanted astroglial cells isolated from GFP mice (Okabe et al., 1997) into wild type animals. Twenty-days post transplantation (dpt), animals were perfused and grafted cells were analyzed for their morphology and chemical markers (Figure 5). We observed that virtually all CtxAstro population nucleofected with control plasmid kept astroglial morphologies (Figure 5A and C; n=1145 GFP+ cells). Interestingly, astroglial cells adopted morphologies similar to endogenous astrocytes in the grey and white matter (Figure S5A-B; Emsley and Macklis, 2006). In sharp contrast, about one-fifth of CtxAstro nucleofected with Neurog2 and transplanted into the postnatal cerebral cortex adopted neuronal morphologies in all animals analyzed (n=2338 GFP+ cells, 18±7%) (Figure 5B-O). Intriguingly, we noted that about half of iNs settled in the layers II/III of the cerebral cortex (Figure 5D). Induced neurons adopted a pyramidal neuron-like morphology with apical dendrites towards the molecular layer and basal dendrites projecting radially (Figure 5E, F). We could also observe basal axonal processes directed towards the white matter (Figure 5E). Interestingly, some GFP+ processes were observed in the corpus callosum
(Figure S5D-H), where no neurons were observed, suggesting that such processes originated from grey matter iNs. Some iNs showed very complex morphologies with several secondary and tertiary dendrites (Figure 5G) and the majority of these cells also showed dendritic spines (Figure 5I, 66±8% of pyramidal-like iNs, n=29 cells). Most cells with neuronal morphology also expressed NeuN+ (Figure 5J-L) and about 60% of iNs in layer II/III expressed the TF Cux1 (Figure 5M-O, n=15 cells). These data suggest that cortical astroglia reprogrammed with Neurog2 can differentiate into pyramidal-like iNs in vivo.

In contrast, however, we found only a very small number of GFP+ cells with neuronal morphology following transplantation of CtxAstro or CerebAstro reprogrammed with Ascl1 (Figure S5I-O; >500 cells counted/animal). However, different from iNs derived from CtxAstro reprogrammed with Neurog2, those iNs displayed morphologies reminiscent of non-spiny GABAergic interneurons and some also expressed calbindin (Figure S5J-N), suggesting a GABAergic phenotype. These observations suggest that reprogramming and survival of iNs following transplantation are affected by both the origin of the reprogrammed cell and TF used.
Figure 5. Cortical astroglia nucleofected with Neurog2 integrate as pyramidal cell like iNs in vivo. (A-B) Examples of GFP+ CtxAstro 20 days post transplantation in the postnatal mouse cortex. Observe the astrocytic morphology of CtxAstro nucleofected with control plasmid (Ctrl) in host layer II cortex (A), and the presence of GFP+ cells with neuronal morphology in animals transplanted with CtxAstro nucleofected with Neurog2 (B, arrowhead). (C) Quantification of
cells showing astroglial or neuronal morphology 20 days after transplantation. (D-H) Examples of GFP+ pyramidal-like iNs observed in the cerebral cortex following transplantation of CtxAstro nucleofected with Neurog2. Note the typical pyramidal cell morphology of iNs in layers II-III of the host cerebral cortex (D, dashed boxes). (E, F) Magnification of cells shown in D revealing the apical dendrite (white arrowheads), basal dendrites (white arrows) and axonal process of iNs (yellow arrowheads). (G) Example of an iNs in the layer II of the cerebral cortex showing spiny dendrites (dashed box, magnified in H) suggestive of a glutamatergic identity. (I) Quantification of spiny and non-spiny iNs in the cerebral cortex of host animals. (J-L) GFP+ iN expressing the mature neuronal marker NeuN. (M-O) GFP+ iN expressing the supragranular neuronal marker Cux1 (M-O). Nuclei are stained with either DAPI (blue) or TO-PRO3 (red). (Student t-test was performed in C, Mean±SD). Scale bars: 50µm. (See also Figure S5 and S6).

