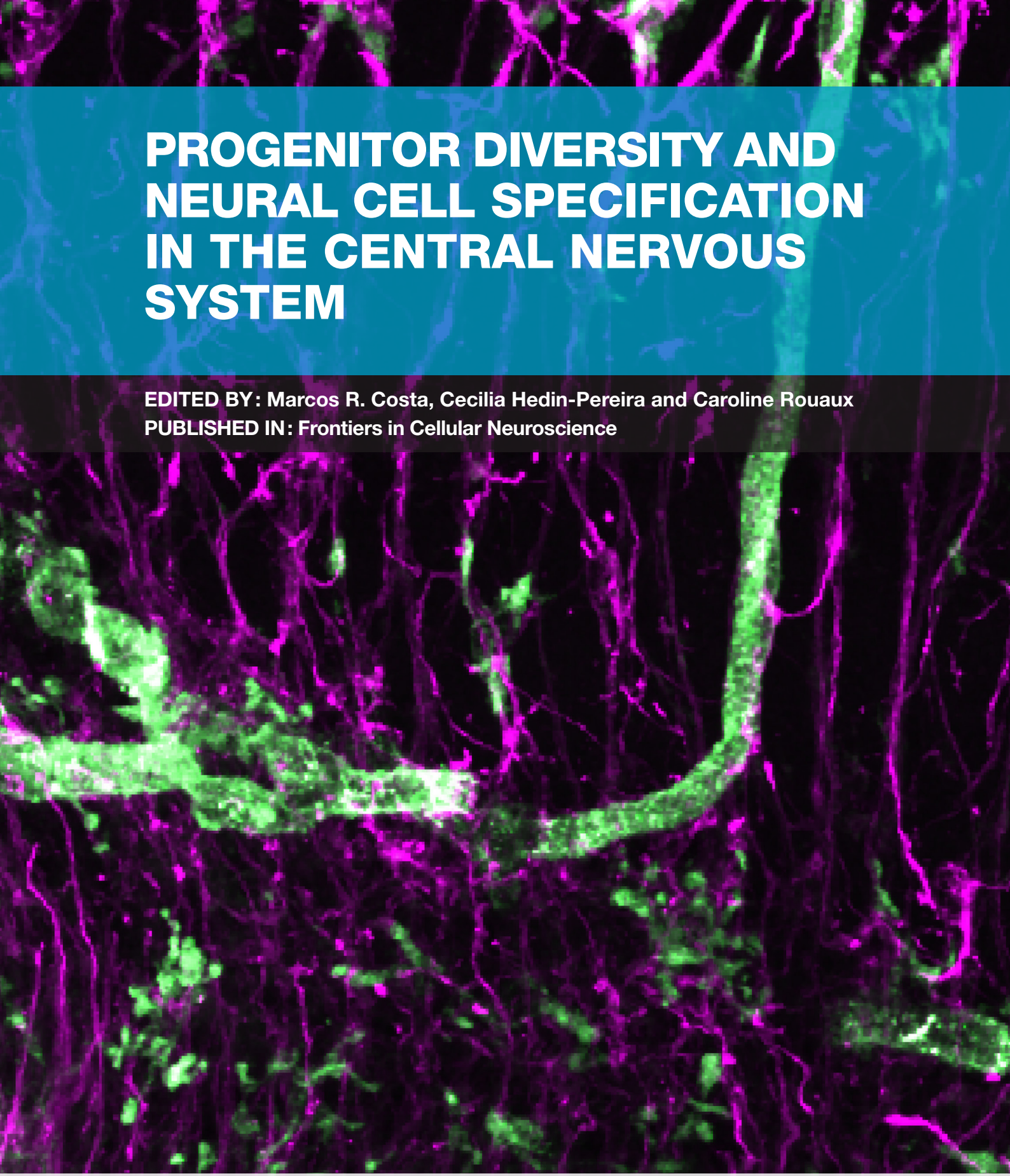
PROGENITOR DIVERSITY AND NEURAL CELL SPECIFICATION IN THE CENTRAL NERVOUS SYSTEM

EDITED BY: Marcos R. Costa, Cecilia Hedin-Pereira and Caroline Rouaux
PUBLISHED IN: Frontiers in Cellular Neuroscience



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88919-683-8
DOI 10.3389/978-2-88919-683-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.

Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.

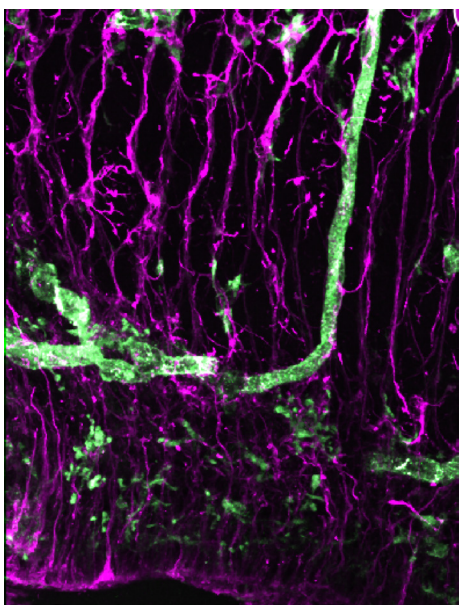
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

PROGENITOR DIVERSITY AND NEURAL CELL SPECIFICATION IN THE CENTRAL NERVOUS SYSTEM

Topic Editors:
Marcos R. Costa, Federal University of Rio Grande do Norte, Natal, Brazil
Cecilia Hedin-Pereira, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
Caroline Rouaux, Université de Strasbourg, France



The cover image shows an optical sectioning of immunolabeled postnatal mouse subventricular zone and cortical parenchyma. GFAP-positive radial glia processes (magenta) and F4/80-positive blood vessels (green), spanning the entire cortical parenchyma, serve as scaffolds for the migration of F4/80-positive microglial cells (green) within the cortical parenchyma.

Image taken from: Xavier et al., (2015). Ontogeny of CX3CR1-EGFP expressing cells unveil microglia as an integral component of the postnatal subventricular zone. Front. Cell. Neurosci. 9,37. doi: 10.3389/fncel.2015.00037.

The central nervous system continuously perceives, integrates, processes and generates information. These complex functions rely on the detailed elaboration of its cellular network and on the myriads of individual, highly differentiated and specialized cell types, classically subdivided into neurons, astrocytes and oligodendrocytes. The specification of these individual populations begins early during development with less differentiated, yet already partly restricted, progenitor cells. Anatomically located in dedicated germinative niches, neural progenitors perceive the influence of diffusible molecules of various natures and concentrations. These signals result in the initial specialization of cohorts of progenitors that express unique combinations of tran-

scription factors. It is now clearly established that both extrinsic and intrinsic signals act in concert to determine the fate potentials of these progenitor cohorts. This limitation increases over time, adult neural progenitors being more restricted than their developmental counterparts. Nevertheless, recent data have shown that the fate restriction of neural progenitors, as well as that of their progenies, can be overwritten upon selected intrinsic factor expression, not only during development but also in adulthood. This e-book is a collection of original research studies along with review articles that, together, provide insights into the vast spatiotemporal diversity of neural progenitors, and the various factors that govern their fate potential.

Citation: Costa, M. R., Hedin-Pereira, C., Rouaux, C., eds. (2015). Progenitor Diversity and Neural Cell Specification in the Central Nervous System. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-683-8

Table of Contents

frontiers in Cellular Neuroscience

EDITORIAL
published: 09 September 2015
doi: 10.3389/fncel.2015.00358

Editorial: Progenitor diversity and neural cell specification in the central nervous system

Caroline Rouaux[1], *Cecília Hedin-Pereira*[2] *and Marcos R. Costa*[3]*

[1] *Institut National de la Santé et de la Recherche Médical U1118, Fédération de Médecine Translationnelle de Strasbourg, Université de Strasbourg, Strasbourg, France,* [2] *Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil,* [3] *Brain Institute, Federal University of Rio Grande do Norte, Natal, Brazil*

Keywords: central nervous system, cellular diversity, progenitor cells, neurons, glial cells, fate specification

The central nervous system (CNS) harbors an enormous diversity of neuronal and glial cell types, which can be identified according to morphological, chemical, and electrical properties. This variety of cell types is generated from progenitor cells located in different germinative niches, where distinct molecular signalings prompt distinctive transcription factors expression. In the last two decades, it has been acknowledged that such progenitor diversity is important for the generation of different subtypes of neurons, astrocytes and oligodendrocytes. Genetic fate-mapping studies have provided direct evidence for the contribution of separate cohorts of progenitor cells to generate individual subtypes of neurons and/or glial cells. Additionally, genetic deletions of single transcription factors and forced expression of ectopic transcription factor genes have pointed out the leading role of such molecules on the specification of different, individual cell types. However, other sources of data indicate that the environment plays important roles in cellular specification during CNS development, eventually overriding the influence of early transcription factors expression. These observations suggest that genetic determinants for both neuronal and glial specification would be changeable. However, the time-window during which neuronal and glial lineage genetic programs could be overwritten by external signals remains to be determined, along with exact signals that could perform such a fate modification. This research topic gathers a number of articles highlighting the role of a wide panel of intrinsic and extrinsic factors that contribute to the generation of such diversity. More importantly, the possible interplay between intrinsic and extrinsic factors is here considered in a new light, in which intrinsic and extrinsic signals act not only in concert, but also within a critical time-window, to determine cell fate.

Focusing on the specification of glutamatergic and GABAergic neurons in the developing cerebral cortex, Costa & Müller and Brandão & Romcy-Pereira discuss the evidences supporting the existence of genetically committed progenitor populations contributing to the generation of discrete neuronal subtypes (Costa and Muller, 2014; Brandão and Romcy-Pereira, 2015). In the first article, the authors discuss classical experiments of cell-lineage tracing in the developing cerebral cortex and try to conciliate data from these first studies with more recent genetic fate-mapping works that suggest the existence of multipotent and fate-restricted progenitors for glutamatergic cortical neurons. The second article reviews evidences for the generation of discrete GABAergic cortical neurons from separate progenitor territories. In addition, both articles discuss the important role of environmental signals and activity-dependent mechanisms in regulating the fate of cortical neurons, supporting the view that intrinsic genetic mechanisms are not the sole determinants of neuronal phenotypes. According to this notion, the article by Vasconcelos and Castro highlights a previously unexpected degree of complexity provided by the oscillatory expression of proneural genes in response to external signals (Vasconcelos and Castro, 2014).

This complex scenario of interactions is not restricted to developmental neurogenesis but extends to adulthood. Sequerra discusses the maintenance of morphogenetic gradients in the adult

OPEN ACCESS

Edited and reviewed by:
Egidio D'Angelo, University of Pavia, Italy

***Correspondence:**
Marcos R. Costa, mrcosta@neuro.ufrn.br

Received: *07 August 2015*
Accepted: *26 August 2015*
Published: *09 September 2015*

Citation:
Rouaux C, Hedin-Pereira C and Costa MR (2015) Editorial: Progenitor diversity and neural cell specification in the central nervous system. Front. Cell. Neurosci. 9:358. doi: 10.3389/fncel.2015.00358

subventricular zone (SVZ), regulating the expression of genes involved in neuronal specification (Sequerra, 2014). He also points out the higher complexity of the adult neurogenic milieu as compared to the embryonic, which could widen the range of possible cellular phenotypes. In consonance with this article, Bernal and colleagues show mechanisms of extrinsic lipid signaling regulating the expression of transcription factors involved in the balance between neural progenitor cell proliferation and differentiation in the adult SVZ (Bernal et al., 2015).

Neuronal diversity may only be the tip of the iceberg, considering the emerging complexity of glial cells as revealed in the articles by Schitine et al. and Tomassy and Fossati (Tomassy and Fossati, 2014; Schitine et al., 2015). Astrocyte heterogeneity, regarding morphology and functions, is still a matter of debate in the literature. The first article reviews the diverse origins of these cells and the possibility that this may reflect in heterogenous astrocyte types, making the specific contributions of these cells to the brain functions even more complex (Schitine et al., 2015). The authors also review the morphological changes and peculiar responses of astrocytic subpopulations associated to different diseases, unveiling the relation between astrocytic diversity and environmental differences in pathological conditions. Supporting the notion that extrinsic signals may be pivotal in controlling astroglial progenitor behavior through the regulation of intrinsic genetic mechanisms, Stipursky et al., in their original paper, reveal the role TGF-beta in controlling glial progenitor differentiation (Stipursky et al., 2014). The authors show that TGF-beta down regulates the expression of FoxG1 and causes premature transformation of radial glial cells, which also leads to alterations in the laminar distribution of neurons. Likewise, Schmid et al. show that the disruption of the radial glia scaffold upon genetic deletion of alpha-catenin leads to a severe disruption of the laminar organization of cortical neurons, mimicking the human subcortical band heterotopia (Schmid et al., 2014).

The review by Tomassy and Fossati discusses the different origins of oligodendrocyte progenitors and puts forward the appealing question as to which extent this diverse origin could result in the existence of diverse populations of mature oligodendrocytes (Tomassy and Fossati, 2014). Interestingly, the authors also discuss recent evidence indicating that neuronal populations modulate the functions of oligodendrocytes, regulating for instance their capacity to produce myelin.

Not only cells from the neural lineage, but also microglial cells emerge as diverse and plastic players in the nervous system. The original article by Xavier et al. reveals a rich heterogeneous microglial population within the SVZ neurogenic niche, raising intriguing possibilities of interactions between microglia and neural progenitor cells (Xavier et al., 2015).

Therefore, this issue covers progenitor diversity in the generation of neurons (embryonic and postnatal), astrocytes and oligodendrocytes, their sites of origin and the spatio-temporal cues regulating their appearance and cell fate determination. The issue also highlights the interplay between intrinsic and extrinsic factors on cell type commitment, opening new perspectives to our understanding of neurodevelopmental disorders, CNS response to lesions, and possible regenerative approaches.

Acknowledgments

CR is the holder of a European Research Council (ERC) Starting Grant ("CorticALS": No. 639737); CH is supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ); MC is supported by CNPq, CAPES and Fundação de Amparo à Pesquisa do Rio de Grande do Norte (FAPERN).

References

Bernal, C., Araya, C., Palma, V., and Bronfman, M. (2015). PPARbeta/delta and PPARgamma maintain undifferentiated phenotypes of mouse adult neural precursor cells from the subventricular zone. *Front. Cell. Neurosci.* 9:78. doi: 10.3389/fncel.2015.00078

Brandão, J. A., and Romcy-Pereira, R. N. (2015). Interplay of environmental signals and progenitor diversity on fate specification of cortical GABAergic neurons. *Front. Cell. Neurosci.* 9:149. doi: 10.3389/fncel.2015.00149

Costa, M. R., and Muller, U. (2014). Specification of excitatory neurons in the developing cerebral cortex: progenitor diversity and environmental influences. *Front. Cell. Neurosci.* 8:449. doi: 10.3389/fncel.2014.00449

Schitine, C., Nogaroli, L., Costa, M. R., and Hedin-Pereira, C. (2015). Astrocyte heterogeneity in the brain: from development to disease. *Front. Cell. Neurosci.* 9:76. doi: 10.3389/fncel.2015.00076

Schmid, M. T., Weinandy, F., Wilsch-Brauninger, M., Huttner, W. B., Cappello, S., and Gotz, M. (2014). The role of alpha-E-catenin in cerebral cortex development: radial glia specific effect on neuronal migration. *Front. Cell. Neurosci.* 8:215. doi: 10.3389/fncel.2014.00215

Sequerra, E. B. (2014). Subventricular zone progenitors in time and space: generating neuronal diversity. *Front. Cell. Neurosci.* 8:434. doi: 10.3389/fncel.2014.00434

Stipursky, J., Francis, D., Dezonne, R. S., Bérgamo de Araújo, A. P., Souza, L., Moraes, C. A., et al. (2014). TGF-beta1 promotes cerebral cortex radial glia-astrocyte differentiation *in vivo*. *Front. Cell. Neurosci.* 8:393. doi: 10.3389/fncel.2014.00393

Tomassy, G. S., and Fossati, V. (2014). How big is the myelinating orchestra? Cellular diversity within the oligodendrocyte lineage: facts and hypotheses. *Front. Cell. Neurosci.* 8:201. doi: 10.3389/fncel.2014.00201

Vasconcelos, F. F., and Castro, D. S. (2014). Transcriptional control of vertebrate neurogenesis by the proneural factor Ascl1. *Front. Cell. Neurosci.* 8:412. doi: 10.3389/fncel.2014.00412

Xavier, A. L., Lima, F. R., Nedergaard, M., and Menezes, J. R. (2015). Ontogeny of CX3CR1-EGFP expressing cells unveil microglia as an integral component of the postnatal subventricular zone. *Front. Cell. Neurosci.* 9:37. doi: 10.3389/fncel.2015.00037

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

MINI REVIEW ARTICLE
published: 02 December 2014
doi: 10.3389/fncel.2014.00412

Transcriptional control of vertebrate neurogenesis by the proneural factor Ascl1

Francisca F. Vasconcelos and Diogo S. Castro*

Molecular Neurobiology, Instituto Gulbenkian de Ciência, Oeiras, Portugal

Edited by:
Marcos R. Costa, Federal University of Rio Grande do Norte, Brazil

Reviewed by:
Joao R. L. Menezes, Universidade Federal do Rio de Janeiro, Brazil
Carol Schuurmans, University of Calgary, Canada

***Correspondence:**
Diogo S. Castro, Molecular Neurobiology, Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, P-2780-156 Oeiras, Portugal
e-mail: dscastro@igc.gulbenkian.pt

Proneural transcription factors (TFs) such as Ascl1 function as master regulators of neurogenesis in vertebrates, being both necessary and sufficient for the activation of a full program of neuronal differentiation. Novel insights into the dynamics of Ascl1 expression at the cellular level, combined with the progressive characterization of its transcriptional program, have expanded the classical view of Ascl1 as a differentiation factor in neurogenesis. These advances resulted in a new model, whereby Ascl1 promotes sequentially the proliferation and differentiation of neural/stem progenitor cells. The multiple activities of Ascl1 are associated with the activation of distinct direct targets at progressive stages along the neuronal lineage. How this temporal pattern is established is poorly understood. Two modes of Ascl1 expression recently described (oscillatory *vs.* sustained) are likely to be of importance, together with additional mechanistic determinants such as the chromatin landscape and other transcriptional pathways. Here we revise these latest findings, and discuss their implications to the gene regulatory functions of Ascl1 during neurogenesis.

Keywords: Ascl1/Mash1, neurogenesis, proneural gene, transcription, Notch signaling

INTRODUCTION

Neurogenesis in the developing mammalian brain is a highly complex process that requires neural progenitor cells to progress through a succession of distinct cellular states. These developmental steps have been particularly well defined in the embryonic telencephalon, where distinct types of progenitors have been identified during the neurogenesis period (Kriegstein and Alvarez-Buylla, 2009). Radial glial (RG) cells in the ventricular zone (VZ) have characteristic features of neural stem/progenitor cells, as they self-renew by asymmetric division and have the potential to differentiate into both neurons and glial cells (Götz and Huttner, 2005). Upon cell division, RG cells give rise to another RG cell and either a post-mitotic neuron, or an intermediate progenitor that can divide further to amplify the lineage (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Pilz et al., 2013). These various progenitor types are differentially segregated between two germinal layers. Most RG cells divide at the apical surface of the VZ, while most intermediate progenitors divide more basally in the sub-ventricular zone (SVZ).

Proneural transcription factors (TFs) of the bHLH family including Ascl1 (also called Mash1) and members of the Neurogenin family are the main regulators of vertebrate neurogenesis. Both gain and loss-of-function analyses have shown they are both required and sufficient to induce a complete program of neuronal differentiation (Bertrand et al., 2002; Wilkinson et al., 2013). While genetic ablation of Ascl1 in mice results in neural developmental defects associated with reduced generation of neurons (Casarosa et al., 1999; Horton et al., 1999; Marin et al., 2000), overexpression of this TF in neural progenitors induces cell-cycle exit and full neuronal differentiation and specification (Nakada et al., 2004; Castro et al., 2006; Berninger et al., 2007b; Geoffroy et al., 2009). In line with its master regulatory role in the neuronal lineage, recent studies have revealed the ability of Ascl1 to convert various non-neural somatic cells (e.g., fibroblasts) into induced neurons (Berninger et al., 2007a; Vierbuchen et al., 2010; Karow et al., 2012), renewing interest in understanding how this important TF works at the molecular level.