**CerebAstro and CtxAstro iNs integrate as olfactory bulb interneurons upon transplantation in the SVZ**

Next, we set out to test whether integration of iNs could be facilitated by transplantation into a neurogenic milieu. Toward this aim, we transplanted CerebAstro and CtxAstro in the SVZ of postnatal animals following their nucleofection with either Ascl1 or DsRed. Thirty days after transplantation we observed a substantial number of GFP+ cells in the rostral migratory stream (RMS) and olfactory bulb (OB) of host animals (Figure 6A-F). In the control group, virtually all GFP+ cells nucleofected with control plasmid retained astrocytic morphologies in the SVZ, RMS and OB (Figure S6A-B, n>300 cells). In contrast, a significant fraction of CtxAstro and CerebAstro nucleofected with Ascl1 adopted morphologies typical of bona fide OB neurons (Figure 6). Notably, iNs adopted typical morphologies of neurons of the granule cell layer (GCL) or periglomerular (PGL) (Figure 6H-S) and expressed the mature neuronal marker NeuN (Figure 6Q-S). However, the ratio of iNs in the GCL and PGL varied depending on the reprogrammed astroglia (Figure 6G). While iNs derived from CtxAstro mostly adopted the fate of GCL neurons (type I and III) (CtxAstro+Ascl1: 68±14%, n=117 cells; CerebAstro+Ascl1: 28±10%, n=87 cells), iNs derived from CerebAstro preferentially integrated as PGL neurons (CerebAstro+Ascl1: 71±10%; CtxAstro+Ascl1: 31±14%). We also observed a small number of
iNs with granular- and periglomerular-like morphologies after transplantation of CtxAstro nucleofected with Neurog2 in the SVZ (Figure S6C-D).
Figure 6. Cerebellar and cortical astroglia derived iNs integrate in the postnatal olfactory bulb. (A-F) Coronal sections obtained from a mouse brain 30 dpt of CerebAstro nucleofected with Ascl1. GFP+ cells grafted in the SVZ (A), anterior RMS (B), and OB (C). Dashed boxes in A-C are magnificed in D-F. (G) Quantification of iNs o in the OB GCL or PGL following
transplantation of CerebAstro or CtxAstro nucleofected with Ascl1 in the SVZ. (H-L) Examples of GFP+ OB iNs derived from CerebAstro nucleofected with Ascl1. Note the typical morphology of granular (H) or periglomerular (I) OB neurons adopted by iNs. Observe also the expression of NeuN in OB iN (J-L, white arrowhead). (M-S) Examples of GFP+ OB iNs derived from CtxAstro nucleofected with Ascl1. Observe the typical morphologies of iNs in the PGL (M,N) and GCL (O,P). Example of a granular like iN expressing NeuN (Q-S). SVZ: subventricular zone; RMS: rostral migratory stream, OB: olfactory bulb, GL: glomerular layer, EPL: external plexiform layer, GCL: granule cell layer. Nuclei are stained with DAPI (blue). (Two-way ANOVA, followed by Bonferroni post test, Mean ±SD). Scale bars: 50µm. (See also Figure S7).

Finally, we transplanted Ascl1 lineage-reprogrammed CtxAstro in the adult SVZ. We observed that, similar to our experiments in the postnatal brain, GFP cells also migrated throughout the RMS and reached the OB. However, many cells albeit with neuronal morphology, remained in the RMS (Figure 7C-D, 41±8%). Amongst the cells that differentiate within the OB, we observed exclusively iNs in the GCL with morphologies of granular neurons (Figure 7A-G, 58.9±8%, n=96 counted cells, 2 animals). Some of these cells expressed GABA (Figure 7F-G).

Still different from transplantation in the postnatal SVZ, we did not detect GFP+ cells with astroglial morphology (Figure S6A-B) in the OB following transplantation of CtxAstro in the adult SVZ.

Altogether, these data suggest that the neurogenic environment in the SVZ plays an instructive role in the phenotypic specification of iNs. Yet, the origin of astroglial cells and the TF used for reprogramming still interfere with the final fate of iNs.
Figure 7. Cortical astroglia are reprogrammed into iNs in the adult SVZ-RMS-OB. (A-G) Immunostainings showing GFP+ CtxAstro nucleofected with Ascl1 30 dpt in the adult SVZ. Example of granular cell-like iNs observed in the OB (A, white arrowhead; magnified in B). Example of iNs found in the anterior RMS (C, yellow arrowhead; magnified in D). Example of a GFP+ granular cell-like iN (E) expressing GABA (F, G). Quantification of the localization of iNs found in the GCL (white bar) and RMS (grey bar). GCL= granule cell layer, RMS=rostral migratory stream, OB=olfactory bulb, d.p.t: days post transplantation. Nuclei are stained with DAPI (blue). (Student t-test was used in H, Mean±SD). Scale bars: 50µm.
DISCUSSION

Our data indicate for the first time that, similar to their CtxAstro counterparts, postnatal CerebAstro can be efficiently lineage-reprogrammed into iNs using a single TF. More importantly, we reveal that astroglial cells isolated from different regions and reprogrammed by overexpression of Neurog2 or Ascl1 generate iNs with different morphological and neurochemical phenotypes. Finally, we demonstrate that integration of iNs after transplantation depends on several factors such as the origin of astroglia population, the TF used, the region of transplant and the age of the transplanted animal.

Studies focused on astroglial cells reprogramming into neurons exclusively used CtxAstro as starting cell population (Berninger et al., 2007; Blum et al., 2011; Heinrich et al., 2010). However, it remained unclear whether astroglial cells isolated from other regions of the central nervous system could be reprogrammed into iNs using the same TFs. Here, we show that postnatal cerebellum astroglia can be reprogrammed into neurons following overexpression of Neurog2 or Ascl1 at similar ratios to postnatal cortical astroglia. This study therefore extended neurogenic potential to a new astroglial cell population, not studied before, suggesting that neurogenic potential may be a hallmark of every astroglia population of the CNS. We chose to use cerebellum because Neurog2- and Ascl1-lineages contribute different neuronal phenotypes compared to the cerebral cortex.

In fact, Ascl1 and Neurog2 are TFs belonging to the bHLH family and expressed in different regions of the developing central nervous system. In the telencephalon, Ascl1 is mostly expressed by progenitors in the ganglionic eminences and contribute to the generation of cortical GABAergic neurons, whereas Neurog2 is mostly expressed by dorsal progenitors that generate
glutamatergic neurons (Fode et al., 2000; Parras et al., 2002; Schuurmans and Guillemot, 2002). These developmental roles of TFs have been suggested to explain the phenotype of lineage-reprogrammed CtxAstro iNs (Heinrich et al., 2010). However, the very same TFs instruct distinctive neuronal phenotypes in other CNS regions. For instance, Ascl1 and Neurog2 are expressed by non-overlapping progenitor populations in the ventricular zone of the cerebellum, the germinative neuroepithelium that gives rise to GABAergic progenitors fated to contribute to the cerebellar nuclei, the Purkinje cells and the inhibitory interneurons of the cerebellar cortex (Zordan et al., 2008). Fate-mapping studies show that Ascl1-progenitor lineage contribute GABAergic neurons in deep cerebellar nuclei and Purkinje cells (Kim et al., 2008).

According to these roles in the developing cerebellum, we show that Ascl1 and Neurog2 reprogram CerebAstro mostly into GABAergic iNs. Moreover, expression of calcium-binding proteins reveals that Ascl1 and Neurog2 lineage-reprogrammed CerebAstro iNs are distinct: while Ascl1 induces mostly calbindin expression, Neurog2 induces parvalbumin. These observations are in line with previous suggestions of these TFs instructing distinct neuronal phenotypes in the developing cerebellum (Zordan et al., 2008). Moreover, we observed that most iNs displaying complex morphologies after Ascl1 also expressed calbindin and CTIP2, which are typical hallmarks of Purkinje cells.

Considering that Ascl1 and Neurog2 reprogram CtxAstro into iNs adopting mostly a GABAergic or glutamatergic phenotype, respectively (Heinrich et al., 2010 and our own data), which is reminiscent of the roles of those TFs in the developing telencephalon, a parsimonious explanation for these data is that astroglial cells retain a molecular memory of the region from where they were isolated. In fact, corroborating with this idea, many recent data indicate that reprogrammed somatic cells retain residual DNA methylation signatures characteristic of their
somatic tissue of origin. These are called “memory” of origin and indeed favors their differentiation towards lineages related to the donor cells (Hu et al., 2010; Kim et al., 2010; Polo et al., 2010; Tian et al., 2011). Astroglial cells from separate regions of the CNS may present different chromatin modifications in genes targeted by neurogenic TFs. These modifications are likely to occur in early progenitor cells, under influence of distinct morphogenetic signals at different domains of the developing CNS (Kiecker and Lumsden, 2005; Lupo et al., 2006), before generation of neurons and glial cells. This patterning contributes to generate neuronal diversity but would also be inherited by astroglial cells within the same lineage (Costa et al., 2009; Gao et al., 2014). Alternatively, astroglial cells obtained from different regions could express different sets of microRNAs or IncRNAs involved in the specification of neuronal fates (Flynn and Chang, 2014; Jönsson et al., 2015). Future experiment should help to elucidate the exact molecular machinery controlling the acquisition of neuronal phenotypes during lineage reprogramming. It will also be interesting to test whether astroglial cell types isolated from other CNS regions, such as the spinal cord and retina, generate iNs phenotypically similar to neurons of these regions.

In accordance with our observations in vitro, iNs derived from Neurog2 lineage-reprogrammed CtxAstro and transplanted in the postnatal cerebral cortex mostly adopted a phenotype of pyramidal spiny neurons, which are glutamatergic in this region (Shepherd, 2003). Similarly, previous data in the literature have shown that NeuroD1, a downstream target of Neurog2, converts cerebral cortex reactive astrocytes into Tbr1+ iNs in situ (Guo et al., 2014). However, iNs morphologies described here are much more elaborate, showing typical apical and basal dendrites, as well as long-distance axonal projections. One possible explanation for this thorough differentiation could be that NEUROG2 targets genes important for morphological maturation of
cortical pyramidal cells that are not regulated by NeuroD1. In fact, it has been shown that phosphorylation of a single tyrosine residue (T241) of NEUROG2 is essential and sufficient to control radial migration, neuronal polarity and dendritic morphology of pyramidal neurons (Hand et al., 2005). We assume that NEUROG2 phosphorylation happens in the host-developing cortex and permits therefore the development of a mature pyramidal morphology. This could also help to explain the preferential localization of transplanted cells in layers II/III of the cerebral cortex, which are under formation at the time of transplantation. In contrast, however, CerebAstro expressing Neurog2 and transplanted in the postnatal cerebral cortex differentiate into a very small number of iNs with GABAergic interneuron phenotypes. Thus, Neurog2 expression alone is not sufficient to reprogram all astroglial populations into pyramidal-like iNs in vivo. Similarly, we found very few cells with GABAergic interneuron-like morphologies in the cerebral cortex of animals transplanted with cortical or cerebellum astroglia nucleofected with Ascl1, further supporting the notion that both the origin of the astroglial cell and the TF used for reprogramming are important to determine the final iN fate in vivo.