PRONEURAL FACTORS AND THE NOTCH SIGNALING PATHWAY

While driving neuronal differentiation, proneural factors also activate the Notch signaling pathway in neighboring progenitors, a process that is highly reminiscent of the lateral inhibition model proposed for *Drosophila* neurogenesis (Louvi and Artavanis-Tsakonas, 2006). Proneural factors directly activate the transcription of Notch ligands such as Dll1 (Castro et al., 2006; Henke et al., 2009), which interact with a transmembrane Notch receptor in neighboring cells. This event results in the cleavage and release of the Notch intracellular domain (NICD) from the cell membrane into the nucleus, where it forms a complex with the DNA-binding TF Rbpj and additional coactivators. Direct targets of this complex include the bHLH transcriptional repressors Hes1 and Hes5, which in turn bind to the promoters of proneural genes, repressing their expression and thereby inhibiting neuronal differentiation (Kageyama et al., 2005). Thus, proneural genes are both regulators and regulated by the Notch

signaling pathway, a network that functions in parallel to the differentiation program to keep—even if only transiently—adjacent cells undifferentiated. Such lateral inhibition results in proneural factors being expressed in a "salt-and-pepper" pattern and prevents simultaneous differentiation of all progenitors, ensuring that an appropriate number is maintained during embryonic development.

A REVISED VIEW OF LATERAL INHIBITION IN VERTEBRATES

It is known that in a variety of cell types (e.g., fibroblasts), Hes1 expression levels regularly alternate over time due to its ability to behave as an intrinsic oscillator (Hirata et al., 2002; Masamizu et al., 2006; Kobayashi et al., 2009). Hes1 represses its own promoter in a feedback mechanism, which associated with short-lived Hes1 transcript and protein, results in autonomous oscillations of its expression with a 2–3 h period. It was recently shown that Hes1 also oscillates in neural progenitors (Shimojo et al., 2008). Because Hes1 and proneural factors display complementary patterns of expression, one possibility is that oscillation of Hes1 results in the oscillation of proneural genes. This is indeed the case for both Neurog2 and Ascl1, as shown by a variety of approaches (Shimojo et al., 2008; Imayoshi et al., 2013). Most notably was the generation of transgenic mice bearing a bacterial artificial chromosome (BAC) containing the Ascl1 regulatory regions driving the expression of Ascl1 fused to either luciferase or green fluorescent protein (GFP), where the activity of the reporter monitors faithfully the expression of the endogenous Ascl1 protein (Imayoshi et al., 2013). Proneural proteins are direct activators of Dll1, resulting in its oscillation and mutual activation of Notch signaling in neighboring progenitors (Castro et al., 2006; Shimojo et al., 2008). There is evidence that Hes1 activity both promotes and inhibits the cell cycle and therefore its oscillation may be required for cell proliferation (Castella et al., 2000; Hartman et al., 2004; Sang et al., 2008). Oscillatory expression of TFs with lineage specification functions has also been observed in other systems, and the function of such oscillations is still a matter of debate. As opposed to steady-state mode, oscillatory expression may generate heterogeneity of response of an apparently homogeneous progenitor cell population to a given input signal. In addition, different inputs may affect different parameters of the oscillation (e.g., period, amplitude) and, hence, trigger different functional outcomes (Mengel et al., 2010; Pina et al., 2012; Sequerra et al., 2013).

In light of these recent findings, the "salt-and-pepper" expression pattern of proneural factors is perceived as a snapshot of a dynamic mode of expression. Proneural factors are therefore expressed in neural progenitors at different stages of differentiation, and not only in committed progenitors that will soon become post-mitotic, as was previously thought (**Figure 1**).

OSCILLATORY VS. SUSTAINED MODE OF EXPRESSION

Time-lapse imaging of individual neural progenitors in culture revealed that during neuronal induction, Ascl1 and Neurog2 switch from an oscillatory to a sustained mode of expression after the last cell division, followed by the expression of the neuronal marker doublecortin 6–8 h later (Shimojo et al., 2008; Imayoshi et al., 2013). This suggested the ability of proneural factors to trigger differentiation may require their expression to be sustained. Such a causal link was established upon the use of an optogenetic approach where Ascl1 expression is regulated by a light-switchable transactivator (Imayoshi et al., 2013). This system was introduced in an Ascl1 null background to investigate the consequence of different dynamics of Ascl1 expression induced by different pulses of light. An oscillatory mode with a 3 h periodicity increased proliferation, compensating the lower proliferation rate observed in Ascl1 null progenitors in culture. By contrast, sustained expression of Ascl1 for 6 h resulted in cell-cycle exit and neuronal differentiation. The same approach used in slice cultures of the dorsal telencephalon where Ascl1 is usually expressed at very low levels reached similar conclusions. What determines the transition to a sustained mode of Ascl1 expression remains an open question, but it was suggested that varying levels of NICD may play a role in this step (Imayoshi et al., 2013).

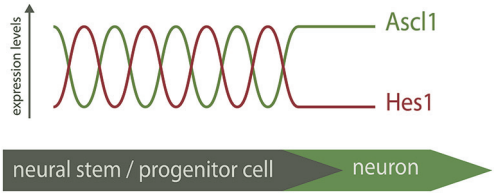


FIGURE 1 | Distinct modes of expression of Ascl1 in neural stem/progenitor cells. Hes1 and Ascl1 oscillate in neural progenitors with a 2–3 h period. The inverse correlation of expression of Hes1 and Ascl1 proteins suggest these TFs oscillate out-of-phase. At the onset of differentiation, Hes1 expression is extinguished and Ascl1 expression becomes sustained. The "salt-and-pepper" expression of Ascl1 in the neural tube is therefore the result of a dynamic pattern of expression, and Ascl1 expression is not necessarily an indicative of differentiation.

Overall, these findings explain why most evidence based on Ascl1 gain-of-function (sustained expression) points to a role in promoting differentiation (with concomitant cell cycle-exit) of progenitors (Nakada et al., 2004; Castro et al., 2006; Geoffroy et al., 2009). By contrast, knock-down of Ascl1 levels upon expression of sequence-specific shRNA decreased proliferation of neural progenitors in culture (Castro et al., 2011), while acute knock-out of Ascl1 in the ventral telencephalon caused premature cell-cycle withdrawal of both VZ and SVZ progenitors (Castro et al., 2011; Pilz et al., 2013), suggesting a role in maintaining proliferation in both RG cells and intermediate progenitors.

CHARACTERIZATION OF Ascl1 TARGET GENES

Proneural factors function primarily as transcriptional activators, binding in heterodimeric complexes with bHLH E-proteins to the regulatory regions of their target genes (Bertrand et al., 2002). A major leap forward in our understanding of the molecular mechanisms underlying Ascl1 function has been the progressive characterization of its transcriptional program. The advent of genomic approaches based on chromatin immunoprecipitation allowed the characterization of a large number of genes directly controlled by Ascl1 in a neurogenesis context. Two studies have

used chromatin immunoprecipitation followed by hybridization to DNA arrays (ChIP-chip), or massive parallel sequencing (ChIP-seq), to characterize the Ascl1 transcriptional program in ventral telencephalon and dorsal spinal cord of the developing mouse embryo, respectively (Castro et al., 2011; Borromeo et al., 2014). A common theme of both studies was the diversity of biological functions of Ascl1 target genes, indicating Ascl1 directly controls various stages of neurogenesis, including neuronal differentiation, migration, axon guidance and synapse formation. In the ventral telencephalon, the region of the murine brain with the largest SVZ during the neurogenic period, the pro-proliferation activity of Ascl1 extends beyond the maintenance of Notch/Hes1 oscillations through activation of Dll1, and includes the activation of genes required for cell-cycle progression such as E2F1 and Foxm1 (Castro et al., 2011).

The Ascl1 targets are associated with progressive functions along neurogenesis and have distinct onsets of expression along the neuronal lineage in this brain region, as indicated by their expression patterns. The expression of the largest group mirrors that of Ascl1 itself in both germinal layers and includes genes expected to promote cell proliferation (e.g., E2F1), whereas that of a smaller but significant group of targets is restricted to the mantle zone (e.g., MAP2) (**Figure 2**).

All the above observations resulted in a model whereby Ascl1 sequentially promotes proliferation and differentiation of progenitors along the neuronal lineage, with the concomitant activation of partially distinct transcriptional programs. This reconciles the classical view of Ascl1 as a differentiation factor with the fact that this proneural factor is expressed mostly in cycling cells. The different kinetics of Ascl1 targets may result from the integration of various mechanistic determinants, including its two modes of expression as briefly discussed below.

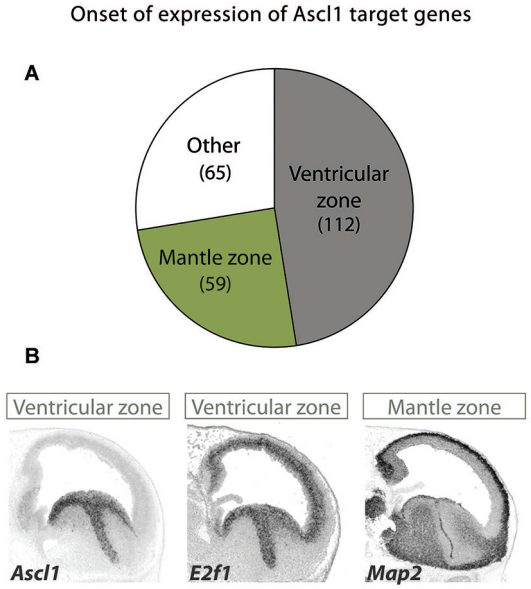


FIGURE 2 | Different patterns of expression of Ascl1 target suggest distinct kinetics of gene activation. (A) A large screening of expression patterns of Ascl1 direct targets in embryonic mouse ventral telencephalon revealed distinct patterns of expression (Castro et al., 2011). Three distinct groups can be identified. Genes in the largest set display an onset of expression at the VZ (which includes mostly neural stem cells), while in another the onset occurs at the mantle zone (here defined as including SVZ and all other outer layers, comprising intermediate progenitors and post-mitotic neurons). A third group includes genes with a uniform or more complex pattern of expression. Number of genes in each set is indicated in between brackets. **(B)** Examples of two Ascl1 direct targets with distinct patterns of expression revealed by *in situ* hybridization on sagittal sections of mouse telencephalon at E13.5 stage of embryonic development. Expression of the proliferation gene E2f1 in VZ/SVZ mirrors that of Ascl1. By contrast, the neuronal differentiation gene MAP2 is expressed in post-mitotic neurons. Images are from Allen Developing Mouse Brain Atlas.[1]

REGULATION OF Ascl1 PROTEIN LEVELS AND TRANSCRIPTIONAL ACTIVITY

One simple possibility is that increasing levels of Ascl1 activity, resulting from changes in the Ascl1 mode of expression (oscillatory *vs.* sustained), protein levels and/or its transcriptional function, will result in the sequential activation of promoters with an increasing threshold of response. Although not formally demonstrated, it is likely that some Ascl1 targets respond differently to the oscillatory or sustained expression of Ascl1. One possible model invokes the function of a feed-forward-loop (FFL), a motif highly enriched in transcriptional networks. A variant of such motif called coherent FFL (whereby one TF activates another TF, and both co-activate target genes) has been shown to allow for a discriminated response of the target gene triggered by transient or sustained input signals from the first TF (Shen-Orr et al., 2002; Mangan and Alon, 2003). Considering the large number of TFs found amongst Ascl1 targets, it would be of interest to investigate if any may establish with this proneural factor such a network motif, providing a mechanistic basis for differential activation of Ascl1 targets upon its two modes of expression.

Some observations suggest Ascl1 protein levels may also play a role at the onset of neuronal differentiation. Time-lapse imaging of Ascl1/luciferase expressing neural progenitors in culture revealed an increase in Ascl1 levels in 90% of the cells that undergo asymmetrical (neurogenic) cell division, against 30% of the cells that undergo a proliferative symmetric division (Imayoshi et al., 2013). Thus, although not being strictly required or sufficient, an increase in Ascl1 protein levels before cell division does bias cells towards the neuronal fate.

A few signaling pathways have been implicated in the regulation of Ascl1 protein levels in different cellular contexts (Sriuranpong et al., 2002; Viñals et al., 2004; Oishi et al., 2009). In the most striking example, varying Ascl1 protein levels regulated by retinoic acid, result in the generation of different types of neurons at the p3 domain of hindbrain and spinal cord (Jacob et al., 2013). In all cases studied however, it is unclear if and how Ascl1 levels affect the activation of its target genes. Thus, while all these results indicate that various pathways converge to control Ascl1 protein levels, their relevance to the differential regulation of subsets of Ascl1 targets remains to be explored.

[1] Available at: http://developingmouse.brain-map.org

Two recent studies provided evidence that Ascl1 function can be modulated by phosphorylation at multiple serine-proline sites. During cortical development, manipulating RAS/ERK signaling to abnormal high level induces a Neurog2 to Ascl1 switch of expression and modifies Ascl1 activity by direct phosphorylation. As a result, Ascl1 drives a glioblast-like differentiation program reminiscent of its function in oligodendrogenesis. Another work showed that Ascl1 phosphorylation is sensitive to levels of Cdk/Cdk inhibitors, and diminishes its ability to drive primary neurogenesis in *Xenopus* embryos, providing a direct link between the cell-cycle machinery and regulation of neurogenesis (Ali et al., 2014). It is currently not known how phosphorylation impacts the neurogenic activity of Ascl1. Possible mechanisms include differential binding to DNA (as shown with Neurog2 phosphorylation) (Ali et al., 2011), or co-factor recruitment. Strikingly, phosphorylation affects the ability of Ascl1 to up-regulate the late/differentiation targets Myt1 and neural β-tubulin, while having little effect on *Dll1* induction (Ali et al., 2014). Moreover, a differential effect is also observed on the ability of Ascl1 to transactivate the promoters of various target genes (Li et al., 2014). Differential sensitivity of promoters to Ascl1 phosphorylation may thus be one important mechanism determining which targets Ascl1 regulates in proliferating vs. differentiating progenitors.

IMPORTANCE OF CHROMATIN LANDSCAPE AND Ascl1 BINDING SITES

Distinct thresholds of response to Ascl1 may result from differences in requirements for chromatin remodeling across its target genes. Very little is known however, on the impact that Ascl1 and proneural factors in general may have on the chromatin landscape when regulating gene transcription. Expression of a dominant negative form of Brg1, a catalytic component of SWI/SNF chromatin remodeling complex, blocks neuronal differentiation of P19 cells mediated by Neurog3 (Seo et al., 2005). In addition, it inhibits Neurog3 activation of the promoter of *NeuroD2*, the paradigm of a late/differentiation target of Neurogenins. In spite of this suggestive example, the importance of chromatin remodeling to the overall temporal patterning of the transcriptional program downstream of proneural factors remains to be investigated.

The chromatin landscape can contribute to restrict the accessibility of a TF to its target sites. A study of Ascl1 mediated neuronal reprogramming has recently shown that Ascl1 binds to its *bona fide* target sites when ectopically expressed in fibroblasts, even to those located within closed chromatin context, as defined by FAIRE-seq (Wapinski et al., 2013). The term "on target" pioneer factor was coined to indicate the ability of Ascl1 to recognize its cognate binding sites when ectopically expressed, as opposed to other TFs in iPS reprogramming (Soufi et al., 2012). Although the ability to bind nucleosomal DNA may argue against a dominant role of the chromatin structure in controlling Ascl1 function, it remains possible that Ascl1 accessibility to its target sites may be different in proliferating vs. differentiating progenitors.

The affinity of a TF binding site, determined by the DNA sequence, can dictate the kinetics of response of its direct targets. A striking example of how such mechanism can establish the temporal pattern of a developmental program is the activation of pharyngeal genes by the forkhead factor PHA4 at different developmental stages in *Caenorhabditis elegans* (Gaudet and Mango, 2002). Binding site mutations result in abnormalities in the timing of target gene expression *in vivo*, according to the resulting affinity to PHA4 binding. Concerning bHLH TFs, and in addition to the two central bases of the E-box, which provide specificity to distinct factors, residues flanking the hexamer sequence contribute to modulate binding affinity (Blackwell and Weintraub, 1990; Fisher et al., 1993). A study investigating the regulation of the Dll1 gene by Ascl1 has shown that residues at each side and immediately adjacent to the CAGSTG E-box determine binding affinity of Ascl1 *in vitro*, and strength of response in transcriptional assays (Castro et al., 2006). Although a possibility, the contribution of varying affinities for its binding sites to the kinetics of Ascl1 targets remains to be investigated.

FUNCTIONAL INTERACTIONS WITH OTHER TRANSCRIPTIONAL NETWORKS

Transcriptional programs are at the intersection of multiple transcriptional networks. Thus, a comprehensive view of the dynamics of the Ascl1 program will require its integration within other transcriptional pathways operating in neural progenitors. Very few studies have so far identified functional interactions between Ascl1 and other TFs. The forkhead factor FOXO3 regulates neural stem cell maintenance and is required to preserve the neural stem cell pool in adult mice (Renault et al., 2009). Recently, it has been shown that FOXO3 inhibits Ascl1-induced neuronal differentiation in cultured neural progenitors and direct neuronal conversion in fibroblasts (Webb et al., 2013). Although the molecular basis for this antagonism is not yet clear, it is likely to make use of the large number of regulatory regions co-bound by both TFs, many of which regulate Notch pathway related genes. Also SOX1B factors (Sox1/2/3) have been previously shown to counteract proneural proteins in gain-of-function experiments in the chick neural tube (Bylund et al., 2003). Thus, the intertwining of transcriptional networks regulating neural progenitor maintenance and differentiation may be a recurrent feature to be explored in future studies.

One immediate example is the Notch pathway. Within the large number of Ascl1 targets identified in ventral telencephalon, the Rbpj consensus binding sequence is enriched at the vicinity of Ascl1 binding sites, specifically in targets that promote proliferation (Castro et al., 2011). Previous studies of neurogenesis in *Drosophila* provide important clues on how the two factors may interact at the molecular level (Nellesen et al., 1999; Cave et al., 2005). In co-bound regulatory regions with a specific cis-architecture, efficient transactivation is only achieved upon the simultaneous activation of both proneural and Notch pathways. A similar synergy between Ascl1 and Rbpj can be observed in transcriptional assays in murine cells (Cave et al., 2005), although it remains to be shown whether such interaction does take place in gene regulatory regions. RG cells can be distinguished from intermediate progenitors in ventral telencephalon based on their high levels of canonical Notch signaling

(Mizutani et al., 2007), thus in principle such a mechanism could be used to differentially activate Ascl1 targets in the two types of progenitors.

PERSPECTIVE

Proneural TFs such as Ascl1 have been seen as master regulators of the neuronal lineage that play an important regulatory role at the onset of differentiation. Recent findings have uncovered a far more complex picture in which Ascl1 plays sequential functions in proliferating and differentiating neural stem/progenitor cells, with the concomitant regulation of distinct target genes. How sub-sets of the Ascl1 transcriptional program are differentially activated along the neuronal lineage is poorly understood, and will certainly result from the combination of distinct mechanistic determinants. This important question will surely remain a subject of intense research for the foreseeable future.

ACKNOWLEDGMENTS

We thank A. Raposo for critically reading this manuscript. Our work is supported by grants PTDC/SAU-BID/117418/2010 and PTDC/NEU-NMC/0315/2012 from FCT and a Marie Curie CIG to DSC. FFV and DSC are recipients of SFRH/BD/51178/2010 and IF/00413/2012 from FCT.