The environment in the postnatal cerebral cortical may not be permissive for all lineage-reprogrammed astroglial cells to differentiate into iNs. In fact, after transplantation in the neurogenic subventricular zone, we observed that both cerebellar and cortical astroglia nucleofected with Ascl1 could migrate throughout the RMS and differentiate in the OB as GCL- and PGL-like interneurons. This suggests that the milieu in the postnatal SVZ is not only more permissive to lineage-reprogrammed astroglia iNs, but also play instructive roles in the phenotype of the iNs. Interestingly, however, despite this instructive role of environment, Ascl1 lineage-reprogrammed cortical and cerebellar astroglia iNs generated GCL and PGL-like interneurons at different ratios, suggesting that the origin of the astroglial cell still play some role
in fate determination. A few iNs were also detected in the OB after transplantation of cortical astroglia nucleofected with Neurog2. One possible explanation for this difference could be the distinct roles played by Ascl1 and Neurog2 in the postnatal SVZ. While the former is required for generation of most OB interneurons, specially granule cells (Parras et al., 2004), Neurog2 contributes to the generation of a very small portion of juxtaglomerular neurons (Winpenny et al., 2011).

Noteworthy, we observed GFP+ cells with astrocytic morphologies in the RMS and OB of all animals transplanted with both cortical and cerebellar astroglia at postnatal stages, regardless of the plasmid used for transfection (control-, Neurog2- or Ascl1-DsRed). Most of these cells did not express DsRed (non-transfected cell) and some did (transfected but not reprogrammed). This observation suggests that transplanted astrocytic cells can also respond to migration cues in the SVZ-RMS-OB system and integrate in the OB. Accordingly, it has been recently shown that astrocytes are constantly added to the OB after generation in the SVZ (Sohn et al., 2015).

Finally, we also observed that integration in the adult brain is more limited than in the postnatal brain. In fact, we could not observe integration of iNs after transplantation in the adult cerebral cortex (data not shown). In the adult OB, we could detect some iNs following transplantation of cortical astroglia transfected with Ascl1. Interestingly, an important fraction of iNs in the adult OB seems to have a problem to reach the GCL.

Altogether, our results indicate that lineage reprogramming of astroglial cells into neurons by neurogenic TFs is more complex than previously thought. In fact, they show that a same TF can induce the generation of glutamatergic or GABAergic iNs, which would have a completely different role in a neuronal circuitry. Moreover, they demonstrate that both the origin and the region of integration play an important role in the phenotypic specification of iNs. These results
have critical relevance for future cell-based therapies, either using transplantation of exogenous lineage-reprogrammed astroglial cells or direct in situ lineage reprogramming of resident astroglia.