REFERENCES

Ali, F. R., Cheng, K., Kirwan, P., Metcalfe, S., Livesey, F. J., Barker, R. A., et al. (2014). The phosphorylation status of Ascl1 is a key determinant of neuronal differentiation and maturation in vivo and in vitro. *Development* 141, 2216–2224. doi: 10.1242/dev.106377

Ali, F., Hindley, C., McDowell, G., Deibler, R., Jones, A., Kirschner, M., et al. (2011). Cell cycle-regulated multi-site phosphorylation of neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. *Development* 138, 4267–4277. doi: 10.1242/dev.067900

Berninger, B., Costa, M. R., Koch, U., Schroeder, T., Sutor, B., Grothe, B., et al. (2007a). Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. *J. Neurosci.* 27, 8654–8664. doi: 10.1523/jneurosci.1615-07.2007

Berninger, B., Guillemot, F., and Götz, M. (2007b). Directing neurotransmitter identity of neurones derived from expanded adult neural stem cells. *Eur. J. Neurosci.* 25, 2581–2590. doi: 10.1111/j.1460-9568.2007.05509.x

Bertrand, N., Castro, D. S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* 3, 517–530. doi: 10.1038/nrn874

Blackwell, T. K., and Weintraub, H. (1990). Preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* 250, 1104–1110. doi: 10.1126/science.2174572

Borromeo, M. D., Meredith, D. M., Castro, D. S., Chang, J. C., Tung, K.-C., Guillemot, F., et al. (2014). A transcription factor network specifying inhibitory versus excitatory neurons in the dorsal spinal cord. *Development* 141, 2803–2812. doi: 10.1242/dev.105866

Bylund, M., Andersson, E., Novitch, B. G., and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1–3 activity. *Nat. Neurosci.* 6, 1162–1168. doi: 10.1038/nn1131

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* 126, 525–534.

Castella, P., Sawai, S., Nakao, K., Wagner, J. A., and Caudy, M. (2000). HES-1 repression of differentiation and proliferation in PC12 cells: role for the helix 3-helix 4 domain in transcription repression. *Mol. Cell. Biol.* 20, 6170–6183. doi: 10.1128/mcb.20.16.6170-6183.2000

Castro, D. S., Martynoga, B., Parras, C., Ramesh, V., Pacary, E., Johnston, C., et al. (2011). A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. *Genes Dev.* 25, 930–945. doi: 10.1101/gad.627811

Castro, D. S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I. J., Parras, C., Hunt, C., et al. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. *Dev. Cell* 11, 831–844. doi: 10.1016/j.devcel.2006.10.006

Cave, J. W., Loh, F., Surpris, J. W., Xia, L., and Caudy, M. A. (2005). A DNA transcription code for cell-specific gene activation by notch signaling. *Curr. Biol.* 15, 94–104. doi: 10.1016/j.cub.2004.12.070

Fisher, F., Crouch, D. H., Jayaraman, P. S., Clark, W., Gillespie, D. A., and Goding, C. R. (1993). Transcription activation by Myc and Max: flanking sequences target activation to a subset of CACGTG motifs in vivo. *EMBO J.* 12, 5075–5082.

Gaudet, J., and Mango, S. E. (2002). Regulation of organogenesis by the Caenorhabditis elegans FoxA protein PHA-4. *Science* 295, 821–825. doi: 10.1126/science.1065175

Geoffroy, C. G., Critchley, J. A., Castro, D. S., Ramelli, S., Barraclough, C., Descombes, P., et al. (2009). Engineering of dominant active basic helix-loop-helix proteins that are resistant to negative regulation by postnatal central nervous system antineurogenic cues. *Stem Cells* 27, 847–856. doi: 10.1002/stem.17

Götz, M., and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* 6, 777–788. doi: 10.1038/nrm1739

Hartman, J., Müller, P., Foster, J. S., Wimalasena, J., Gustafsson, J.-A., and Ström, A. (2004). HES-1 inhibits 17beta-estradiol and heregulin-beta1-mediated upregulation of E2F-1. *Oncogene* 23, 8826–8833. doi: 10.1038/sj.onc.1208139

Haubensak, W., Attardo, A., Denk, W., and Huttner, W. B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl. Acad. Sci. U S A* 101, 3196–3201. doi: 10.1073/pnas.0308600100

Henke, R. M., Meredith, D. M., Borromeo, M. D., Savage, T. K., and Johnson, J. E. (2009). Ascl1 and Neurog2 form novel complexes and regulate Delta-like3 (Dll3) expression in the neural tube. *Dev. Biol.* 328, 529–540. doi: 10.1016/j.ydbio.2009.01.007

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., et al. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. *Science* 298, 840–843. doi: 10.1126/science.1074560

Horton, S., Meredith, A., Richardson, J. A., and Johnson, J. E. (1999). Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. *Mol. Cell. Neurosci.* 14, 355–369. doi: 10.1006/mcne.1999.0791

Imayoshi, I., Isomura, A., Harima, Y., Kawaguchi, K., Kori, H., Miyachi, H., et al. (2013). Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. *Science* 342, 1203–1208. doi: 10.1126/science.1242366

Jacob, J., Kong, J., Moore, S., Milton, C., Sasai, N., Gonzalez-Quevedo, R., et al. (2013). Retinoid acid specifies neuronal identity through graded expression of Ascl1. *Curr. Biol.* 23, 412–418. doi: 10.1016/j.cub.2013.01.046

Kageyama, R., Ohtsuka, T., Hatakeyama, J., and Ohsawa, R. (2005). Roles of bHLH genes in neural stem cell differentiation. *Exp. Cell Res.* 306, 343–348. doi: 10.1016/j.yexcr.2005.03.015

Karow, M., Sánchez, R., Schichor, C., Masserdotti, G., Ortega, F., Heinrich, C., et al. (2012). Reprogramming of pericyte-derived cells of the adult human brain into induced neuronal cells. *Cell Stem Cell* 11, 471–476. doi: 10.1016/j.stem.2012.07.007

Kobayashi, T., Mizuno, H., Imayoshi, I., Furusawa, C., Shirahige, K., and Kageyama, R. (2009). The cyclic gene Hes1 contributes to diverse differentiation responses of embryonic stem cells. *Genes Dev.* 23, 1870–1875. doi: 10.1101/gad.1823109

Kriegstein, A., and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. *Annu. Rev. Neurosci.* 32, 149–184. doi: 10.1146/annurev.neuro.051508.135600

Li, S., Mattar, P., Dixit, R., Lawn, S. O., Wilkinson, G., Kinch, C., et al. (2014). RAS/ERK signaling controls proneural genetic programs in cortical development and gliomagenesis. *J. Neurosci.* 34, 2169–2190. doi: 10.1523/jneurosci.4077-13.2014

Louvi, A., and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat. Rev. Neurosci.* 7, 93–102. doi: 10.1038/nrn1847

Mangan, S., and Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proc. Natl. Acad. Sci. U S A* 100, 11980–11985. doi: 10.1073/pnas.2133841100

Marin, O., Anderson, S. A., and Rubenstein, J. L. (2000). Origin and molecular specification of striatal interneurons. *J. Neurosci.* 20, 6063–6076.

Masamizu, Y., Ohtsuka, T., Takashima, Y., Nagahara, H., Takenaka, Y., Yoshikawa, K., et al. (2006). Real-time imaging of the somite segmentation clock: revelation of unstable oscillators in the individual presomitic mesoderm cells. *Proc. Natl. Acad. Sci. U S A* 103, 1313–1318. doi: 10.1073/pnas.0508658103

Mengel, B., Hunziker, A., Pedersen, L., Trusina, A., Jensen, M. H., and Krishna, S. (2010). Modeling oscillatory control in NF-κB, p53 and Wnt signaling. *Curr. Opin. Genet. Dev.* 20, 656–664. doi: 10.1016/j.gde.2010.08.008

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T., and Ogawa, M. (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* 131, 3133–3145. doi: 10.1242/dev.01173

Mizutani, K., Yoon, K., Dang, L., Tokunaga, A., and Gaiano, N. (2007). Differential notch signalling distinguishes neural stem cells from intermediate progenitors. *Nature* 449, 351–355. doi: 10.1038/nature06090

Nakada, Y., Hunsaker, T. L., Henke, R. M., and Johnson, J. E. (2004). Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus neuronal cell-type specification. *Development* 131, 1319–1330. doi: 10.1242/dev.01008

Nellesen, D. T., Lai, E. C., and Posakony, J. W. (1999). Responsiveness of enhancer of split complex genes to common transcriptional activators. *Dev. Biol.* 53, 33–53.

Noctor, S. C., Martínez-Cerdeño, V., Ivic, L., and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* 7, 136–144. doi: 10.1038/nn1172

Oishi, K., Watatani, K., Itoh, Y., Okano, H., Guillemot, F., Nakajima, K., et al. (2009). Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1. *Proc. Natl. Acad. Sci. U S A* 106, 13064–13069. doi: 10.1073/pnas.0808400106

Pilz, G.-A., Shitamukai, A., Reillo, I., Pacary, E., Schwausch, J., Stahl, R., et al. (2013). Amplification of progenitors in the mammalian telencephalon includes a new radial glial cell type. *Nat. Commun.* 4:2125. doi: 10.1038/ncomms3125

Pina, C., Fugazza, C., Tipping, A. J., Brown, J., Soneji, S., Teles, J., et al. (2012). Inferring rules of lineage commitment in haematopoiesis. *Nat. Cell. Biol.* 14, 287–294. doi: 10.1038/ncb2442

Renault, V. M., Rafalski, V. A., Morgan, A. A., Salih, D. A. M., Brett, J. O., Webb, A. E., et al. (2009). FoxO3 regulates neural stem cell homeostasis. *Cell Stem Cell* 5, 527–539. doi: 10.1016/j.stem.2009.09.014

Sang, L., Coller, H. A., and Roberts, J. M. (2008). Control of the reversibility of cellular quiescence by the transcriptional repressor HES1. *Science* 321, 1095–1100. doi: 10.1126/science.1155998

Seo, S., Richardson, G. A., and Kroll, K. L. (2005). The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD. *Development* 132, 105–115. doi: 10.1242/dev.01548

Sequerra, E. B., Costa, M. R., Menezes, J. R. L., and Hedin-Pereira, C. (2013). Adult neural stem cells: plastic or restricted neuronal fates?. *Development* 140, 3303–3309. doi: 10.1242/dev.093096

Shen-Orr, S. S., Milo, R., Mangan, S., and Alon, U. (2002). Network motifs in the transcriptional regulation network of Escherichia coli. *Nat. Genet.* 31, 64–68. doi: 10.1038/ng881

Shimojo, H., Ohtsuka, T., and Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron* 58, 52–64. doi: 10.1016/j.neuron.2008.02.014

Soufi, A., Donahue, G., and Zaret, K. S. (2012). Facilitators and impediments of the pluripotency reprogramming factors' initial engagement with the genome. *Cell* 151, 994–1004. doi: 10.1016/j.cell.2012.09.045

Sriuranpong, V., Borges, M. W., Christopher, L., Nakakura, E. K., Watkins, D. N., Christine, M., et al. (2002). Notch signaling induces rapid degradation of achaete-scute homolog 1 notch signaling induces rapid degradation of achaete-scute homolog 1. *Mol. Cell. Biol.* 22, 3129–3139. doi: 10.1128/mcb.22.9.3129-3139.2002

Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Südhof, T. C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature* 463, 1035–1041. doi: 10.1038/nature08797

Viñals, F., Reiriz, J., Ambrosio, S., Bartrons, R., Rosa, J. L., and Ventura, F. (2004). BMP-2 decreases Mash1 stability by increasing Id1 expression. *EMBO J.* 23, 3527–3537. doi: 10.1038/sj.emboj.7600360

Wapinski, O. L., Vierbuchen, T., Qu, K., Lee, Q. Y., Chanda, S., Fuentes, D. R., et al. (2013). Hierarchical mechanisms for direct reprogramming of fibroblasts to neurons. *Cell* 155, 621–635. doi: 10.1016/j.cell.2013.09.028

Webb, A. E., Pollina, E. A., Vierbuchen, T., Urbán, N., Ucar, D., Leeman, D. S., et al. (2013). FOXO3 shares common targets with ASCL1 genome-wide and inhibits ASCL1-dependent neurogenesis. *Cell Rep.* 4, 477–491. doi: 10.1016/j.celrep.2013.06.035

Wilkinson, G., Dennis, D., and Schuurmans, C. (2013). Proneural genes in neocortical development. *Neuroscience* 253, 256–273. doi: 10.1016/j.neuroscience.2013.08.029

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 August 2014; accepted: 12 November 2014; published online: 02 December 2014.

Citation: Vasconcelos FF and Castro DS (2014) Transcriptional control of vertebrate neurogenesis by the proneural factor Ascl1. Front. Cell. Neurosci. ***8****:412. doi: 10.3389/fncel.2014.00412*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in Cellular Neuroscience

GENERAL COMMENTARY
published: 18 June 2015
doi: 10.3389/fncel.2015.00232

Corrigendum: TGF-β1 promotes cerebral cortex radial glia-astrocyte differentiation *in vivo*

*Joice Stipursky *, Daniel Francis, Rômulo S. Dezonne, Ana P. B. de Araújo, Lays Souza, Carolina A. Moraes and Flávia C. A. Gomes **

Programa de Biologia Celular e do Desenvolvimento, Laboratório de Neurobiologia Celular, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro - Centro de Ciências da Saúde, Rio de Janeiro, Brazil

Keywords: radial glia, TGF-β, gliogenesis, neurogenesis, cerebral cortex

A Corrigendum on

TGF-β1 promotes cerebral cortex radial glia-astrocyte differentiation *in vivo*
by Stipursky, J., Francis, D., Dezonne, R. S., Bérgamo de Araújo, A. P., Souza, L., Moraes, C. A., et al. (2014). Front Cell Neurosci. 8:393. doi: 10.3389/fncel.2014.00393

The last author Flavia Carvalho Alcantara Gomes appears with the incorrect citation name in this article (Stipursky et al., 2014). The correct citation name for this author is Gomes FC.

OPEN ACCESS

Edited by:
Marcos R. Costa, Federal University of Rio Grande do Norte, Brazil

****Correspondence:***
Joice Stipursky, jstipursky@gmail.com; Flávia C. A. Gomes, fgomes@icb.ufrj.br

Received: *22 April 2015*
Accepted: *26 May 2015*
Published: *18 June 2015*

Citation:
Stipursky J, Francis D, Dezonne RS, de Araújo APB, Souza L, Moraes CA and Gomes FCA (2015) Corrigendum: TGF-β1 promotes cerebral cortex radial glia-astrocyte differentiation in vivo. Front. Cell. Neurosci. 9:232. doi: 10.3389/fncel.2015.00232

Funding

Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Stipursky, J., Francis, D., Dezonne, R. S., Bérgamo de Araújo, A. P., Souza, L., Moraes, C. A., et al. (2014). TGF-β1 promotes cerebral cortex radial glia-astrocyte differentiation *in vivo*. *Front Cell Neurosci*. 8:393. doi: 10.3389/fncel.2014.00393

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 12 January 2015
doi: 10.3389/fncel.2014.00449

Specification of excitatory neurons in the developing cerebral cortex: progenitor diversity and environmental influences

Marcos R. Costa*[1] ***and Ulrich Müller*[2]***

[1] *Brain Institute, Federal University of Rio Grande do Norte, Natal, Brazil*
[2] *Dorris Neuroscience Center and Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA*

Edited by:
Enrico Cherubini, International School for Advanced Studies, Italy

Reviewed by:
Hongyu Sun, University of Pennsylvania, USA
Antonello Mallamaci, International School for Advanced Studies, Italy

****Correspondence:***
Marcos R. Costa, Brain Institute, Federal University of Rio Grande do Norte, Av. Nascimento de Castro 2155, Lagoa Nova, Natal 59056-450, Brazil
e-mail: mrcosta@neuro.ufrn.br;
Ulrich Müller, Dorris Neuroscience Center and Department of Cell Biology, The Scripps Research Institute, 3215 Merryfield Row, La Jolla, CA 92121, USA
e-mail: umueller@scripps.edu

The mature cerebral cortex harbors a heterogeneous population of glutamatergic neurons, organized into a highly intricate histological architecture. Classically, this mixed population of neurons was thought to be generated sequentially from a seemingly homogenous group of progenitors under the influence of external cues. This view, however, has been challenged in the last decade by evidences pointing to the existence of fate-restricted neuronal progenitors in the developing neocortex. Here, we review classical studies using cell transplantation, retroviral labeling and cell culture, as well as new data from genetic fate-mapping analysis, to discuss the lineage relationships between neocortical progenitors and subclasses of excitatory neurons. We also propose a temporal model to conciliate the existence of fate-restricted progenitors alongside multipotent progenitors in the neocortex. Finally, we discuss evidences for a critical period of plasticity among post mitotic excitatory cortical neurons when environmental influences could change neuronal cell fate.

Keywords: cerebral cortex, excitatory neurons, progenitor diversity, neuronal specification, development

CLASSES OF EXCITATORY NEURONS IN THE MAMMALIAN CEREBRAL CORTEX

The mammalian cerebral cortex harbors a heterogeneous population of neurons, which has been classically divided into two major groups: spiny and smooth neurons (Shepherd, 2003). It is accepted that in the adult mammalian cerebral cortex spiny neurons are excitatory neurons, whereas smooth neurons are inhibitory neurons (Migliore and Shepherd, 2005; Petilla Interneuron Nomenclature et al., 2008). Spiny neurons are usually classified according to the lamina where their soma is located and by dendritic morphologies. The latter allows the identification of pyramidal neurons and spiny stellate neurons.

Pyramidal neurons can be distinguished by their long apical dendrite, are found in all cortical layers except layer I and represent the major output neurons of the neocortex. It is estimated that most projections from pyramidal neurons connect different cortical regions, whereas only 1 in 100 fibers would connect subcortical targets (Braitenberg and Schüz, 1991). Pyramidal neurons also participate in local circuitry, representing the major source of excitatory input to the area in which they are found. Based on the differences in connections, pyramidal neurons are further classified as projection neurons with long axons that connect different cortical regions or project to subcortical targets; and interneurons with short axons that most commonly project locally (Shepherd, 2003).

Spiny stellate neurons have several dendrites of similar lengths and are found exclusively in layer IV of the granular cortex, where they represent the major recipient of thalamic inputs. Different from pyramidal neurons, spiny stellate neurons project mostly locally to areas near their cell bodies, although some can occasionally project to more distant cortical areas (Shepherd, 2003).

Cortical projection neurons can be further classified by hodology in associative, commissural and corticofugal subtypes (Molyneaux et al., 2007). Associative projection neurons extend axons within a single hemisphere, whereas commissural projection neurons connect neurons in the two cortical hemispheres either through the corpus callosum or the anterior commissure. Cortifugal projection neurons send axons to target areas outside the cerebral cortex, such as the thalamus (corticothalamic neurons), pons (corticopontine neurons (CPN)), spinal cord (corticospinal neurons), superior colliculus (corticotectal neurons) and striatum (corticostriatal neurons).

Cortical neurons can also be classified according to their main sensory inputs, as for instance, visual neurons, olfactory neurons, auditory neurons, somatosensory neurons and gustatory neurons in primary sensory areas. In more complex sensory

Table 1 | Relative expression of transcription factors in neurons of different neocortical layers.

	Layers II/III	Layer IV	Layer V	Layer VI	References
Cux1/2	+++	+++			Nieto et al. (2004), Zimmer et al. (2004)
Svet1	+++	+++			Tarabykin et al. (2001)
Satb2	+++	+	++	+	Alcamo et al. (2008), Britanova et al. (2008)
Lmo4	+++		+		Bulchand et al. (2003), Arlotta et al. (2005)
Brn2	+++		+		McEvilly et al. (2002), Sugitani et al. (2002)
RorB		+++			Schaeren-Wiemers et al. (1997)
Ctip2			+++	++	Leid et al. (2004), Arlotta et al. (2005), Chen et al. (2005)
Fezf2			++	+	Arlotta et al. (2005), Chen et al. (2005), Molyneaux et al. (2005)
Foxp2			+	+++	Ferland et al. (2003), Hisaoka et al. (2010)
Tle4			+	+++	Hack et al. (2007)
Er81			+++		Hevner et al. (2003), Yoneshima et al. (2006)

+++ highly expressed; ++ expressed; + weakly expressed.

areas, neurons can be classified as bi-modal neurons (role in the processing of two different sensory modalities) and multi-modal neurons (role in the processing of many different sensory modalities). Physiological classes of cortical neurons can also be distinguished according to their electrical intrinsic properties (Connors and Gutnick, 1990). For instance, while regular-spiking pyramidal neurons are observed in cortical layers II to VI, intrinsically bursting neurons are restricted to layers IV and V (Connors and Gutnick, 1990). Another difference can be observed in interspike interval (ISI). Whereas layer IV neurons usually evoke a short burst of action potentials with ISI <40 ms, layer V neurons have a longer first ISI (De la Rossa et al., 2013).