Acknowledgments

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Figure S1. Related to Figure 1. Characterization of cerebral cortex and cerebellum astroglial cells cultures and neuronal reprogramming of CtxAstro. (A-D) Images showing CerebAstro and CtxAstro cell cultures 2h after passage and immunostained for GFAP (A and C, red) and BIII-tubulin (B and D). Quantification of GFAP+ and BIII-tubulin+ cells in both CerebAstro and CtxAstro cultures (E). (F-H) Example of CerebAstro culture 24H after
nucleofection with *Ascl1*. Note that all RFP+ nucleofected cells (red) are GFAP+ (green) (H). Example of a CerebAstro culture nucleofected with control plasmid 7dpn with *DsRed* (I), note that all RFP+ cells do not express BIII-tubulin (J). (K-P) CtxAstro cultures 7dpn with *DsRed* (K-L), *Ascl1* (M-N) or *Neurog2* (O-P) and immunostained for BIII-tubulin (L, N and P, green). Note that RFP+/BIII-tubulin+ cells were found only in cultures nucleofected with *Ascl1* (N) or *Neurog2* (P). Quantification of RFP+/BIII-tubulin+ cells amongst all RFP+ cells in CtxAstro 7 dpn with *Ascl1* (black bar), *Neurog2* (grey bar) or *DsRed* (white bar) (Q). N.F: not found, ns: not significant, dpn: days post nucleofection. (Student t-test in Q, Mean±SD). Nuclei are stained with DAPI (blue). Scale bars: 25µm.
Figure S2. Related to Figure 2. Morphological properties of iNs depend on astroglia origin and TF used. (A-C) Sholl analyses comparing morphological complexity of iNs 15 dpn with Ascl1 or Neurog2. Graphs show Sholl analyses of iNs derived from CerebAstro and CtxAstro nucleofected with Ascl1 (A), CerebAstro and CtxAstro nucleofected with Neurog2 (B), and CtxAstro nucleofected with Ascl1 or Neurog2 (C). Note that iNs derived from CerebAstro nucleofected with Ascl1 are more complex than iNs derived from CtxAstro reprogrammed with the same TF (A). The contrary is observed for iNs derived from those astroglial populations reprogrammed with Neurog2 (B). (D) Cartesian plot representing the polarity of iNs derived from CtxAstro nucleofected with Ascl1 or Neurog2. Observe that CtxAstro reprogrammed with Neurog2 generate iNs more polarized than those converted by Ascl1. (E,F) Representative images showing the morphology of iNs derived from CtxAstro nucleofected with Ascl1 (E) and Neurog2 (F). (Two-way ANOVA followed by Bonferroni post test, n>15 iNs/condition, Mean±SEM). Nuclei are stained with DAPI (blue). Scale bars: 100µm (A-D), 20µm (F-P).
Figure S3. Related to Figure 3. Expression of glutamatergic and GABAergic markers in iNs derived from lineage-converted astroglia. (A-L) Images of CtxAstro and CerebAstro cultures 7 dpn with Neurog2 (A-I) or Ascl1 (J-L). (A-C) Example of an iN derived from CtxAstro nucleofected with Neurog2 co-staining for RFP (red) and BIII-tubulin (green) and Tbr1 (white, arrowhead). (D-F) Example of an iN derived from CerebAstro nucleofected with Neurog2 co-staining for RFP (red) and BIII-tubulin (green) but not Tbr1 (E, arrowhead). (G-I) Example of a RFP+ iN (red) derived from CtxAstro reprogrammed with Neurog2 expressing glutamate (green). (J-L) Example of a RFP+ iN (red) derived from CtxAstro reprogrammed with Ascl1 expressing GABA (green). Nuclei are stained with DAPI (blue). dpn: days post nucleofection. Scale bars: 20µm.
Figure S4. Related to Figure 4. Most iNs derived from CerebAstro reprogrammed with Ascl1 express the Purkinje-cell markers calbindin and Ctip2. (A-D) Example of an RFP+ iNs derived from CerebAstro 20 dpn with Ascl1 (A, red) expressing calbindin (B, green) and Ctip2 (white). Images are merged in panel D. (E) Quantification of Ctip2+ cells amongst RFP+/Calbindin+ iNs. (Student t-test, Mean±SD). Nuclei are stained with DAPI (blue). Scale bars: 20µm.
Figure S5. Related to Figure5. Induced neurons project long-distance axons in the host brain. (A-E) Coronal sections of brains transplanted with CtxAstro nucleofected with Neurog2 showing GFP+ axonal processes reaching (A) and within the corpus callosum (B-E). Note that some processes are found in the hemisphere ipsilateral to the transplant (A,B). CC: corpus callosum, gcc: genus of the corpus callosum, MC: motor cortex, PD: dorsal peduncular cortex. Nuclei are stained with DAPI (blue). Scale bar: 20µm.
Astroglial cells nucleofected with control plasmid or Ascl1 adopts distinct phenotypes in host postnatal cortex. (A-C) Pictures showing GFP+ CtxAstro and CerebAstro nucleofected with control plasmid 20 dpt in postnatal cortex. Note that CtxAstro adopt a protoplasmic-like morphology in the grey matter (A), whereas cells from the same population adopt a fibrous-like morphology in the white matter (B). Note also that CerebAstro do not display morphologies reminiscent of cerebral cortex astrocytes (C). (D-I) Example of CerebAstro nucleofected with Ascl1 20 dpt in the postnatal cortex. Example of iN in layer VI of the cerebral cortex (D, dashed box), showing a GABAergic interneuron-like morphology (E). Note that iN processes are non-spiny (F, magnification of box in E). Example of GFP+ iNs found in layer I of the host cerebral cortex expressing the GABAergic interneuron marker calbindin (G, dashed box). High magnification of dashed box in G showing GFP (H) and calbindin (I) expression in iNs. (J) Example of GFP+ cells CtxAstro nucleofected with Ascl1 30 dpt in host postnatal cortex. Observe the cell with interneuron-like morphology (arrowhead). Roman numbers in D represent the cerebral cortex layers. Scale bar: 20µm.
Figure S7. Related to Figure 6. CtxAstro nucleofected with Neurog2 and transplanted in the postnatal SVZ differentiate into OB-like iNs. (A-D) Coronal sections of the OB of animals 30 dpt of CtxAstro nucleofected with control plasmid (A,B) or Neurog2 (C,D) in the postnatal SVZ. Note that CtxAstro nucleofected with control plasmids migrated to OB and maintained astroglial morphology and expressed GFAP (A-B, white arrowhead), but not NeuN (B, red). In contrast, GFP+ cells adopted typical morphologies of granular (C) and periglomerular (D) OB neurons in animals transplanted with CtxAstro nucleofected with Neurog2 (C). Nuclei are stained with DAPI (blue). dpt: days post transplantation. Scale bars: 50μm.
Supplemental Experimental Procedures

Isolation and expansion of astroglial cells

Cerebellum and cerebral cortex tissues were mechanically triturated and plated in T75 culture flasks containing Astromedium, composed of DMEM/F12 (Gibco), 3.5 mM glucose (Sigma), 10% fetal bovine serum (Gibco), 5% horse serum (Gibco), 100U/ml penicillin/streptomycin (Gibco), 2 % B27 (Gibco), 10 ng/mL epidermal growth factor (EGF, Gibco) and 10ng/mL fibroblast growth factor 2 (FGF2, Gibco). Cultures were incubated at 5% CO₂ and 37°C without moving. After 3-4 days, cultures were washed vigorously with PBS in order to remove unattached and weakly attached cells and medium was replaced with fresh Astromedium. After 7-10 days, astroglial cells reached up to 90% of confluence and were used for transfection.

Astroglial cell passage and transfection

Astroglial cells were chemically detached from T75 culture flasks using trypsin/EDTA (Gibco) 0,5% for 10 minutes at 37°C. In order to stop trypsin action, an equal amount of medium with 10% FBS was added to the detaching cells. Cells were then centrifuged at 1500rpm, 4°C for 5 min. Supernatant was removed and cells were resuspended at 5 x 10⁴ cells/µl in the P3 solution (Lonza) containing 1-2 µg of plasmid DNA. Cell/DNA suspension was dropped in the nucleofection well to receive an electrical shock with the program EM110 for mammalian glial cells (Lonza). Astroglial cells were nucleofected with either pCAG-Neurog2-IRES-DsRed, pCAG-Ascl1-IRES-DsRed or the control plasmid pCAG-IRES-DsRed. Next, cells were plated at a density of 70.000 to 100.000 cells/well in poly-D-lysine coated 24-well tissue plates containing Astromedium. 24 hours after nucleofection, medium was replaced with differentiation medium composed of DMEM/F12, 3.5 mM glucose, penicillin/streptomycin and 2% B27. Brain-derived
neurotrophic factor (BDNF, Sigma) was added at 20ng/mL every fifth day during the differentiation process.