Furthermore, there is correlation between the laminar position of cortical neurons and their connection patterns (Douglas and Martin, 2004). Commissural neurons, for example, are mostly found in layers II, III and V, whereas corticothalamic neurons tend to be located in layer VI and subcerebral neurons in layer V. Differences in the projection patterns of subtypes of cortical neurons have been ingeniously exploited by the Mackli's laboratory to identify specific molecular features of neurons settled in different cortical layers of the mouse brain (Arlotta et al., 2005). Using microinjection of fluorescent microspheres into distinct axonal tracts, the authors retrogradely labeled three neuronal populations: corticospinal motoneurons (CSMN), callosal neurons (CN) and CPN. These cell populations were then isolated at four different developmental time points (E17, P3, P6 and P14) by fluorescent-activated cell sorting (FACS) and their gene expression profile was studied by gene-chip analysis. This approach led to the identification of genes involved in the specification, morphologic maturation and connectivity of layer V CSMN, such as Fezl (Fezf2) and Ctip2 (Arlotta et al., 2005; Chen et al., 2005, 2008; Molyneaux et al., 2005). Other studies have described genes involved in the specification of callosal projection neurons (Alcamo et al., 2008; Britanova et al., 2008) and corticothalamic neurons (McKenna et al., 2011).

Many other molecules are expressed in a layer-specific pattern in cortical neurons (Molyneaux et al., 2007) but their expression may not correlate with specific subtypes of excitatory neurons. **Table 1** summarizes some of these neuronal molecules, which will be of interest for our following discussion. Importantly, however, expression of these molecules is frequently variable across different cortical areas, leading to three important caveats: (i) neurons from the same cortical layer not necessarily express the same molecular marker; (ii) lack of expression of a given layer-marker not necessarily means that neurons do not belong to that layer; and (iii) molecules expressed in a layer-specific manner into a particular cortical area can be expressed in a different fashion into another areas. These limitations are particularly important for the interpretation of experimental data, as we shall discuss in the next chapters.

GENERATION OF EXCITATORY CORTICAL NEURONS

Cortical neurons in the mammalian cerebral cortex are generated in a limited period of development, varying from days to months depending on the species. In humans, cortical neurogenesis starts at gestational week (GW) 5 and finishes around GW20 (Bystron et al., 2008). In rodents, neurogenic intervals are much shorter lasting from embryonic day (E) 13 to E21 in rats (Berry and Rogers, 1965; Bayer et al., 1991) and E11 to E19 in mice (Angevine and Sidman, 1961; Caviness, 1982; Takahashi et al., 1993). These periods were defined by the administration to pregnant females of molecules that are incorporated into DNA during the S-phase of the cell cycle, such as tritiated thymidine (TH^3) or BrdU. Later, the neuronal fate and laminar position of cells labeled with such molecules was determined by auto-radiography (TH^3) or immunohistochemistry (BrdU).

These population studies showed that neurons destined for different cortical layers are generated in a temporal sequence, such that deep layer neurons are generated before upper layer neurons. Although these experiments did not distinguish between excitatory and inhibitory neurons, it is widely accepted that excitatory cortical neurons of layers II to VI generally follow this inside-out pattern (Greig et al., 2013). However, it should be noted that this does not hold true when one analyzes the birth data of neurons with similar projection patterns. This is most obvious for corticocortical and callosal projection neurons, which share the expression of molecular markers and are located predominantly in layer II-III but are also found in substantial numbers in deep layers. These molecularly and functionally similar neurons are

born over an extended time window ranging between ∼E11.5 and E15.5 (Greig et al., 2013).

Two main progenitor populations in the dorsal telencephalon are responsible for the generation of cortical excitatory neurons: (i) ventricular zone (VZ) progenitors or radial glia cells (RGC); and (ii) subventricular zone (SVZ) or intermediate progenitors. VZ progenitors are the first cells in the developing telencephalon and generate SVZ progenitors and neurons (Takahashi et al., 1995; Malatesta et al., 2000, 2003; Miyata et al., 2001, 2004; Noctor et al., 2001, 2002, 2004). SVZ progenitors were first described as gliogenic (Takahashi et al., 1995), but later acknowledged as an important source of cortical neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004).

More recently, genetic fate-mapping experiments using the Cre/LoxP system have provided direct evidence for the generation of glutamatergic cortical neurons from a discrete population of progenitors located in the dorsal telencephalon (Gorski et al., 2002). By crossing Emx1-Cre transgenic mice to a Cre-reporter mouse, the authors could show that Emx1-expressing progenitors are confined to the dorsal telencephalon and contribute glutamatergic neurons to all cortical layers, but not GABAergic cortical neurons.

The contribution of subtypes of progenitors to subtypes of excitatory neurons in different cortical layers remained unknown until 10 years ago, when studies in mice suggested that SVZ progenitors could contribute preferentially to the generation of upper layer neurons (Tarabykin et al., 2001; Nieto et al., 2004; Zimmer et al., 2004; Sessa et al., 2008, 2010; Dominguez et al., 2013). However, SVZ progenitors are present in mice at early and late stages of cortical neurogenesis, and RGCs generate directly only 10% of all excitatory neurons in the cerebral cortex (Kowalczyk et al., 2009). Since IPCs are generated from RGCs (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004), these two cell types likely represent different progenitor states along a developmental time line rather than separate fate-restricted lineages. Indeed, a recent fate-mapping study using a Tbr2-Cre mouse line show that $Tbr2^+$ cells, i.e., SVZ progenitors, contribute neurons to all cortical layers (Vasistha et al., 2014). Thus, SVZ progenitors likely represent an intermediate stage between VZ progenitors and cortical glutamatergic neurons during all cortical development. As a consequence, expansion of the SVZ in primates could reflect a homogeneous amplification of cell numbers in the cerebral cortex, rather than a selective expansion of upper cortical layers during evolution (Smart et al., 2002; Martinez-Cerdeno et al., 2006; Hansen et al., 2010).

ARE PROGENITOR CELLS SPECIFIED TO THE GENERATION OF PARTICULAR SUBTYPES OF EXCITATORY CORTICAL NEURONS?

Although our knowledge about the generation of cortical glutamatergic neurons from a population point of view dates from several decades, much debate still exists on the possible relations between individual progenitors or subpopulation of progenitors and the generation of specific subclasses of cortical glutamatergic neurons.

Pioneer experiments from the laboratory of Susan McConnell addressed the potential of cortical progenitors from different developmental stages by transplanting these cells iso- or heterochronically into the developing cerebral cortex (McConnell, 1988; McConnell and Kaznowski, 1991; Desai and McConnell, 2000). These experiments showed that when progenitors were isolated from animals at late stages of corticogenesis, during times when layers II and III are generated, and transplanted into the brain of animals of a similar age (isochronic transplantation), they generated neurons of layers II/III and astrocytes (McConnell, 1985, 1988), thus resembling the fate of endogenously generated neurons. Next, presumptive layer V/VI progenitors were transplanted into the brain of animals of later developmental age (heterochronic transplantation), when layer II/III neurons are generated (McConnell, 1988). Most transplanted cells (80%) failed to migrate out from the injection site and the remainder (20%) differentiated into neurons in layers V and VI (57%) and II/III (43%). Based on these findings, the author concluded that "at least a subpopulation of embryonically generated neurons appears to be committed to a deep layer fate prior to migration" (McConnell, 1988).

Next, similar heterochronic transplantation experiments of presumptive layer V/VI progenitors into brains of animals of later developmental age were done using cells isolated at different stages of the cell cycle (McConnell and Kaznowski, 1991). The authors showed that cells isolated in S-phase generated neurons for layers II/III, similar to host cells. In contrast, cells isolated at later stages of the cell cycle generated neurons for layers V and VI, thus resembling the behavior of progenitors at the time of isolation. Together, these experiments suggested that environmental cues are important to specify the laminar fate of cortical neurons, but progenitors have a time-window to respond to such cues.

Other experiments showed that the capacity of cortical progenitors to respond to external cues is reduced during development (Desai and McConnell, 2000). Progenitors isolated at the stage when layer IV neurons are generated and transplanted into animals of later stages adopt the same fate as neurons generated in the host brain from endogenous progenitors, i.e., layers II/III neurons. In contrast, when transplanted into animals of earlier stages when layer VI neurons are generated, cells migrated to layer IV, the position typical of their origin.

Collectively, these transplantation experiments suggest that (i) environmental cues are important to determine the laminar fate of glutamatergic neurons; (ii) specification occurs at the level of progenitors; (iii) early progenitors respond to late extrinsic signal, but not the contrary; and (iv) post mitotic neurons are specified according to the environment where they are generated and do not change layer identity when exposed to new extrinsic signals. More generally, these experiments are cited as evidence of restriction in the fate potential of progenitor cells over developmental age. However, the data are also consistent with the existence of multiple progenitors, but where early and late progenitors behave different in different environments such as the late environment is conductive for the survival and differentiation of late-stage progenitors, while only the early environment sustains early progenitors.

Cell culture systems were also employed to study the potential of cortical progenitors to generate neurons and macroglial cells (Costa et al., 2009). However, only one study has accessed the lineage-relations among neurons bearing molecular hallmarks of individual cortical layers (Shen et al., 2006). This work showed that isolated cortical progenitor maintain the timing for generation of neurons destined to different layers, i.e., deep layer neurons first and upper layer neurons later. However, the authors could not show that a single progenitor could generate clones with both deep and upper layer neurons. Instead, they show examples of clones containing only upper layer Cux2$^+$ neurons alongside neurons that do not label for other markers used. Since the panel of layer-markers available at the time to label subtypes of neurons was very restricted (Foxp2, Tle4, Er81 and Cux1), data could be interpreted in at least two ways: (1) Progenitors for deep and upper layer neurons comprise two different populations; or (2) Single progenitors can generate deep and upper layer neurons, but the markers used could not reveal this phenomenon.

Finally, the potential of single cortical progenitors to generate different types of cells was also assessed by retroviral-mediated fate mapping (reviewed by Costa et al., 2009). These studies rely on the infection of low number of progenitors and the identification of their progeny in the adult brain. Several technical issues complicate the interpretation of such studies, including silencing of retroviral vectors and labeling of GABAergic neurons that invade the cerebral cortex by tangential migratory routes (Costa et al., 2009). Nevertheless, fundamental insights into the behavior of progenitor cells and their contribution to the production of glutamatergic neurons of different cortical layers were derived from these studies (Luskin et al., 1993; Reid et al., 1995). In the first study, the authors described only one clone out of nine containing pyramidal (glutamatergic) neurons in both upper and deep layers (II and V) following retroviral injections at E15/16 rats. The other clones were restricted to layers II/III and IV (Luskin et al., 1993). Similarly, Reid et al. (1995) described clones containing pyramidal neurons either in deep layers (3 out of 15) or upper layers (12 out of 15) after retroviral injections of E15 rats. Using a combination of two retrovirus carrying plasmids encoding for green fluorescent protein (GFP) or red fluorescent protein (RFP) injected in E13 mice (Costa et al., 2009), we observed similar results, namely a bimodal distribution for clones of pyramidal neurons in upper or deep layers (**Figure 1**). Collectively, these experiments suggest that neurons destined to layers II-IV or V-VI tend to be generated from two different sets of progenitors.

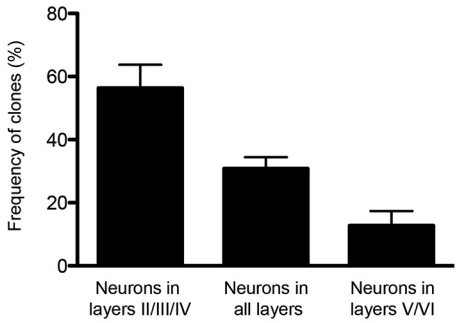


FIGURE 1 | Clones of spiny neurons, derived from neocortical progenitors labeled with retrovirus at E13, were classified according to the laminar position of clonally related neurons at P15. The graphic shows the quantification of clones containing spiny neurons only in the upper cortical layers (II/III and IV), only lower cortical layers (V and VI) or both (n = 64 clones from 3 animals). Observe that 1/3 of clones contain neurons in both deep and upper layers.

LINEAGE RESTRICTED PROGENITORS FOR SUBTYPES OF NEOCORTICAL PROJECTION NEURONS

Cre-Lox mediated genetic lineage tracing studies have recently provided new insights into the fate-potential of VZ progenitors. These lineage-tracing strategies depend on suitable genetic loci that allow for the expression of Cre in progenitor cells. Significantly, Cux2 is expressed in subsets of progenitor cells in the VZ (Franco et al., 2012) and SVZ (Nieto et al., 2004; Zimmer et al., 2004) already during earliest stages of neocortical development when layer VI and V neurons are born. At postnatal ages, Cux2 expression is most prominent in neurons within layers II-IV, but also found in subsets of neurons in deep layers (Nieto et al., 2004; Zimmer et al., 2004). The Cux2 expression pattern suggested a lineage relationship between Cux2$^+$ progenitors and layer II-IV neurons. However, the co-existence of progenitors for late-born layer II-IV and early-born layer V-VI neurons early during cortical development is contradictory to a model of progressive restriction in the lineage potential of a common progenitor for all cortical projection neurons (Shen et al., 2006; Okano and Temple, 2009).

To carry out lineage-tracing studies, Cux2-Cre mice were generated by a knock-in strategy and crossed with several Cre-reporter mouse lines (Franco et al., 2012). The majority of labeled neurons (∼75%) of the Cux2-Cre lineage were present in upper cortical cell layers of the mature cerebral cortex, but significant numbers (∼25%) were also localized in deep layers. Consistent with the known expression pattern of Cux2 (Zimmer et al., 2004), many but not all of the neurons in deep layers were interneurons (Franco et al., 2012). Further analysis of the labeled projection neurons with molecular markers revealed that the majority expressed Satb2 (Franco et al., 2012), a marker for corticocortical projection neurons that are predominantly present in upper layers, but are also found in deep layers (Greig et al., 2013). Analysis of the expression of Ctip2, a marker for a subset of subcerebral projection neurons (Arlotta et al., 2005) revealed its expression in a small subset of neurons labeled by Cux2-Cre; some of them co-expressed Ctip2 and Satb2 (Franco et al., 2012). Thus, these data demonstrated that the majority of projection neurons in the Cux2-Cre lineage are Satb2$^+$ cells.

Constitutively active Cre is an important read-out to reveal the full complement of cells expressing the gene under study at any time in a developing or mature tissue. Likewise, it allows identifying the cell types that do not express the gene under study. Thus, lineage tracing studies with Cux2-Cre revealed that a large fraction of Satb2$^+$ cells but only very few Ctip2$^+$ cells express

Cux2 at any time during their developmental history. However, to define whether recombination occurred in progenitors, migrating cells and/or post mitotic neuron, temporal fate mapping studies are important. Therefore mice expressing tamoxifen-inducible CreERT2 from the Cux2 locus were generated, thereby conferring temporal control over Cre activity (Franco et al., 2012). The findings from these studies demonstrated that at E10.5 $Cux2^+$ RGCs are specified to generate $Satb2^+$ projection neurons in upper and deep layers of the neocortex. Importantly, further analysis demonstrated that $Cux2^+$ progenitors are primarily proliferative during phases of lower layer neurogenesis and start to generate significant numbers of upper layer neurons only at later developmental time points. When the progenitors were forced to prematurely leave the cell cycle, they prematurely generated neurons expressing markers for upper layer neurons. Similarly, when progenitors were forced to differentiate *in vitro*, $Cux2^+$ progenitors predominantly generated neurons expressing upper layer markers. Taken together, these findings suggest that a population of $Cux2^+$ RGC cells is restricted in their fate potential to mostly generate $Satb2^+$ projections neurons (Franco et al., 2012).

Recently, the model that $Cux2^+$ progenitors are specified to generate $Satb2^+$ projection neurons was challenged. Using Fezf2-CreERT2 mice, the authors proposed the existence of a multipotent progenitor for all neocortical projection neurons (Guo et al., 2013). Using the same tamoxifen inducible Cux2-CreERT2 mouse line used previously by Franco et al. (2012) the authors showed that neurons derived from the Cux2 lineage occupy at P0 both upper and deep neocortical cell layers (Guo et al., 2013). However, this result is expected since the formation of neocortical cell layers is not complete by P0. Many of the cells within the Cux2-lineage had at P0 the morphology of radially migrating neurons (Guo et al., 2013). In addition, Cux2-Cre traces not only $Satb2^+$ cells in deep and upper cortical cell layers, but also a subset of interneurons especially in deep layers (Franco et al., 2012).

To further support their conclusion, the authors analyzed the phenotype of the neurons with molecular markers. These experiments were also carried out at P0 prior to the final maturation of cortical neurons. Many of the Cux2-CreERT2-derived projection neurons in deep layers express at P0 Ctip2, which is strongly expressed in layer V neurons that project to subcerebral targets (Arlotta et al., 2005). However, during early stages of differentiation, neurons frequently co-express genes that at later stages preferentially label subtypes of projection neurons with different layers position and projection patterns (Alcamo et al., 2008; Bedogni et al., 2010; Srinivasan et al., 2012; Deck et al., 2013). Furthermore, Ctip2 is also expressed at lower levels in other projection neuron subtypes (Arlotta et al., 2005), and the expression of other markers such as Satb2 was not evaluated. Thus the apparent discrepancy between the two studies is potentially explained by the fact that Guo et al. analyzed neuronal positioning and molecular phenotype during developmental time points with a limited set of markers, and markers such as Ctip2 where expression is not all-or-none but various in intensity between subtypes of neurons.

A recent study used a different strategy to analyze the potential of RGCs to generate neocortical projection neurons. The authors used Mosaic Analysis with Double Markers (MADM) to analyze the neuronal output from single RGCs (Gao et al., 2014). In MADM, Cre-recombinase induces interchromosomal recombination that reconstitutes fluorescent markers and allows tracing the progeny of progenitors where Cre was active. Using Emx1-CreERT2 mice and Nestin-CreERT2 mice, the authors induce recombination in progenitors between E10 and E13 and analyze their fate potential. Neurons in the Cre lineages occupied for the most part all neocortical cell layers. Studies with molecular markers suggested that most clones contain neurons with upper and deep layer identity. How can these data be reconciled with the findings from lineage studies using Cux2-Cre mice? Two points should be considered. First, the extent to which the MADM strategy is unbiased is unclear. Cre is active in a time window during mitosis and recombination thus likely depends on the length of the cell cycle of a particular progenitor and thus may not capture all progenitors. Perhaps more likely and interesting, Emx1-CreERT2 might label a multipotent progenitor that generates lineage restricted progenitors such as those labeled by Cux2-CreERT2 (**Figure 2**). This model is also consistent with retrovirus lineage tracing studies, which show that mulitpotent and restricted progenitors coexist within the cortical VZ (**Figure 1**).

Collectively, the new findings are consistent with a model where neuronal subtype specification occurs in part already at the level of progenitor cells. Perhaps, the cortical VZ is a mosaic of progenitors with different fate potentials, where a multipotent progenitor gives rise to lineage restricted progenitors (**Figure 2**). Of course, the cortex consists of many neuronal subtypes and there is heterogeneity even within each neocortical cell layer population (Britanova et al., 2008). Thus, further specification events are necessary to generate the immense diversity within neocortical projection neurons. Some of this diversity is established at the level of post mitotic neurons (Greig et al., 2013), suggesting that mechanisms acting both at the level of progenitors and post mitotic cells cooperate to generate subtypes of neocortical projection neurons.