**Co-culture of lineage-reprogrammed astroglial cells with hippocampal neurons**

Due to the decreasing survival rate of iNs starting from 20 days post transfection, for analyses performed after this period we co-cultured transfected cells 5 days after nucleofection with hippocampal neurons isolated from P0 pups. Briefly, hippocampal tissues were dissected and dissociated in trypsin/ EDTA for 15 min. Cells were then centrifuged (1000 rpm, 4°C) and suspended in a serum-containing medium to stop trypsin activity. Next, cells were centrifuged again, suspended in serum-free medium and added at a density of 50,000 to 70,000 cells/well.

**Tissue preparation and histology**

Cell cultures were fixed with 4% PFA for 10 minutes at room temperature and stored in PBS. For anti-glutamate staining, we also added 0.3% glutaraldehyde to the fixative solution. Primary antibodies were prepared in a solution of 0.5% Triton X-100, 10% normal goat serum (NGS) and 2% bovine serum albumin (BSA). Samples were incubated with primary antibody solution at 4°C overnight. The following primary antibodies were used: polyclonal anti-green fluorescent protein (GFP, chicken, 1:500, Aves Labs, GFP-1020), polyclonal anti-Glial Fibrillary Acidic Protein (GFAP, rabbit, 1:4000, DakoCytomation, Z0334), polyclonal anti-Red Fluorescent Protein (RFP, rabbit, Rockland, 1:1000, 600-401-379), monoclonal anti-Microtubule Associated Protein 2 (MAP2, mouse IgG1, 1:200, Sigma-Aldrich, M4403), monoclonal anti-NeuN (mouse, 1:500, Millipore, MAB377), monoclonal anti-synapsin 1 (mouse IgG2, 1:2000, Synaptic Systems, 106001), polyclonal anti-Tbr1 (rabbit, 1:500, Abcam, ab51502), monoclonal anti-bIII tubulin (mouse IgG2b, 1:500, Sigma, T5076), monoclonal anti-calbindin 28K (mouse IgG1, 1:2000,
Swant), monoclonal anti-parvalbumin (mouse IgG1, 1:2000, Sigma, p3088), monoclonal anti-Cux1 (mouse IgG1, 1:500, ABCAM). For some nuclear staining, TO-PRO-3 (1:2000, Invitrogen) was incubated together with secondary antibody solution. After washing with PBS cells were incubated with appropriate species secondary AlexaFluor (Invitrogen) antibodies for 2 hours at room temperature. After 3 washes in PBS, cell nuclei were stained with DAPI and coverslips were mounted on glass slides with an anti-fading mounting medium (Aqua Poly/Mount).

Tissue fixation was performed 20 to 30 days post transplantation. For that, animals were deeply anesthetized with THIOPENTAX (Cristalia) and transcardially perfused using a ventricular catheter with 0.9% saline solution for 15 min and 4% phosphate-buffered saline-buffered paraformaldehyde (PFA) for another 15 min. Brains were removed and kept in phosphate-buffered saline (PBS) overnight. The next day, brains were kept in 30% sucrose solution for cryoprotection before freezing procedure. Brains were cut in slices going from 40 to 50µm of thickness using a cryostat (Leica). Subsequently, slices were mounted on gelatin-coated slides and stored at -20ºC until immunohistochemistry (see previous paragraph).

Quantifications

For the in vitro study, cells were quantified for colocalization of DsRed and BIII-tubulin immunoreactivity at 7 days post nucleofection (dpn). Colocalization of DsRed, Map-2 and NeuN was performed 15 dpn. Calbindin and parvalbumin expression were analyzed at 20 dpn whereas expression of GABA, Glutamate and synapsin was analyzed later, at 30 dpn. Induced neurons (iNs) or RFP+ neurons terms refer to DsRed positive cells that have a clear neuronal morphology. Morphological analysis was performed using the plugin “Sholl Analysis” (version 3.4.5 June 2015) in ImageJ version 1.49 (NIH) at 15 dpn. Polarization of iNs was quantified
using a Cartesian distribution of neuronal processes. For the in vivo experiments, we studied GFP+ cells for their morphology, hodology and neurochemical markers such as NeuN and Cux1. For each transplant, cells were quantified through the entire brain 20 or 30 days post transplantation.
GENERAL DISCUSSION

Discovering the neurogenic potential of cerebellum astroglia

During the last decade, cell reprogramming gained ground in the field of neuroscience. Many studies revealed the possibility to convert somatic cells in neuron-like cells by simply overexpressing neurogenic TFs (Amamoto and Arlotta, 2014). Amongst many used cell types, astrocytes, even isolated from humans (Corti et al., 2012), were revealed to be amongst the best candidates (Berninger et al., 2007; Heinrich et al., 2010). In vitro, cortical astrocytes can be reprogrammed into different types of neurons. Several studies reported their high efficiency conversion into glutamatergic and GABAergic neurons (Heinrich et al., 2010) or with a lower efficiency into dopaminergic neurons (Addis et al., 2011). In our present study, we could broaden the neurogenic potential to a new population of astroglial cells: cerebellum astroglia. As explained in the introduction, neurogenic potential of cortical astrocytes may be related to the fact that astrocytes and neurons of the forebrain share a same ancestral progenitor. Our question here is whether cerebellum astroglia neurogenic potential have a similar explanation. It is known that in the germinal niches of the cerebellum, (1) the ventricular zone (VZ) and (2) the rhombic lip (RL), progenitors with astroglial features give rise to all the glutamatergic and GABAergic neurons (Mori et al., 2006; Carletti and Rossi, 2007). Interestingly, several studies revealed that these cells can be considered as radial glial cells and additionally, as cortical RGCs, are bipotent progenitors for both interneurons and astrocytes of the cerebellum (Mori et al., 2006; Silbereis et al., 2009; Sudarov et al., 2011; Parmigiani et al., 2015). Our results concerning the neurogenic potential of cerebellar astroglia can therefore be explained in light of these studies. We assume that cerebellar astroglia keep a molecular or genetic signature of the neurogenic RGCs from which they are generated. Another important point to highlight is the phenotype of the iNs
generated from cerebellum astroglia. We observed that both Ascl1 and Neurog2 induced the conversion of cerebellum astroglia into mainly GABAergic neurons. On the other hand, in parallel experiments on cortical astroglia, Ascl1 and Neurog2 induced GABAergic and glutamatergic phenotypes respectively. These results suggest that these neurogenic TFs have different instructing function on different astroglial cell populations. Ascl1 and Neurog2 role is still poorly studied in the hindbrain. Nevertheless, some studies showed that both TFs are expressed in the progenitors of the VZ that give rise to distinct GABAergic interneurons of the cerebellum (Zordan et al., 2008; Sudarov et al., 2011; Florio et al., 2012) suggesting that cerebellar astroglia may keep some of the molecular signatures as progenitors of the cerebellum.

**Astroglia reprogramming in vivo: an incomplete puzzle**

In vitro results point to the fact that the source cell origin may be more important than expected in the determination of the iNs phenotypes. Yet, many primordial questions still remain unresolved, especially concerning environmental factors. At the beginning of our research, we raised many questions and proposed some experiments in order to better understand astroglia conversion into neurons (Chouchane and Costa, 2012). One of our main concerns was to study the neuronal differentiation of reprogrammed astrocytes in vivo. We considered that studying the integration of iNs inside an already established system would provide us with valuable information about the complete reprogramming process. We proposed that targeting the expression of TFs directly in resident astrocytes or transplanting astrocytes already transduced with neurogenic TFs into the brain could be a good approach. Following our report, many studies used *in situ* targeting of resident astroglial cells to convert them into neurons. Quiescent and reactive astrocytes in the cortex, striatum and spinal cord have been targeted with virus expressing several transcription factors, amongst which Ascl1, Brn2, Sox2, NeuroD1, and
reported to convert into neurons (Guo et al., 2014; Heinrich et al., 2014; Su et al., 2014). Despite
the demonstration that iNs expressed the early neuronal marker doublecortin (DCX) and only
occasionally more mature markers, such as GABA and NeuN, a general limitation of those
studies is the lack of an extensive characterization of reprogrammed cells. Moreover, we do not
yet have clues about the role of the host environment in which cell fate conversion takes place.
Some studies revealed that tissue damage prior to cell reprogramming has a boosting effect by
probably inducing plasticity in the cellular context (Guo et al., 2014; Su et al., 2014). Other
considerable observations suggest that a regional difference of permissiveness for glia to neuron
conversion seems to exist between striatal and cortical astrocytes (Magnusson et al., 2014). All
these studies raised the questions of whether different conversion efficiencies are due to cell
intrinsic differences or/and result from environmental influences that promote or hinder cell fate
conversion.

In our study, we decided to use a different approach: isolation of astroglial cell in vitro,
transfection with genes of interest and transplantation into a neurogenic and a non-neurogenic
environment. We suggest that such approach contributes to control important variables: 1) which
cell population is, in fact, reprogrammed; 2) whether transplanted cells survive in the host
environment and to which extent the phenotype of the cell (astroglia versus iN) affect survival;
and 3) whether cells originated from a complete different brain region can contribute neurons
with similar properties of cells in the region of integration.