PLASTICITY OF EARLY-POST MITOTIC NEURONS

Indeed, there are compelling evidences for an additional degree of plasticity at the level of post mitotic neocortical projection neurons. Both connectivity and electrical properties of neocortical neurons are affected by manipulations of sensory inputs after the neocortical neurogenic interval (Van der Loos and Woolsey, 1973; Sur et al., 1988), suggesting that the final fate of those neurons could be influenced by environmental cues acting at the level of post mitotic cells. Recently, Días-Alonso et al. have shown that conditional deletion of the cannabinoid receptor CB1 in post mitotic neurons decreases the numbers of $Ctip2^+$ subcerebral projection neurons (Díaz-Alonso et al., 2012).

According to the notion that post mitotic neocortical projection neurons are plastic, two inspiring studies have shown that forced expression of a single transcription factor can shift fates of early post mitotic neurons (De la Rossa et al., 2013; Rouaux and Arlotta, 2013). Both groups have used genetic

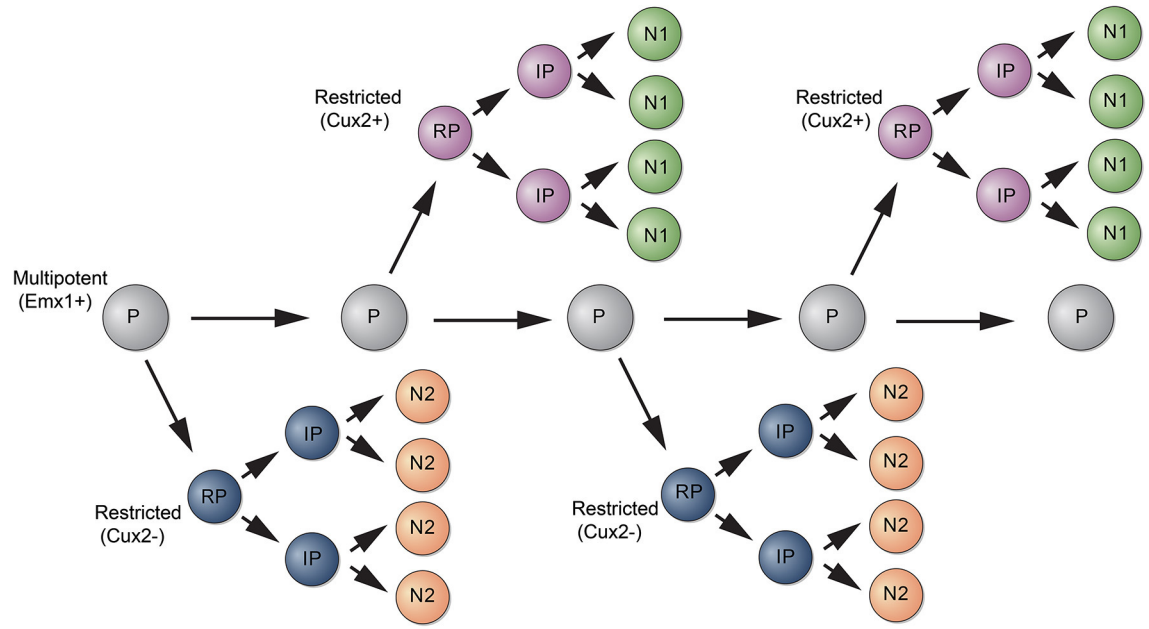


FIGURE 2 | Model for the generation of subtypes of projection neurons. A multipotent progenitor (P, gray) persists in the cortical VZ and generates subtypes of restricted progenitors (RP, purple and blue). RPs generate intermediate progenitors (IPs) that differentiate into subtypes of neurons (N1, N2).

strategies to ectopically express Fezf2 in spiny neurons from layer IV (De la Rossa et al., 2013) and layers II/III (Rouaux and Arlotta, 2013), which in response acquired molecular identity, morphology, physiology and functional input-output connectivity of layer V projection neurons. Interestingly, the number of layer II/III neurons reprogrammed into layer V neurons by forced expression of Fezf2 is highest during the earliest stages of post mitotic differentiation (Rouaux and Arlotta, 2013). At later time-points, this plasticity decreases and is eventually abolished, suggesting the existence of a critical period of nuclear plasticity for post mitotic neurons. However, neuronal plasticity at late post mitotic stages can be partially rescued by combining Fezf2 expression with axonal sectioning (Rouaux and Arlotta, 2013). Together, these data indicate that cortical glutamatergic neurons retain some degree of plasticity, which is likely regulated by interplay between intrinsic transcriptional control and extrinsic network control (Russ and Kaltschmidt, 2014).

ENVIRONMENTAL SIGNALS CONTROLLING THE GENERATION OF UPPER LAYER NEURONS

The temporal sequence for generation of neurons towards different cortical layers and/or lineages is also regulated by environmental cues. The neurotrophin Ntf3 acts as a feedback signal from post mitotic neurons to progenitors, promoting the generation of upper layer at the expense of deep layer neurons (Parthasarathy et al., 2014). Ntf3 gene is a target for Sip1, expressed at high levels in post mitotic neocortical neurons (Seuntjens et al., 2009). Conditional deletion of Sip1 in post mitotic neurons induces premature generation of upper layer neurons, also at the expense deep layer neurons (Seuntjens et al., 2009). However, down-regulation of Ntf3 produces an increase in layer VI neurons but does not rescue the Sip1 mutant phenotype, indicating that other signals are also involved in the control of cortical progenitor cell fate (Parthasarathy et al., 2014).

Genetic ablation of deep layer neurons also affects the fate of cortical progenitors, inducing *de novo* generation of deep layer neurons at the expense of upper layer neurons (Toma et al., 2014). However, it is not clear whether this effect is due to the lack of feedback signals from post mitotic neurons to progenitor cells, which would then resume generation of deep layer neurons, or by a direct fate conversion of post mitotic neurons. Future studies are needed to address the possibility of environmental signals contributing to the specification of neocortical projection neurons directly through regulation of transcriptional networks in both progenitors and post mitotic neurons.

CONCLUSIONS

Generation of the large variety of neocortical spiny neurons starts at the level of progenitors in the neocortical VZ with the generation of at least two major classes of progenitors identified by expression or absence of Cux2. These progenitors are likely derived from a multipotent progenitor population and environmental cues may contribute for the establishment and balance of these populations.

Specification programs are inherited by post mitotic neurons and contribute to the laminar organization of the neocortex.

The role of environment in the specification of neocortical spiny neurons at a post mitotic level requires more investigation, but is a potential mechanism to further refine the neocortical cytoarchitecture.

REFERENCES

Alcamo, E. A., Chirivella, L., Dautzenberg, M., Dobreva, G., Fariñas, I., Grosschedl, R., et al. (2008). Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* 57, 364–377. doi: 10.1016/j.neuron.2007.12.012

Angevine, J. B. Jr., and Sidman, R. L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* 192, 766–768. doi: 10.1038/192766b0

Arlotta, P., Molyneaux, B. J., Chen, J., Inoue, J., Kominami, R., and Macklis, J. D. (2005). Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron* 45, 207–221. doi: 10.1016/j.neuron.2004.12.036

Bayer, S. A., Altman, J., Russo, R. J., Dai, X. F., and Simmons, J. A. (1991). Cell migration in the rat embryonic neocortex. *J. Comp. Neurol.* 307, 499–516. doi: 10.1002/cne.903070312

Bedogni, F., Hodge, R. D., Elsen, G. E., Nelson, B. R., Daza, R. A., Beyer, R. P., et al. (2010). Tbr1 regulates regional and laminar identity of postmitotic neurons in developing neocortex. *Proc. Natl. Acad. Sci. U S A* 107, 13129–13134. doi: 10.1073/pnas.1002285107

Berry, M., and Rogers, A. W. (1965). The migration of neuroblasts in the developing cerebral cortex. *J. Anat.* 99, 691–709.

Braitenberg, V., and Schüz, A. (1991). *Anatomy of the Cortex: Statistics and Geometry.* New York City: Springer.

Britanova, O., de Juan Romero, C., Cheung, A., Kwan, K. Y., Schwark, M., Gyorgy, A., et al. (2008). Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. *Neuron* 57, 378–392. doi: 10.1016/j.neuron.2007.12.028

Bulchand, S., Subramanian, L., and Tole, S. (2003). Dynamic spatiotemporal expression of LIM genes and cofactors in the embryonic and postnatal cerebral cortex. *Dev. Dyn.* 226, 460–469. doi: 10.1002/dvdy.10235

Bystron, I., Blakemore, C., and Rakic, P. (2008). Development of the human cerebral cortex: boulder committee revisited. *Nat. Rev. Neurosci.* 9, 110–122. doi: 10.1038/nrn2252

Caviness, V. S. Jr. (1982). Development of neocortical afferent systems: studies in the reeler mouse. *Neurosci. Res. Program Bull.* 20, 560–569.

Chen, B., Schaevitz, L. R., and McConnell, S. K. (2005). Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex. *Proc. Natl. Acad. Sci. U S A* 102, 17184–17189. doi: 10.1073/pnas.0508732102

Chen, B., Wang, S. S., Hattox, A. M., Rayburn, H., Nelson, S. B., and McConnell, S. K. (2008). The Fezf2-Ctip2 genetic pathway regulates the fate choice of subcortical projection neurons in the developing cerebral cortex. *Proc. Natl. Acad. Sci. U S A* 105, 11382–11387. doi: 10.1073/pnas.0804918105

Connors, B. W., and Gutnick, M. J. (1990). Intrinsic firing patterns of diverse neocortical neurons. *Trends Neurosci.* 13, 99–104. doi: 10.1016/0166-2236(90)90185-d

Costa, M. R., Bucholz, O., Schroeder, T., and Götz, M. (2009). Late origin of glia-restricted progenitors in the developing mouse cerebral cortex. *Cereb. Cortex* 19(Suppl. 1), i135–i143. doi: 10.1093/cercor/bhp046

Deck, M., Lokmane, L., Chauvet, S., Mailhes, C., Keita, M., Niquille, M., et al. (2013). Pathfinding of corticothalamic axons relies on a rendezvous with thalamic projections. *Neuron* 77, 472–484. doi: 10.1016/j.neuron.2012.11.031

De la Rossa, A., Bellone, C., Golding, B., Vitali, I., Moss, J., Toni, N., et al. (2013). In vivo reprogramming of circuit connectivity in postmitotic neocortical neurons. *Nat. Neurosci.* 16, 193–200. doi: 10.1038/nn.3299

Desai, A. R., and McConnell, S. K. (2000). Progressive restriction in fate potential by neural progenitors during cerebral cortical development. *Development* 127, 2863–2872.

Díaz-Alonso, J., Aguado, T., Wu, C. S., Palazuelos, J., Hofmann, C., Garcez, P., et al. (2012). The CB(1) cannabinoid receptor drives corticospinal motor neuron differentiation through the Ctip2/Satb2 transcriptional regulation axis. *J. Neurosci.* 32, 16651–16665. doi: 10.1523/JNEUROSCI.0681-12.2012

Dominguez, M. H., Ayoub, A. E., and Rakic, P. (2013). POU-III transcription factors (Brn1, Brn2 and Oct6) influence neurogenesis, molecular identity and migratory destination of upper-layer cells of the cerebral cortex. *Cereb. Cortex* 23, 2632–2643. doi: 10.1093/cercor/bhs252

Douglas, R. J., and Martin, K. A. (2004). Neuronal circuits of the neocortex. *Annu. Rev. Neurosci.* 27, 419–451. doi: 10.1146/annurev.neuro.27.070203.144152

Ferland, R. J., Cherry, T. J., Preware, P. O., Morrisey, E. E., and Walsh, C. A. (2003). Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain. *J. Comp. Neurol.* 460, 266–279. doi: 10.1002/cne.10654

Franco, S. J., Gil-Sanz, C., Martinez-Garay, I., Espinosa, A., Harkins-Perry, S. R., Ramos, C., et al. (2012). Fate-restricted neural progenitors in the mammalian cerebral cortex. *Science* 337, 746–749. doi: 10.1126/science.1223616

Gao, P., Postiglione, M. P., Krieger, T. G., Hernandez, L., Wang, C., Han, Z., et al. (2014). Deterministic Progenitor behavior and unitary production of neurons in the Neocortex. *Cell* 159, 775–788. doi: 10.1016/j.cell.2014.10.027

Gorski, J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L., and Jones, K. R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J. Neurosci.* 22, 6309–6314.

Greig, L. C., Woodworth, M. B., Galazo, M. J., Padmanabhan, H., and Macklis, J. D. (2013). Molecular logic of neocortical projection neuron specification, development and diversity. *Nat. Rev. Neurosci.* 14, 755–769. doi: 10.1038/nrn3586

Guo, C., Eckler, M. J., Mckenna, W. L., Mckinsey, G. L., Rubenstein, J. L., and Chen, B. (2013). Fezf2 expression identifies a multipotent progenitor for neocortical projection neurons, astrocytes and oligodendrocytes. *Neuron* 80, 1167–1174. doi: 10.1016/j.neuron.2013.09.037

Hack, I., Hellwig, S., Junghans, D., Brunne, B., Bock, H. H., Zhao, S., et al. (2007). Divergent roles of ApoER2 and Vldlr in the migration of cortical neurons. *Development* 134, 3883–3891. doi: 10.1242/dev.005447

Hansen, D. V., Lui, J. H., Parker, P. R., and Kriegstein, A. R. (2010). Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature* 464, 554–561. doi: 10.1038/nature08845

Haubensak, W., Attardo, A., Denk, W., and Huttner, W. B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl. Acad. Sci. U S A* 101, 3196–3201. doi: 10.1073/pnas.0308600100

Hevner, R. F., Daza, R. A., Rubenstein, J. L., Stunnenberg, H., Olavarria, J. F., and Englund, C. (2003). Beyond laminar fate: toward a molecular classification of cortical projection/pyramidal neurons. *Dev. Neurosci.* 25, 139–151. doi: 10.1159/000072263

Hisaoka, T., Nakamura, Y., Senba, E., and Morikawa, Y. (2010). The forkhead transcription factors, Foxp1 and Foxp2, identify different subpopulations of projection neurons in the mouse cerebral cortex. *Neuroscience* 166, 551–563. doi: 10.1016/j.neuroscience.2009.12.055

Kowalczyk, T., Pontious, A., Englund, C., Daza, R. A., Bedogni, F., Hodge, R., et al. (2009). Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex. *Cereb. Cortex* 19, 2439–2450. doi: 10.1093/cercor/bhn260

Leid, M., Ishmael, J. E., Avram, D., Shepherd, D., Fraulob, V., and Dolle, P. (2004). CTIP1 and CTIP2 are differentially expressed during mouse embryogenesis. *Gene Expr. Patterns* 4, 733–739. doi: 10.1016/j.modgep.2004.03.009

Luskin, M. B., Parnavelas, J. G., and Barfield, J. A. (1993). Neurons, astrocytes and oligodendrocytes of the rat cerebral cortex originate from separate progenitor cells: an ultrastructural analysis of clonally related cells. *J. Neurosci.* 13, 1730–1750.

Malatesta, P., Hack, M. A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F., et al. (2003). Neuronal or glial progeny: regional differences in radial glia fate. *Neuron* 37, 751–764. doi: 10.1016/S0896-6273(03)00116-8

Malatesta, P., Hartfuss, E., and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* 127, 5253–5263.

Martinez-Cerdeno, V., Noctor, S. C., and Kriegstein, A. R. (2006). The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. *Cereb. Cortex* 16(Suppl. 1), i152–i161. doi: 10.1093/cercor/bhk017

McConnell, S. K. (1985). Migration and differentiation of cerebral cortical neurons after transplantation into the brains of ferrets. *Science* 229, 1268–1271. doi: 10.1126/science.4035355

McConnell, S. K. (1988). Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. *J. Neurosci.* 8, 945–974.

McConnell, S. K., and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* 254, 282–285. doi: 10.1126/science.1925583

McEvilly, R. J., De Diaz, M. O., Schonemann, M. D., Hooshmand, F., and Rosenfeld, M. G. (2002). Transcriptional regulation of cortical neuron migration by POU domain factors. *Science* 295, 1528–1532. doi: 10.1126/science.1067132

McKenna, W. L., Betancourt, J., Larkin, K. A., Abrams, B., Guo, C., Rubenstein, J. L., et al. (2011). Tbr1 and Fezf2 regulate alternate corticofugal neuronal identities during neocortical development. *J. Neurosci.* 31, 549–564. doi: 10.1523/JNEUROSCI.4131-10.2011.

Migliore, M., and Shepherd, G. M. (2005). Opinion: an integrated approach to classifying neuronal phenotypes. *Nat. Rev. Neurosci.* 6, 810–818. doi: 10.1038/nrn1769

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* 31, 727–741. doi: 10.1016/s0896-6273(01)00420-2

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T., and Ogawa, M. (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* 131, 3133–3145. doi: 10.1242/dev.01173

Molyneaux, B. J., Arlotta, P., Hirata, T., Hibi, M., and Macklis, J. D. (2005). Fezl is required for the birth and specification of corticospinal motor neurons. *Neuron* 47, 817–831. doi: 10.1016/j.neuron.2005.08.030

Molyneaux, B. J., Arlotta, P., Menezes, J. R., and Macklis, J. D. (2007). Neuronal subtype specification in the cerebral cortex. *Nat. Rev. Neurosci.* 8, 427–437. doi: 10.1038/nrn2151

Nieto, M., Monuki, E. S., Tang, H., Imitola, J., Haubst, N., Khoury, S. J., et al. (2004). Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex. *J. Comp. Neurol.* 479, 168–180. doi: 10.1002/cne.20322

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409, 714–720. doi: 10.1038/35055553

Noctor, S. C., Flint, A. C., Weissman, T. A., Wong, W. S., Clinton, B. K., and Kriegstein, A. R. (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J. Neurosci.* 22, 3161–3173.

Noctor, S. C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* 7, 136–144. doi: 10.1038/nn1172

Okano, H., and Temple, S. (2009). Cell types to order: temporal specification of CNS stem cells. *Curr. Opin. Neurobiol.* 19, 112–119. doi: 10.1016/j.conb.2009.04.003

Parthasarathy, S., Srivatsa, S., Nityanandam, A., and Tarabykin, V. (2014). Ntf3 acts downstream of Sip1 in cortical postmitotic neurons to control progenitor cell fate through feedback signaling. *Development* 141, 3324–3330. doi: 10.1242/dev.114173

Petilla Interneuron Nomenclature, G., Ascoli, G. A., Alonso-Nanclares, L., Anderson, S. A., Barrionuevo, G., Benavides-Piccione, R., et al. (2008). Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat. Rev. Neurosci.* 9, 557–568. doi: 10.1038/nrn2402

Reid, C. B., Liang, I., and Walsh, C. (1995). Systematic widespread clonal organization in cerebral cortex. *Neuron* 15, 299–310. doi: 10.1016/0896-6273(95)90035-7

Rouaux, C., and Arlotta, P. (2013). Direct lineage reprogramming of post-mitotic callosal neurons into corticofugal neurons in vivo. *Nat. Cell Biol.* 15, 214–221. doi: 10.1038/ncb2660.

Russ, J. B., and Kaltschmidt, J. A. (2014). From induction to conduction: how intrinsic transcriptional priming of extrinsic neuronal connectivity shapes neuronal identity. *Open Biol.* 4:140144. doi: 10.1098/rsob.140144

Schaeren-Wiemers, N., Andre, E., Kapfhammer, J. P., and Becker-Andre, M. (1997). The expression pattern of the orphan nuclear receptor RORbeta in the developing and adult rat nervous system suggests a role in the processing of sensory information and in circadian rhythm. *Eur. J. Neurosci.* 9, 2687–2701. doi: 10.1111/j.1460-9568.1997.tb01698.x

Sessa, A., Mao, C. A., Colasante, G., Nini, A., Klein, W. H., and Broccoli, V. (2010). Tbr2-positive intermediate (basal) neuronal progenitors safeguard cerebral cortex expansion by controlling amplification of pallial glutamatergic neurons and attraction of subpallial GABAergic interneurons. *Genes Dev.* 24, 1816–1826. doi: 10.1101/gad.575410

Sessa, A., Mao, C. A., Hadjantonakis, A. K., Klein, W. H., and Broccoli, V. (2008). Tbr2 directs conversion of radial glia into basal precursors and guides neuronal amplification by indirect neurogenesis in the developing neocortex. *Neuron* 60, 56–69. doi: 10.1016/j.neuron.2008.09.028

Seuntjens, E., Nityanandam, A., Miquelajauregui, A., Debruyn, J., Stryjewska, A., Goebbels, S., et al. (2009). Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. *Nat. Neurosci.* 12, 1373–1380. doi: 10.1038/nn.2409

Shen, Q., Wang, Y., Dimos, J. T., Fasano, C. A., Phoenix, T. N., Lemischka, I. R., et al. (2006). The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. *Nat. Neurosci.* 9, 743–751. doi: 10.1038/nn1694

Shepherd, G. M. (2003). *The Synaptic Organization of the Brain.* Oxford: Oxford University Press.