Our results show that cortical but not cerebellar astroglia nucleofected with Neurog2 and
transplanted in the postnatal cerebral cortex integrate as iNs. Not only this, cortical derived iNs
were very similar to endogenous pyramidal cells found mostly in layer II of the cortex. Interestingly, some cells send proper projections to the contralateral cortical hemisphere and
possibly subcortical targets through the internal capsule. Neurog2 has been shown to be sufficient and necessary in the fate specification of glutamatergic neurons during telencephalon development (Fode et al., 2000; Schuurmans et al., 2004; Mattar et al., 2008). Not only this, it is also implicated in other developmental processes of cortical projection neurons such as their radial migration by regulating neural polarity and development of leading process (Hand et al., 2005). Interestingly also, it has been shown as discussed earlier and confirmed by our in vitro results, that Neurog2 is sufficient to convert other cell types than cortical progenitors such as cortical astrocytes into glutamatergic iNs (Heinrich et al., 2010). In light of these establishments, we can therefore explain our results concerning the in vivo integration of Neurog2-iNs generated from cortical astroglia. We suggest that Neurog2 promotes the integration of pyramidal cell like iNs generated from cortical astroglia in the cortex of the host brain. Our results corroborate with a previous study showing that NeuroD1, a gene acting downstream of Neurog2, could convert reactive astrocytes in situ into Tbr1+ iNs (Guo et al., 2014). Nevertheless, neuronal morphology of iNs was not as developed as observed in our study. This can be explained by the fact that Neurog2 plays an important role in the dendritic morphology of cortical pyramidal cells. In fact, it has been shown that phosphorylation of a single tyrosine residue (T241) of Neurog2 is essential and sufficient for specifying the dendritic morphology of these cells (Hand et al., 2005). We assume that Neurog2 phosphorylation may happen in the host-developing cortex and permits therefore the development of a mature pyramidal morphology. Our results, however, do not imply that Neurog2 is sufficient for in vivo integration of iNs given that transplantation of these same cells in adult cortex (data not shown) did not result in any neuronal integration. On the other hand, it suggests that the environment of the developing cortex is offering other necessary
ingredients for neural integration of iNs. Nevertheless, our results illustrate once more the instructive and/or permissive strength of Neurog2 on cortical cells in a cortical context.

On the other hand, we observed that Neurog2, unlike in vitro, had no neuronal instruction on cerebellar astroglia in the cortex. This may be explained by the following two conflicting phenomena (1) Neurog2 induces GABAergic fate in the cerebellar progenitors as well as, illustrated in our study, in cerebellar astroglia (Zordan et al., 2008) (2) In the developing telencephalon Neurog2 represses the differentiation program of GABAergic neurons (Kovach et al., 2013). Thus, we suggest that these two paradoxical events may impede the neuronal differentiation from cerebellar astroglia nucleofected with Neurog2 in the cortex. This observation theorizes that the cell type and the context are equally important in the fate determination of the iNs. Interestingly, some studies propose that Neurog2 have a permissive action that must act in combination with other factors to specify neuronal phenotypes (Parras et al., 2002). In light of this statement we can also assume that in a cortical context Neurog2 does not find the appropriate factors for the differentiation of cerebellar astroglia into iNs.

Ascl1 did not induce neuronal integration of cortical astroglia in the cortex, and did it at a very low extent with cerebellar astroglia. In 2014, Heinrich et al., assessed whether Sox2 and Ascl1 could induce reprogramming of reactive astroglia in the cortex following an acute stab wound lesion. They found that expression of both TFs, but also of only Sox2 induced the generation of DCX+ cells (Heinrich et al., 2014). In a previous in vitro study, mature neurons could be obtained only if Ascl1 was combined with Dlx (Berninger et al., 2007; Heinrich et al., 2010). These data corroborate with our suggestion that Ascl1 may not be sufficient or not instructive enough to complete a full reprogramming event in vivo. Moreover, corroborating with our results in vitro, we showed that even though Ascl1 induces more GABAergic like iNs, a
considerable fraction of the cells were glutamatergic. Thus, in our case, Ascl1 must probably be combined with other developmental TFs to generate interneurons in the host cortex.

Would a more permissive region enable Ascl1 to have a stronger instruction? In order to investigate this possibility we transplanted Ascl1-transduced cells in the SVZ of the developing brain. We observed that both astroglial cells behaved as endogenous progenitors, migrated though the RMS and generated OB interneurons. This suggests that a neurogenic permissive region could bring some additional ingredients, absent in the cortex, which permit or induce the generation of iNs from Ascl1-transduced astroglia. Corroborating with our data, some studies showed that Ascl1 is expressed in neuronal progenitors of the SVZ that give rise to interneurons in the postnatal and adult olfactory bulb (Parras et al., 2004b; Kim et al., 2008, 2011). Therefore, we could assume that the combination of endogenous expression of Ascl1 and the permissive environment of the SVZ had sufficient influence to induce the generation of typical forebrain neurons from cerebellar astroglia.

Conclusion: how similar is the copy to the authentic?

The neurogenic potential of astrocytes is nowadays doubtless. Our study in sum with others, lead to say that cell intrinsic as well as environmental factors play a determinant role in the fate decision of the newly induced neuron. In this study we could show that different astroglial populations give rise to different populations of iNs. This important piece of data can eventually explain the low efficiency of cortical astrocytes conversion to dopaminergic neurons obtained in previous studies (Addis et al., 2011). This opens the questions of whether isolating astrocytes from the midbrain, a region rich in dopaminergic neurons, would favor a more efficient generation of this type of cells. Choosing the right combination of TFs would also be an important step for generating a specific subtype of neuron considering for example that
GABAergic interneurons subtypes express different subset of TFs (Wonders and Anderson, 2006; Hernández-Miranda et al., 2010).

In sum, the question titling this paragraph is only partially responded. So far, we could realize that what may be important for a full iN integration is a harmonious interaction between the transcriptional instruction, the molecular intrinsic context of the source cell and the environmental cues. More studies should be done in order to understand the full reprogramming process and integration of iNs in already established systems.
REFERENCES


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