Smart, I. H., Dehay, C., Giroud, P., Berland, M., and Kennedy, H. (2002). Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cereb. Cortex* 12, 37–53. doi: 10.1093/cercor/12.1.37

Srinivasan, K., Leone, D. P., Bateson, R. K., Dobreva, G., Kohwi, Y., Kohwi-Shigematsu, T., et al. (2012). A network of genetic repression and derepression specifies projection fates in the developing neocortex. *Proc. Natl. Acad. Sci. U S A* 109, 19071–19078. doi: 10.1073/pnas.1216793109

Sugitani, Y., Nakai, S., Minowa, O., Nishi, M., Jishage, K., Kawano, H., et al. (2002). Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons. *Genes Dev.* 16, 1760–1765. doi: 10.1101/gad.978002

Sur, M., Garraghty, P. E., and Roe, A. W. (1988). Experimentally induced visual projections into auditory thalamus and cortex. *Science* 242, 1437–1441. doi: 10.1126/science.2462279

Takahashi, T., Nowakowski, R. S., and Caviness, V. S. Jr. (1993). Cell cycle parameters and patterns of nuclear movement in the neocortical proliferative zone of the fetal mouse. *J. Neurosci.* 13, 820–833. doi: 10.1093/cercor/13.6.592

Takahashi, T., Nowakowski, R. S., and Caviness, V. S. Jr. (1995). Early ontogeny of the secondary proliferative population of the embryonic murine cerebral wall. *J. Neurosci.* 15, 6058–6068.

Tarabykin, V., Stoykova, A., Usman, N., and Gruss, P. (2001). Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. *Development* 128, 1983–1993.

Toma, K., Kumamoto, T., and Hanashima, C. (2014). The timing of upper-layer neurogenesis is conferred by sequential derepression and negative feedback from deep-layer neurons. *J. Neurosci.* 34, 13259–13276. doi: 10.1523/JNEUROSCI.2334-14.2014

Van der Loos, H., and Woolsey, T. A. (1973). Somatosensory cortex: structural alterations following early injury to sense organs. *Science* 179, 395–398. doi: 10.1126/science.179.4071.395

Vasistha, N. A., Garcia-Moreno, F., Arora, S., Cheung, A. F., Arnold, S. J., Robertson, E. J., et al. (2014). Cortical and Clonal contribution of Tbr2 expressing progenitors in the developing mouse brain. *Cereb. Cortex* doi: 10.1093/cercor/bhu125. [Epub ahead of print].

Yoneshima, H., Yamasaki, S., Voelker, C. C., Molnar, Z., Christophe, E., Audinat, E., et al. (2006). Er81 is expressed in a subpopulation of layer 5 neurons in rodent and primate neocortices. *Neuroscience* 137, 401–412. doi: 10.1016/j.neuroscience.2005.08.075

Zimmer, C., Tiveron, M. C., Bodmer, R., and Cremer, H. (2004). Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons. *Cereb. Cortex* 14, 1408–1420. doi: 10.1093/cercor/bhh102

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 October 2014; accepted: 12 December 2014; published online: 12 January 2015.

*Citation: Costa MR and Müller U (2015) Specification of excitatory neurons in the developing cerebral cortex: progenitor diversity and environmental influences. Front. Cell. Neurosci. **8**:449. doi: 10.3389/fncel.2014.00449*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 07 August 2014
doi: 10.3389/fncel.2014.00215

The role of α-E-catenin in cerebral cortex development: radial glia specific effect on neuronal migration

Marie-Theres Schmid[1], Franziska Weinandy[1], Michaela Wilsch-Bräuninger[2], Wieland B. Huttner[2], Silvia Cappello[1,3]* and Magdalena Götz[1,3]*

[1] *Helmholtz Zentrum München, National Research Center for Environmental Health, Institute of Stem Cell Research, Neuherberg/Munich, Germany*
[2] *Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany*
[3] *Department of Physiological Genomics, Institute of Physiology, University of Munich, Munich, Germany*

Edited by:
Marcos R. Costa, Federal University of Rio Grande do Norte, Brazil

Reviewed by:
Alfredo Cáceres, INEMEC-CONICET, Argentina
Zoltan Molnar, University of Oxford, UK

****Correspondence:***
Silvia Cappello and Magdalena Götz, Helmholtz Zentrum München, National Research Center for Environmental Health, Ingolstaedter Landstrasse 1, 85764 Neuherberg/Munich, Germany; Department of Physiological Genomics, Institute of Physiology, University of Munich, Schillerstrasse 46, 80336 Munich, Germany
e-mail: silvia.cappello@helmholtz-muenchen.de; magdalena.goetz@helmholtz-muenchen.de

During brain development, radial glial cells possess an apico-basal polarity and are coupled by adherens junctions (AJs) to an F-actin belt. To elucidate the role of the actin, we conditionally deleted the key component α-E-catenin in the developing cerebral cortex. Deletion at early stages resulted in severe disruption of tissue polarity due to uncoupling of AJs with the intracellular actin fibers leading to the formation of subcortical band heterotopia. Interestingly, this phenotype closely resembled the phenotype obtained by conditional RhoA deletion, both in regard to the macroscopic subcortical band heterotopia and the subcellular increase in G-actin/F-actin ratio. These data therefore together corroborate the role of the actin cytoskeleton and its anchoring to the AJs for neuronal migration disorders.

Keywords: α-E-catenin, actin, neuronal migration, cell polarity, subcortical band heterotopia, adherens junctions

INTRODUCTION

Malformations of the human neocortex represent a major cause of developmental disabilities and severe epilepsy (Barkovich et al., 2012). It is therefore essential to understand how these diseases develop, find the critical molecules, discover gene networks, and pathways relevant for these malformations and deeply comprehend the cellular functions and the functional consequences.

A feature of the mammalian neocortex is the positioning of functionally distinct neurons in six horizontal layers (Leone et al., 2008). The importance of this arrangement becomes particularly evident in cases of disturbed neocortical development, like in subcortical band heterotopias (SBH), in which neurons remain located below the white matter. This and other malformations are the result of impaired developmental processes such as cell proliferation, differentiation or neuronal migration (Bozzi et al., 2012).

Recent evidence shows that the formation of neuronal heterotopias can also result from defect in the polarity and morphology of radial glial cells (Cappello et al., 2012, 2013; Cappello, 2013), highlighting the emerging role of radial glia polarity and architecture in the etiology of cortical malformations and in particular neuronal migration disorders.

Apical radial glial cells in the developing nervous system possess epithelial features such as junctional coupling at the ventricular surface, localize distinct molecules to the apical and basolateral membrane domain and contact the basement membrane by their radially extending processes (Gotz and Huttner, 2005). On the contrary, the intermediate or basal progenitors, differ from apical radial glial cells as they lack apico-basal polarity and mostly divide once to generate two postmitotic neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Wu et al., 2005). Intrinsic molecules regulating apico-basal polarity in radial glial cells may be involved in orchestrating the complex process of neurogenesis and neuronal migration. Indeed, alterations in cell polarity and adherens junction (AJ) coupling upon conditional deletion of cdc42 from radial glial cells lead to an immediate transition of radial glial cells to basal progenitors (Cappello et al., 2006). However, it is not clear to which extent the phenotype is due to the influence of cdc42 on the Par complex (Costa et al., 2008), on AJ coupling or the random cell localization.

Recently genetic deletion of RhoA in the developing cerebral cortex highlighted the essential role of radial glial cells and AJs localization as it resulted in the formation of subcortical band heterotopia and Lissencephaly type II, with neurons protruding beyond layer I at the pial surface of the brain (Cappello et al., 2012).

We therefore aimed here to selectively examine the significance of junctional coupling on apical radial glial cells in the developing

cerebral cortex. Toward this end, we conditionally deleted the AJ molecule, α-E-catenin which has been shown to dynamically connect the F-actin cytoskeleton to β-catenin (Harris and Tepass, 2010), which in turn is bound to cadherins (Harris and Tepass, 2010). The loss of α-E-catenin was reported to cause AJ disassembly (Vasioukhin et al., 2001; Lien et al., 2006) and widespread deletion of α-E-catenin in the CNS led to profound overproliferation and disturbances in tissue architecture (Lien et al., 2006). Conversely, deletion of α-N-catenin, mostly expressed in differentiated neurons, led to relatively mild defects in the CNS architecture (Park et al., 2002; Uemura and Takeichi, 2006). However, in addition to its role in junctional coupling, α-E-catenin was reported to affect signaling pathways, such as MAP kinase in skin stem cells (Vasioukhin et al., 2001), WNT in HEK293T cells (Merdek et al., 2004) and Sonic Hedgehog (SHH) signaling in radial glial cells (Lien et al., 2006). The deletion of α-E-catenin in the entire nervous system affected amongst others the signaling centers for SHH, the ventral most regions throughout the CNS (Jessell and Sanes, 2000). We choose to delete α-E-catenin specifically in the developing cerebral cortex, avoiding the ventral SHH signaling center, by using the $Emx1^{Cre}$ mouse line (Iwasato et al., 2000; Cappello et al., 2006, 2012). These experiments support a key role of α-E-catenin in coupling AJ complexes to the apical radial glia cells. When this link is abolished, tissue polarity is lost and newly generated neurons fail to reach the cortical plate and accumulate in ectopic positions resulting in the formation of a large subcortical band heterotopia.

Therefore this result strengthens the hypothesis that subcortical band heterotopia is a disorder caused by tissue defects in radial glial cells rather than in the migration of neurons themselves (Cappello et al., 2012; Cappello, 2013).

MATERIALS AND METHODS

ANIMALS

The mouse strains $Catna1^{tm1Efu}$ (Vasioukhin et al., 2001) was obtained from Jackson Laboratory. The $Emx1^{Cre}$ mouse strain was kindly provided by Iwasato et al. (2000). All mouse strains were maintained on a C57BL/6JxCD1 background. Analysis was performed from E10 to P0. Embryonic brains were dissected in HBSS/10 mM HEPES (Gibco-Invitrogen), fixed in 4% paraformaldehyde (PFA), cryoprotected with 30% sucrose and frozen sections were cut at 12 μm thickness for immunohistochemistry or *In Situ*-hybridization.

BrdU-LABELING

Pregnant mice were injected intraperitoneally at E11, E12, and E14 with 5-Bromo-2-deoxyuridine (BrdU) (5 mg/kg bodyweight) and analyzed by immunostaining at P0, (birth dating analysis) as previously shown (Cappello et al., 2012). Quantification was not performed.

F- TO G-ACTIN ANALYSIS AND QUANTIFICATION

F- and G-actin were labeled by Texas-Red labeled Phalloidin (0.165 μM, Invitrogen) and Alexa 488-labeled DNase1 (9 μg/ml, Mol.Probes) respectively. Cryosections (fixed in 4%PFA) were incubated with both reagents for 20 min, washed in PBS and additionally immunolabeled for Tuj1. Images were taken by confocal microscopy and quantitatively analyzed using ImageJ (Java).

IMMUNOHISTOCHEMISTRY AND *IN SITU*-HYBRIDIZATION

Primary antibodies: α-catenin (1:20, BD-Transd.Lab), pan-cadherin (1:200, Sigma), Par3 (1:100, Upstate), Prominin (1:100, eBioscience), Tuj1 (1:100, Sigma), Tbr2 (1:100, Chemicon/Millipore), Calretinin (1:5000, SWant), BrdU (1:100, Abcam), reelin (1:10, Merck), Tbr1 (1:100, Abcam), β-catenin (1:50, BD-Trans.D.), RC2 (provided by P.Leprince), NeuN (1:100, Chemicon/Millipore), L1 (1:2000, Chemicon/Millipore), Nurr1 (1:200, Santa Cruz). Secondary antibodies were used form Jackson Immunoresearch, and Southern Biotechnology. Nuclei were visualized by using DAPI (4′, 6′Diamidino-2-phenylindoline, 0.1 μg/ml,Sigma) or PropidiumIodide (Mol. Probes). Specimens were mounted in Aqua Poly/Mount (Polysciences, Northampton, UK) and analyzed with FV1000 (Olympus) or Axioplan/ApoTome Microscope (Zeiss).

Digoxigenin-labeled RNA probes for ER81, Cux2 (kindly provided by C.Schuurmans), Satb2, (kindly provided by V. Tarabykin), and Rorβ (Armentano et al., 2007) were made and used as described (Chapouton et al., 2001).

ELECTRON MICROSCOPY

Electron microscopy was performed as described previously (Cappello et al., 2006).

WESTERN BLOT

Primary antibodies: Par3 (1:500, Chemicon/Millipore), aPKCλ, i (1:500, Cell Signaling), Par6 (1:500, Santa Cruz), Phospho-aPKCλ (1:500 Cell Signaling).

Cortices from embryonic brains were lysed in RIPA buffer. 20 μg of total protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (Biorad), which were incubated with primary antibodies followed by horseradish peroxidase labeled secondary antibodies (1:25000; Amersham) detected by ECL Western Blotting Detection (Chemicon).

RESULTS

In radial glial cells α-E-catenin localizes to AJs (**Figure 1A**), visible as honeycomb-like structures when viewed from the ventricular surface (**Figure 1B**). We investigated the function of α-E-catenin by deleting exon3 of α-E-catenin (Vasioukhin et al., 2001) in radial glial cells selectively in the cerebral cortex at early stages of neurogenesis by $Emx1^{Cre}$ (Iwasato et al., 2000; Cappello et al., 2006, 2012). Accordingly, α-E-catenin was lost from the anlagen of the cerebral cortex in $Emx1^{Cre/\alpha-cat\Delta ex2fl/fl}$ mutant mice (referred to as α-cat cKO henceforth) but was unaffected in the remaining CNS, including the ganglionic eminence that contains the SHH signaling center (**Figures 1C,D**). Consistent with the onset of Cre-expression in $Emx1^{Cre}$ cortices around E9.5, α-E-catenin mRNA started to decrease at E10 and α-cat cKO cortices lacked α-E-catenin protein at E11 (**Figures 1C,D**). Already at E11 the mutant cortex showed morphological alterations, with cells starting to protrude into the ventricle (**Figures 1C,D**, arrows).

NEURONS MIGRATE IN TWO BANDS IN α-CAT cKO CORTEX AND FORM A DOUBLE-CORTEX

We next examined α-cat cKO cortices at postnatal stages, when most of the neurons have reached the correct final position. At

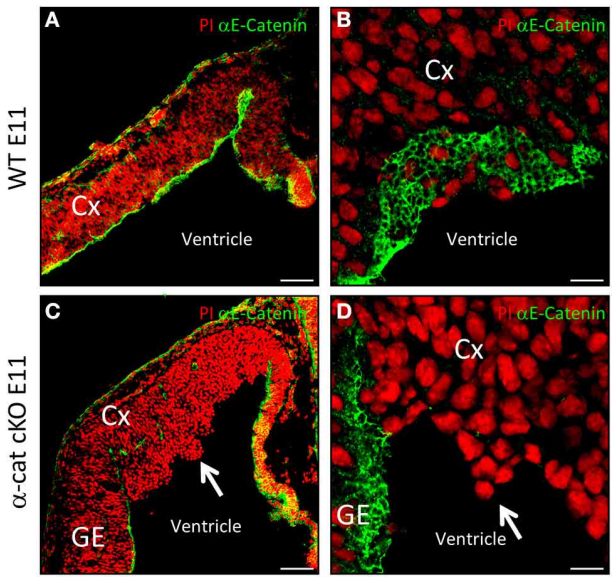


FIGURE 1 | Cortical development upon loss of α-catenin. α-E-catenin immunostaining (green, nuclei labeled with Propidium Iodide, red) in coronal sections of E11 WT **(A,B)** and α-cat cKO **(C,D)** cortices. Note the loss of α-E-catenin upon recombination by $Emx1^{Cre}$ from E11 on, exclusively in the cortex, except in blood-vessels, the meninges, and the Choroid Plexus. Arrows indicates protrusions in the lateral ventricle. CX, cortex; GE, ganglionic eminence. Scale bars: 100 μm **(A,C)**, 10 μm **(B,D)**.

postnatal day 0 (P0) in WT brains, neurons, positive for the neuronal marker NeuN, are located in the cortical plate and separated from the ventricular zone by the white matter containing L1 positive axons (**Figures 2A,B**). α-cat cKO cortices also displayed a similar band of L1 positive axons that separated a thin layer of NeuN positive neurons correctly positioned in the cortical plate but also had a large heterotopia below composed of neurons organized in clusters which were surrounded by L1 positive axons (**Figures 2D,E**). This double band of gray matter neurons (split by white matter) and the additional white matter intermingled in the heterotopia demonstrated that deletion of α-E-catenin in the developing cortex resulted in the formation of a large SBH or double-cortex. Moreover, L1 positive axons did not form a corpus callosum connecting the two cerebral hemispheres (**Figures 2C,F**).

BOTH PARTS OF THE DOUBLE-CORTEX POSSESS NEURONS FROM ALL LAYERS

In order to determine the identity of the neurons located at the different (normal and aberrant) positions, neuronal subtype specific markers were examined after birth (P0). The earliest generated neurons (E10/11) populate layer I and express Calretinin and/or Reelin (Stoykova et al., 2003; Englund et al., 2005). Both in WT and α-cat cKO cortices these neurons were situated at correct positions in layer I beneath the pial surface (**Figures 3A,B,G,H**). However subplate neurons, expressing Nurr1, were distributed both in the upper cortex and the heterotopia (**Figure S1**), suggesting that the neuronal heterotopia is formed very early during

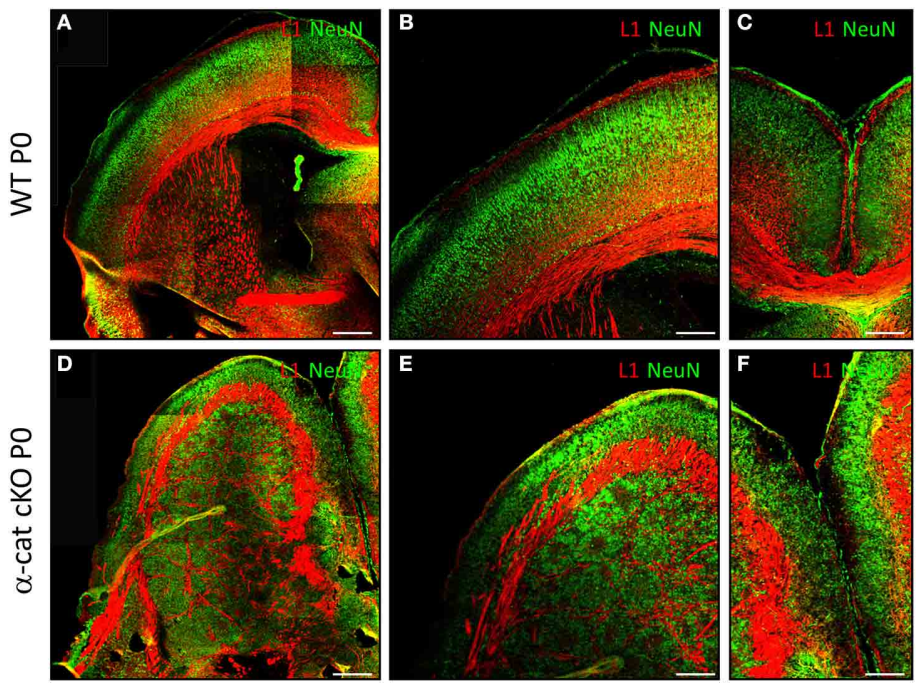


FIGURE 2 | Formation of SBH in the absence of α-E-catenin. Fluorescence micrographs of coronal cortical sections of P0 WT **(A–C)** and α-cat cKO **(D–F)** immunostained as indicated. **(A,B,D,E)**: lateral cortex; **(B,E)**: enlarged view of **(A,D)**; **(C,F)**: connection between the two hemispheres [corpus callosum in **(C)**]. Scale bars: 200 μm **(A,D)**, 100 μm **(B,C,E,F)**.

development. Neurons generated a little later (E12/13), normally settle in layers IV and V and express Rorβ and ER81 (**Figures 3C,D**). These neurons appeared sometimes in clusters in the α-cat cKO cortex (**Figures 3I,J**) and despite being generated early, already showed a distinct distribution in an upper band and clusters located below the white matter. More clearly, neurons generated later, that settle in the upper cortical layers II/III and express Cux2 and Satb2 (including partially layer IV) (Nieto et al., 2004; Zimmer et al., 2004; Britanova et al., 2005) (**Figures 3E,F**), were present in two well defined bands located in the upper cortex and clustered in the heterotopia in the P0 α-cat cKO cortex (**Figures 3K,L**). These data suggest that both, the upper and the lower, cortices were composed by neurons of each layer including the subplate.

In WT, neurons of different layers are generated sequentially at different developmental stages. As some of the above used markers may also be altered in the mutant, we examined whether in the α-cat cKO cortex neurons were still generated in the same order, BrdU was administered to pregnant mice at day E11, E12, or 14 and brains were analyzed at P0. In WT, a large proportion of BrdU-positive cells generated at E11 expressed the deep layer neuronal marker Tbr1 and was located in the deep layers (**Figure 4A**). Neurons generated at E11 in the α-cat cKO were mostly positive for Tbr1 but spread in the entire cortex up to layer I and the pial surface, including a prominent band in the cortical plate (**Figure 4D**). Neurons generated at E12, mostly expressed the deep layer marker Tbr1 in both WT and α-cat cKO cortices (**Figures 4B,E**), whereas neurons generated at E14 in WT were mostly negative for Tbr1 (**Figure 4C**). This colocalization with Tbr1 was similar in the α-cat cKO cortex (**Figure 4F**). Thus, neurons generated from α-E-catenin deficient progenitor cells acquired their neuronal identity according to their time of generation and independently of their position within the cortex. This suggests that the fate of radial glial cells and basal progenitors does not depend on their residence in particular cortical niches.

In contrast to WT however, the vast majority of neurons born at E12 (Tbr1+) or at E14 (Tbr1−) were located in two separated locations in the α-cat cKO cortex (**Figures 4E,F**).

These data together demonstrate that deletion of α-E-catenin in the developing cerebral cortex resulted in the formation of SBH.

NEURAL STEM CELL POSITION AND MORPHOLOGY ARE ALTERED IN α-CAT cKO CORTEX

Previous work suggested that maintenance of radial glial cell polarity is essential to guide neurons toward the cortical plate (Cappello et al., 2012), we therefore analyzed radial glial cells and the localization of newly-generated neurons (Tuj1+) at earlier stages in the α-cat cKO developing cortex. As neurogenesis in the mouse cerebral cortex begins around E10, the position of neurons was investigated 1 day later when α-E-catenin protein was

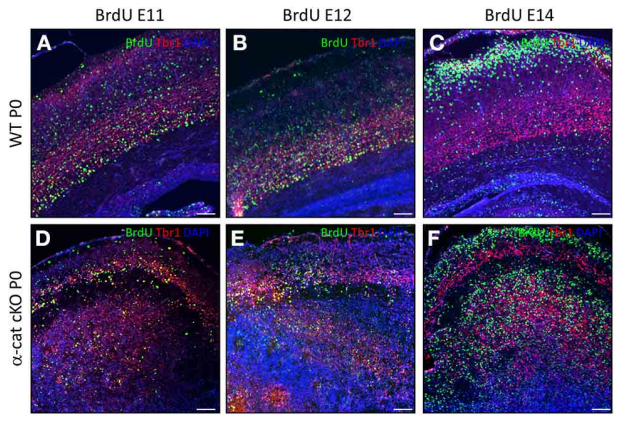


FIGURE 4 | Birth-dating analysis in the α-cat cKO cortex. Fluorescence micrographs of coronal cortical sections of P0 WT **(A–C)** and α-cat cKO **(D–F)** immunostained as indicated. BrdU was injected at E11 **(A,D)**, E12 **(B,E)**, or E14 **(C,F)**. Scale bars: 100 μm.

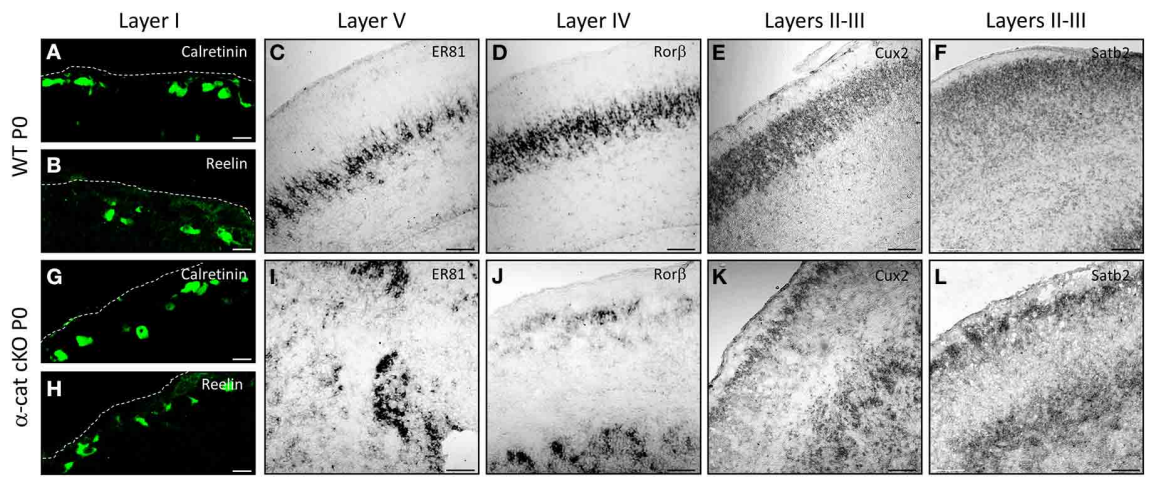


FIGURE 3 | Characterization of the SBH cell identity in the α-cat cKO cortex. Micrographs of coronal sections of P0 WT **(A–F)** and α-cat cKO **(G–L)**. Micrographs depict immunostaining (Calretinin, Reelin) or *in situ* hybridization (ER81, rorβ, cux2, satb2) as depicted in the panels. **(A,B,G,H)**: pial surface is indicated by dashed lines. Scale bars: 10 μm **(A,B,G,F)**; 100 μm **(C–F,I–L)**.

already strongly reduced in the α-cat cKO cortex. At this stage in WT only a thin band of neurons positive for Tuj1 is populating the cortical plate as most of the cells are still proliferating (**Figure 5A**). Later on, at E13, the cortical plate becomes thicker and the newly-generated neurons together with their future axons occupy almost half of the entire cortex (**Figure 5B**). Interestingly already at E11, neurons were localized at ectopic positions in the α-cat cKO, including the ventricular zone (**Figure 5E**, arrow), suggesting that neuronal migration toward the cortical plate was impaired. Two days later, only few Tuj1 positive neurons managed to reach the cortical plate (**Figure 5F**, arrow) while most of them accumulated in ectopic positions scattered in the developing cortex (**Figure 5F**).

As the current hypothesis is that aberrant radial glia polarity is the cause of defective neuronal migration (Cappello et al., 2012; Cappello, 2013), immunostaining for the RC2 isoform of the intermediate filament nestin (Chanas-Sacre et al., 2000) was performed. Immunoreactivity for RC2 was almost absent in the α-cat cKO cortex at E11 (**Figures 5C,G**), and later on, at E13, highlighted a complete disruption of the radial glial morphology in the α-cat cKO suggesting that the loss of α-E-catenin results in an impaired cytoskeletal organization in radial glial cells (**Figures 5D,H**) as in the case of the RhoA cKO (Cappello et al., 2012).

CORTICAL CELLS MAINTAIN CELL-CELL CONTACTS IN THE ABSENCE OF α-E-CATENIN

The cellular alterations, exemplified by the scattering and intermingling of cells, were a prominent aspect of the α-cat cKO phenotype and strongly suggested that the loss of α-E-catenin affected cellular coherence. To better understand the cell biological consequences of the α-E-catenin deletion, cell adhesion, junctional coupling, and the cytoskeleton were then examined in the α-cat cKO cortex. In WT cortices cadherin-immunoreactivity delineated the AJs around progenitors along the ventricle (**Figures 6A–C**) where it co-localized with β-catenin, adjacent to the concentrated rings of F-actin (**Figure 6A**). While disorganized patches of cadherin-staining were irregularly distributed within the tissue of α-cat cKO cortices (**Figures 6E,J**), they still co-localized with β-catenin (**Figure 6F**), suggesting that AJs may still be present between cortical cells, despite the loss of α-E-catenin even though in a random manner. This impression was confirmed at the ultra-structural level where long electron dense structures were present between α-E-catenin-deficient cells (**Figure S2**). However, these structures were not regularly aligned at the ventricular surface as in WT cortices (**Figure S2B**), but rather detected throughout the cortex parenchyma. The cell-cell junctions present in the α-cat cKO cortex were found either dispersed solitarily or in clusters at different sites (**Figures 6F,G**). The largest clusters formed rosette-like structures (data not shown) similar to what has been observed in other mutants (Lien et al., 2006; Kadowaki et al., 2007; Rasin et al., 2007; Cappello et al., 2012).

The dispersal of cells and the absence of a radial cortical architecture suggested disturbances of the cytoskeletal organization in the absence of α-E-catenin. Indeed, despite the presence of AJs, the coupling between AJs and intracellular actin fibers was strongly compromised as already at E11 the accumulation of F-actin was not concentrated at the same side as cadherin (**Figures 6E,J**). This is consistent with the role of α-E-catenin as an anchor to connect the transmembrane AJs to the cytoskeleton by linking the β-catenin/cadherin complex to the intracellular actin fibers (Harris and Tepass, 2010). Thus, while nascent AJs are made, these fail to be anchored to the cytoskeleton.

Several polarity molecules are co-localized together with AJs. We therefore examined whether the alteration in the cytoskeletal organization may have abolished cell polarity. To address this issue we investigated molecules of the apical membrane domain, such as prominin1 or the members of the apical Par complex (Suzuki and Ohno, 2006; Srinivasan et al., 2008). Interestingly, WT and α-cat cKO cortices contained comparable levels of Par3, Par6, and aPKC, as well as phosphorylated aPKC (**Figures 6C,G** and **Figure S3**). Furthermore, Par3, although distributed in

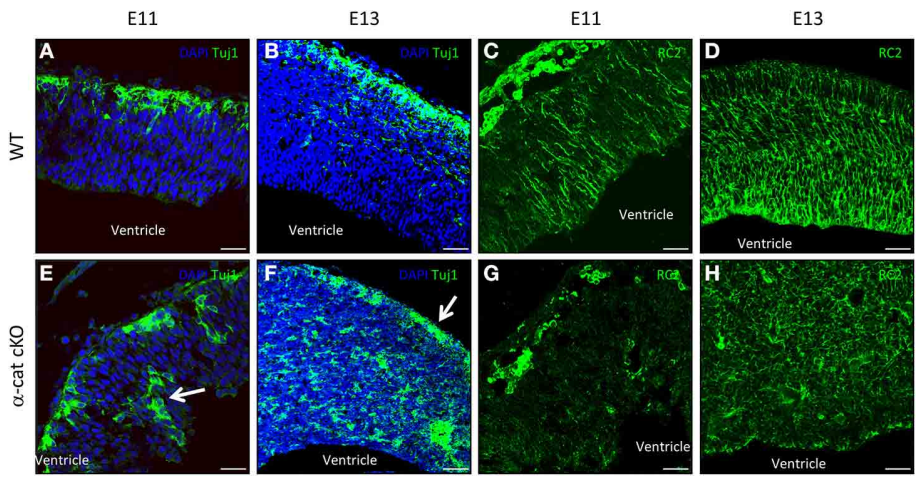


FIGURE 5 | Neuronal position and stem cell morphology upon loss of α-catenin. Fluorescence micrographs of coronal cortical sections of E11 and E13 WT **(A–D)** and α-cat cKO **(E–H)** immunostained as indicated. Arrows indicate ectopic neurons **(E)** or neurons reaching the cortical plate **(F)**. Scale bars: 50 μm **(A,C,E,G)**, 100 μm **(B,D,F,H)**.

patches within the α-cat cKO cortex parenchyma, still localized next to cadherins (**Figures 6C,G**), suggesting that at least some degree of cellular polarity was maintained in the α-cat cKO cortex. This was further supported as Prominin1 (CD133), delineating the ventricular surface in WT (Weigmann et al., 1997), was concentrated in distinct patches along the cell membrane of mutant cells (**Figures 6D,H**), adjacent to β-catenin patches. Moreover, prominin-containing particles released from the apical membrane (Marzesco et al., 2005) were observed close to AJs within the α-cat cKO cortex (data not shown). Thus, α-E-catenin deficient cortical cells possess AJs and concentrate apical membrane components in their proximity, demonstrating that individual cells were able to, at least partially, maintain their polarity despite the intermingling of cell types and tissue architecture in the α-cat cKO.

DELETION OF α-E-CATENIN IMPAIRS THE STABILITY OF F-ACTIN AT THE VENTRICULAR SURFACE

α-E-catenin directly interacts with the cytoskeleton by binding to F-actin at the place of AJs and thereby stabilizing its filamentous form (Pokutta et al., 2008). Therefore it was investigated whether the absence of α-E-catenin may have changed the stability of filamentous actin, thus shifting the ratio between G- and F-actin toward G-actin, as this was the case upon conditional RhoA deletion (Cappello et al., 2012). The ratio of F- to G-actin was assessed by detecting F-actin with fluorescently labeled Phalloidin and G-actin with DNAseI. The fluorescent intensity was then quantified by confocal microscopy. This revealed an increase in G-actin in the α-cat cKO cortical VZ to levels found in the WT only in the cortical plate (**Figures 7A–B″**). The intensities of G- and F-actin were measured for progenitors at the ventricular surface (**Figure 7A**, square1) and within the parenchyma (**Figure 7A**, square2) and for neurons (**Figure 7A**, square3) separately. This revealed a specifically high ratio of F- to G-actin at the ventricular surface of WT cortices, where AJs are positioned. The area of progenitors within the tissue and neurons showed lower ratios of F to G-actin. The pronounced high ratio of F- to G-actin at the ventricular surface was much reduced in the α-cat cKO cortex and F- to G-actin ratios were rather homogeneous throughout the mutant cortex (**Figures 7B,C**). In contrast, F- to G-actin ratios remained unaltered in the GE where α-E-catenin expression is maintained (**Figure 7C**). These results may indicate a generally higher instability of actin filaments in the absence of α-E-catenin. The anchorage of the actin filaments to the AJs by α-E-catenin therefore may be critically required for the maintenance of a radial morphology in cortical progenitor cells.

DISCUSSION

α-E-CATENIN cKO RESULTS IN FORMATION OF DOUBLE-CORTEX

This analysis of α-E-catenin function in the developing cerebral cortex revealed a surprising result, the formation of a prominent double-cortex or SBH that was not reported in previous work deleting α-E-catenin in the entire developing nervous system (Lien et al., 2006, 2008; Stocker and Chenn, 2006, 2009). Interestingly, neurons of all types except layer I were sequentially generated in the α-E-catenin cKO cortex and distributed in both the upper cortex and the lower heterotopia, suggesting that some neurons, even late generated, and destined for the upper

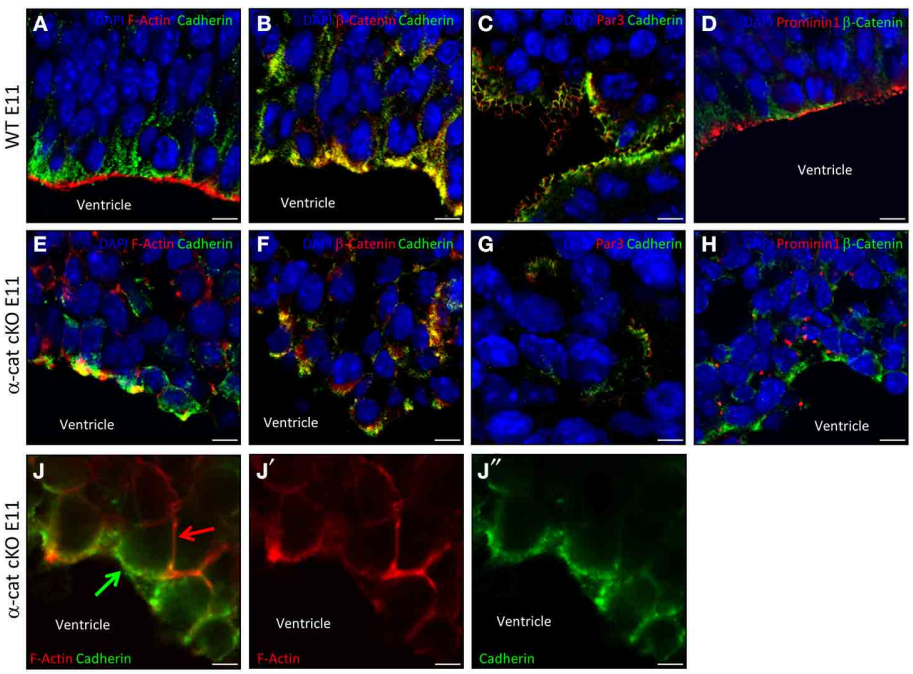


FIGURE 6 | Adhesion and polarity upon loss of α-catenin. Fluorescence micrographs of coronal cortical sections of E11 WT **(A–D)** and α-cat cKO **(E–J)** immunostained as indicated. Arrows indicate uncoupled AJs (green) and F-actin (red) in α-cat cKO cortices **(J)**. Scale bars: 10 μm **(A–H)**, 5 μm **(J–J″)**. Student's *t*-test, $^{*}P < 0.05$.

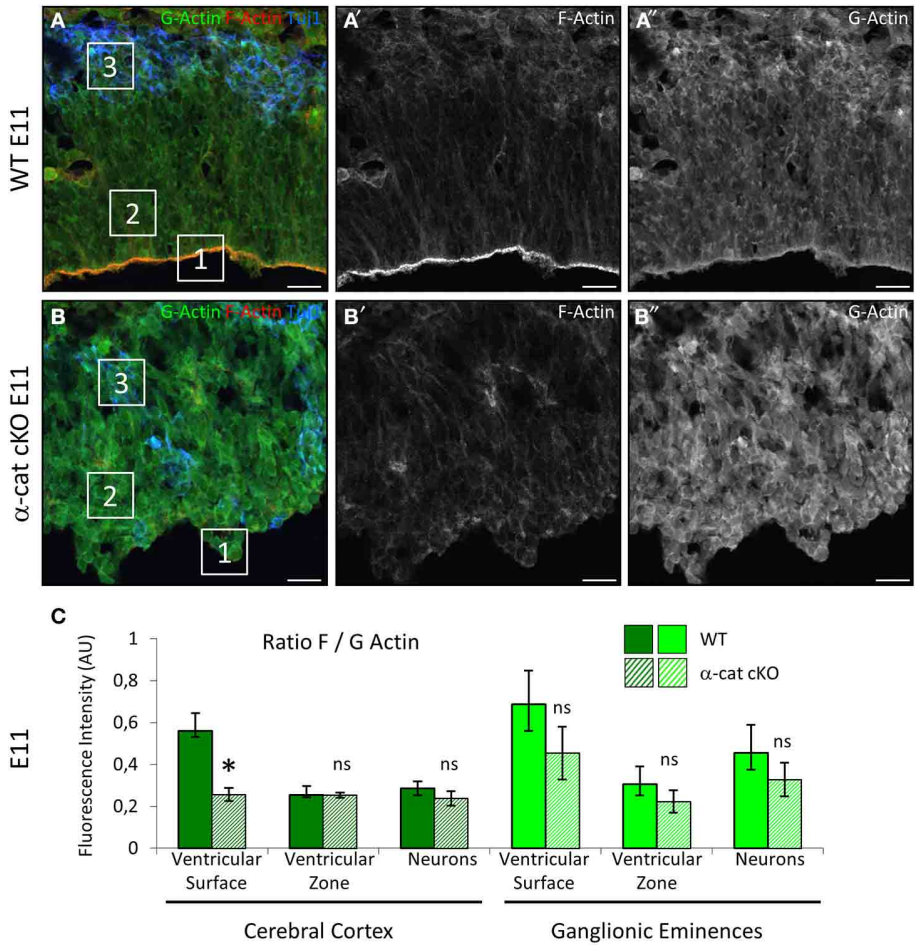


FIGURE 7 | Alterations in in the actin cytoskeleton. Visualization of F- and G-actin in E11 WT and α-cat cKO cortices **(A,B)**. The ratios of F to G-actin were quantified by confocal microscopy **(C)**. Squares in **(A)** and **(B)** exemplify the regions in which the intensity of F- and G actin was measured. Student's *t*-test, $^{*}P < 0.05$. Scale bars: 20 μm.

layers, can reach their position in the normal cortex and migrate on top of the previously generated deep layers. Conversely other neurons generated at the same time settle in a rather more clustered/nuclear manner in the ectopic lower cortex. Interestingly, this phenotype closely resembles the conditional deletion of RhoA (Cappello et al., 2012) and thus suggests common mechanisms at the base of double-cortex formation.

In addition this phenotype also resembles the subcortical band heterotopia described in other mutants, like the HeCo mutant (Croquelois et al., 2009; Kielar et al., 2014) and the conditional mutant RA-GEF-1, a guanine nucleotide exchange factor (GEF) for Rap1 (Bilasy et al., 2009). While the latter mutant has not been yet analyzed in detail, the cellular mechanism of the double-cortex formation appears to be rather comparable in the HeCo, RhoA, and α-E-catenin mutants.

RADIAL GLIAL CELL DEFECTS AT THE BASE OF DOUBLE-CORTEX FORMATION

Both RhoA and α-E-catenin mutants have common morphological defects of radial glial cells. These occur at the apical side due to uncoupling of the AJs and the actin cytoskeleton. Already at E11, 1 day after recombination by $Emx1^{Cre}$, the architecture of the cerebral cortex is disrupted in the α–cat cKO and the morphology of the radial glial cells is strongly affected. Later on, at E13, it is already possible to notice that RC2+ processes are not anymore spanning from the apical to the basal side but start forming round-shape structures, namely rosettes (see **Figure 5H**). These rosettes-like structures persist later in development, at P0 (see **Figure 2D**) and accumulate largely in the heterotopic cortex (see **Figure 2E**). But why do rosettes form?

From the earliest stages of either α-E-catenin or RhoA deletion, the ventricular lining, normally kept together by junctional coupling, starts to get disorganized. In both mutants cadherins can still bind to each other and form nascent AJs as observed by ultrastructural analysis. However these can no longer sustain larger pressure exerted by growth and bulging out of the dorsal telencephalon. In both, the conditional RhoA and α-E-catenin mutants this displaces clusters of radial glial cells that are still coupled to areas of less pressure namely inside the cerebral cortex thereby forming rosettes. Within these rosettes radial glial cells

are still coupled by adherens junctional belts and have an apical membrane domain oriented toward the lumen of the rosette. These cells generate neurons migrating to the outer region of the rosette where they then often settle. This is the basis of the formation of the lower double-cortex with neurons arranged in clusters. Notably, the white matter of these rosettes does not localize, as is the case in the normal cortex, between the cell bodies of radial glial cells and the neurons, but around the rosettes, confining the physical space of a rosette. This may be due to the shorter length of the radial glial cells and radial extension of the rosettes and/or the strength by which the radial processes could resist a fiber tract forming between their basal and apical anchoring (see also below). Indeed, where and how the basal processes of the radial glial cells in the rosettes anchor is still ill understood. One possibility is the basement membrane at blood vessels in the vicinity but this remains to be shown.

Some rosettes, however, also get to be localized at more superficial positions with radial glial cells still extending some shorter processes to the surface (see also Cappello et al., 2012). This allows some neurons even generated at late stages still to reach the upper, normotopic part of the cortex. Importantly, these neurons can then migrate through pre-existing layers settling on top, as both in the RhoA, α-E-catenin cKO, and HeCo mutants neurons in the normotopic cortex are organized in an inside first outside later layering.

Interestingly, the comparison between the RhoA (Cappello et al., 2012) and a-cat cKO shows that the timing of rosette formation seemingly determines the size of the double-cortex. In the α-E-catenin mutant the rupture of the apical surface and rosette formation occurs earlier (already around E11/12), while this reorganization takes longer in the RhoA cKO (Cappello et al., 2012). As a consequence more neurons manage to follow intact radial glial processes in the RhoA cKO and reach the correct position in the cortical plate and therefore a thicker normotopic cortex is formed compared to the α-E-catenin mutant (Cappello et al., 2012). According to the earlier defects the α-E-catenin cortex displays a very large heterotopia and a thin layered cortex which is probably why it was overlooked in earlier studies. Accordingly, in the α-E-catenin double-cortex model, also the early-generated subplate neurons are clearly localized in both the upper cortex and the large heterotopia. On the contrary both the HeCo and RhoA mutants have a larger upper layered cortex and smaller heterotopia compared to the α-E-catenin mutant (Croquelois et al., 2009; Cappello et al., 2012).

EFFECTS ON THE ACTIN CYTOSKELETON AFFECT THE MAINTENANCE OF ADHERENS JUNCTIONS STRENGTH AND RADIAL PROCESSES

The earlier onset of the phenotype in the α-cat cKO compared to the RhoA cKO may be due to the slightly longer perdurance of the RhoA protein (no longer detectable by immunostaining at E12) compared to α-E-catenin (no longer detectable by immunostaining at E11). As the same Cre-line was used for genetic recombination this may be due to protein stability or differences in the efficiency of recombining the different loci. In addition, the functional consequences for the strength of AJ coupling may be more severe when α-E-catenin is not present as the key anchoring molecule between the cadherins at the cell surface and the F-actin belts is lost. RhoA clearly affects the stabilization of actin filaments (Govek et al., 2011; Cappello et al., 2012) but even after complete loss of the protein this may result in less severe defects than the loss of α-E-catenin.

Interestingly, the formation and/or stabilization of actin filaments is also deficient upon loss of α-E-catenin. This may be a consequence of the uncoupling between AJs and actin filaments. As less F-actin is engaged in anchoring to the AJs to the cytoskeleton, actin filaments are disassembled and produce more globular actin. However, actin instability is also the cause for destabilization of the radial processes where no AJs are present, suggesting that the loss of α-E-catenin has effects on the actin filaments throughout the cell. It is also possible that α-E-catenin regulates the dynamics of actin by modulating the activity of Arp2/3 (Drees et al., 2005) or by binding formin-1, an actin nucleator (Kobielak et al., 2004). Indeed, this is the same in the RhoA cKO, where radial glia processes are also destabilized due to changes in the F- to G-actin ratio (Cappello et al., 2012). Upon loss of RhoA also the stabilization of microtubules is affected, suggesting another possible mechanism mediated by microtubules. This is consistent with the requirement of fine-tuning the microtubules stability for migration and process formation in neurons (Creppe et al., 2009; Heng et al., 2010). Moreover, in order to preserve the proper morphology, radial glial cells may require a general stabilization of the cytoskeleton that can be maintained either by actin fibers or stable microtubules (Li et al., 2003; Vreugdenhil et al., 2007) or the intermediate filaments. Intriguingly in both RhoA and α-E-catenin mutants, as well as in the HeCo mutant, Nestin (RC2) was severely downregulated.

Neurons may also change the direction of migration upon cytoskeleton destabilization as for instance Lamellipodin depletion caused pyramidal neurons to adopt a tangential, rather than radial glial guided migration mode by reducing the activity of Serum Response Factor and therefore the ratio of polymerized to unpolymerized actin (Pinheiro et al., 2011).

All together these data suggest that a general destabilization of the cytoskeleton, either actin or microtubules or both, in radial glial cells and neurons can influence their key role in maintaining tissue architecture and their radial processes. Either change can be fatal for neurons that use the glial cells as a guidance to find the cortical plate.

ACKNOWLEDGMENTS

We would like to thank Detlef Franzen, Timucin Öztürk, Angelika Waiser, Andrea Steiner-Mezzadri, Markus Körbs, and Elsa Melo for excellent technical help. This work was supported by the DFG, BMBF and the Helmholtz Association.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00215/abstract

Figure S1 | Characterization of the SBH cell identity in the α-cat cKO cortex. Micrographs of coronal sections of P0 WT **(A,A′)** and α-cat cKO **(B,B′)**. Micrographs depict Nurr1 immuno- and DAPI- staining as indicated in the panels. Scale bars: 100 μm.

Figure S2 | Adherens Junctions upon loss of α-catenin. Electron micrographs of WT **(A)** and α-cat cKO **(B,B′)** E11 cortices revealing electrondense AJs (indicated by arrows). Ventricle is down in **(A)**. **(B′)**: high magnification of boxed area in **(B)**. Scale bars: 500 nm.

Figure S3 | Par complex upon loss of α-catenin. Western Blot of cortical tissue from E11 WT and α-cat cKO. Primary antibodies used for the comparison are indicated above the lanes, lower strips blotted for β-actin serve as loading controls.

REFERENCES

Armentano, M., Chou, S. J., Tomassy, G. S., Leingartner, A., O'Leary, D. D., and Studer, M. (2007). COUP-TFI regulates the balance of cortical patterning between frontal/motor and sensory areas. *Nat. Neurosci.* 10, 1277–1286. doi: 10.1038/nn1958

Barkovich, A. J., Guerrini, R., Kuzniecky, R. I., Jackson, G. D., and Dobyns, W. B. (2012). A developmental and genetic classification for malformations of cortical development: update 2012. *Brain J. Neurol.* 135, 1348–1369. doi: 10.1093/brain/aws019

Bilasy, S. E., Satoh, T., Ueda, S., Wei, P., Kanemura, H., Aiba, A., et al. (2009). Dorsal telencephalon-specific RA-GEF-1 knockout mice develop heterotopic cortical mass and commissural fiber defect. *Eur. J. Neurosci.* 29, 1994–2008. doi: 10.1111/j.1460-9568.2009.06754.x

Bozzi, Y., Casarosa, S., and Caleo, M. (2012). Epilepsy as a neurodevelopmental disorder. *Front. Psychiatry* 3:19. doi: 10.3389/fpsyt.2012.00019

Britanova, O., Akopov, S., Lukyanov, S., Gruss, P., and Tarabykin, V. (2005). Novel transcription factor Satb2 interacts with matrix attachment region DNA elements in a tissue-specific manner and demonstrates cell-type-dependent expression in the developing mouse CNS. *Eur. J. Neurosci.* 21, 658–668. doi: 10.1111/j.1460-9568.2005.03897.x

Cappello, S. (2013). Small Rho-GTPases and cortical malformations: fine-tuning the cytoskeleton stability. *Small GTPases* 4, 6. doi: 10.4161/sgtp.23093

Cappello, S., Attardo, A., Wu, X., Iwasato, T., Itohara, S., Wilsch-Brauninger, M., et al. (2006). The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. *Nat. Neurosci.* 9, 1099–1107. doi: 10.1038/nn1744

Cappello, S., Bohringer, C. R., Bergami, M., Conzelmann, K. K., Ghanem, A., Tomassy, G. S., et al. (2012). A radial glia-specific role of RhoA in double cortex formation. *Neuron* 73, 911–924. doi: 10.1016/j.neuron.2011.12.030

Cappello, S., Gray, M. J., Badouel, C., Lange, S., Einsiedler, M., Srour, M., et al. (2013). Mutations in genes encoding the cadherin receptor-ligand pair DCHS1 and FAT4 disrupt cerebral cortical development. *Nat. Genet.* 45, 1300–1308. doi: 10.1038/ng.2765

Chanas-Sacre, G., Thiry, M., Pirard, S., Rogister, B., Moonen, G., Mbebi, C., et al. (2000). A 295-kDA intermediate filament-associated protein in radial glia and developing muscle cells *in vivo* and *in vitro*. *Dev. Dyn.* 219, 514–525. doi: 10.1002/1097-0177(2000)9999:9999<::AID-DVDY1078>3.0.CO;2-0

Chapouton, P., Schuurmans, C., Guillemot, F., and Gotz, M. (2001). The transcription factor neurogenin 2 restricts cell migration from the cortex to the striatum. *Development* 128, 5149–5159.

Costa, M. R., Wen, G., Lepier, A., Schroeder, T., and Gotz, M. (2008). Par-complex proteins promote proliferative progenitor divisions in the developing mouse cerebral cortex. *Development* 135, 11–22. doi: 10.1242/dev.009951

Creppe, C., Malinouskaya, L., Volvert, M. L., Gillard, M., Close, P., Malaise, O., et al. (2009). Elongator controls the migration and differentiation of cortical neurons through acetylation of alpha-tubulin. *Cell* 136, 551–564. doi: 10.1016/j.cell.2008.11.043

Croquelois, A., Giuliani, F., Savary, C., Kielar, M., Amiot, C., Schenk, F., et al. (2009). Characterization of the HeCo mutant mouse: a new model of subcortical band heterotopia associated with seizures and behavioral deficits. *Cereb. Cortex* 19, 563–575. doi: 10.1093/cercor/bhn106

Drees, F., Pokutta, S., Yamada, S., Nelson, W. J., and Weis, W. I. (2005). alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* 123, 903–915. doi: 10.1016/j.cell.2005.09.021

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bulfone, A., et al. (2005). Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J. Neurosci.* 25, 247–251. doi: 10.1523/JNEUROSCI.2899-04.2005

Gotz, M., and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* 6, 777–788. doi: 10.1038/nrm1739

Govek, E. E., Hatten, M. E., and Van Aelst, L. (2011). The Role of Rho GTPase Proteins in CNS Neuronal Migration. *Dev. Neurobiol.* 71, 528–553. doi: 10.1002/dneu.20850

Harris, T. J. C., and Tepass, U. (2010). Adherens junctions: from molecules to morphogenesis. *Nat. Rev. Mol. Cell Bio.* 11, 502–514. doi: 10.1038/nrm2927

Haubensak, W., Attardo, A., Denk, W., and Huttner, W. B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3196–3201. doi: 10.1073/pnas.0308600100

Heng, J. I., Chariot, A., and Nguyen, L. (2010). Molecular layers underlying cytoskeletal remodelling during cortical development. *Trends Neurosci.* 33, 38–47. doi: 10.1016/j.tins.2009.09.003

Iwasato, T., Datwani, A., Wolf, A. M., Nishiyama, H., Taguchi, Y., Tonegawa, S., et al. (2000). Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the barrel cortex. *Nature* 406, 726–731. doi: 10.1038/35021059

Jessell, T. M., and Sanes, J. R. (2000). Development. The decade of the developing brain. *Curr. Opin. Neurobiol.* 10, 599–611. doi: 10.1016/S0959-4388(00)00136-7

Kadowaki, M., Nakamura, S., Machon, O., Krauss, S., Radice, G. L., and Takeichi, M. (2007). N-cadherin mediates cortical organization in the mouse brain. *Dev. Biol.* 304, 22–33. doi: 10.1016/j.ydbio.2006.12.014

Kielar, M., Tuy, F. P. D., Bizzotto, S., Lebrand, C., Romero, C. D., Poirier, K., et al. (2014). Mutations in Eml1 lead to ectopic progenitors and neuronal heterotopia in mouse and human. *Nat. Neurosci.* 17, 923–933. doi: 10.1038/Nn.3729

Kobielak, A., Pasolli, H. A., and Fuchs, E. (2004). Mammalian formin-1 participates in adherens junctions and polymerization of linear actin cables. *Nat. Cell Biol.* 6, 21–30. doi: 10.1038/ncb1075

Leone, D. P., Srinivasan, K., Chen, B., Alcamo, E., and McConnell, S. K. (2008). The determination of projection neuron identity in the developing cerebral cortex. *Curr. Opin. Neurobiol.* 18, 28–35. doi: 10.1016/j.conb.2008.05.006

Li, H. D., Berlin, Y., Hart, R. P., and Grumet, M. (2003). Microtubules are critical for radial glial morphology: possible regulation by MAPs and MARKs. *Glia* 44, 37–46. doi: 10.1002/glia.10267

Lien, W. H., Klezovitch, O., Fernandez, T. E., Delrow, J., and Vasioukhin, V. (2006). alphaE-catenin controls cerebral cortical size by regulating the hedgehog signaling pathway. *Science* 311, 1609–1612. doi: 10.1126/science.1121449

Lien, W. H., Klezovitch, O., Null, M., and Vasioukhin, V. (2008). alpha E-catenin is not a significant regulator of beta-catenin signaling in the developing mammalian brain. *J. Cell Sci.* 121, 1357–1362. doi: 10.1242/jcs.020537

Marzesco, A. M., Janich, P., Wilsch-Brauninger, M., Dubreuil, V., Langenfeld, K., Corbeil, D., et al. (2005). Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. *J. Cell Sci.* 118, 2849–2858. doi: 10.1242/jcs.02439

Merdek, K. D., Nguyen, N. T., and Toksoz, D. (2004). Distinct activities of the alpha-catenin family, alpha-catulin and alpha-catenin, on beta-catenin-mediated signaling. *Mol. Cell. Biol.* 24, 2410–2422. doi: 10.1128/MCB.24.6.2410-2422.2004

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T., and Ogawa, M. (2004). Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development* 131, 3133–3145. doi: 10.1242/dev.01173

Nieto, M., Monuki, E. S., Tang, H., Imitola, J., Haubst, N., Khoury, S. J., et al. (2004). Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex. *J. Comp. Neurol.* 479, 168–180. doi: 10.1002/cne.20322

Noctor, S. C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* 7, 136–144. doi: 10.1038/nn1172

Park, C., Falls, W., Finger, J. H., Longo-Guess, C. M., and Ackerman, S. L. (2002). Deletion in Catna2, encoding alpha N-catenin, causes cerebellar and hippocampal lamination defects and impaired startle modulation. *Nat. Genet.* 31, 279–284. doi: 10.1038/ng908

Pinheiro, E. M., Xie, Z., Norovich, A. L., Vidaki, M., Tsai, L. H., and Gertler, F. B. (2011). Lpd depletion reveals that SRF specifies radial versus tangential migration of pyramidal neurons. *Nat. Cell Biol.* 13, 989–995. doi: 10.1038/ncb2292

Pokutta, S., Drees, F., Yamada, S., Nelson, W. J., and Weis, W. I. (2008). Biochemical and structural analysis of alpha-catenin in cell-cell contacts. *Biochem. Soc. Trans.* 36, 141–147. doi: 10.1042/BST0360141

Rasin, M. R., Gazula, V. R., Breunig, J. J., Kwan, K. Y., Johnson, M. B., Liu-Chen, S., et al. (2007). Numb and Numbl are required for maintenance of cadherin-based

adhesion and polarity of neural progenitors. *Nat. Neurosci.* 10, 819–827. doi: 10.1038/nn1924

Srinivasan, K., Roosa, J., Olsen, O., Lee, S. H., Bredt, D. S., and McConnell, S. K. (2008). MALS-3 regulates polarity and early neurogenesis in the developing cerebral cortex. *Development* 135, 1781–1790. doi: 10.1242/dev.013847

Stocker, A. M., and Chenn, A. (2006). Differential expression of alpha-E-catenin and alpha-N-catenin in the developing cerebral cortex. *Brain Res.* 1073–1074, 151–158. doi: 10.1016/j.brainres.2005.12.057

Stocker, A. M., and Chenn, A. (2009). Focal reduction of alphaE-catenin causes premature differentiation and reduction of beta-catenin signaling during cortical development. *Dev. Biol.* 328, 66–77. doi: 10.1016/j.ydbio.2009.01.010

Stoykova, A., Hatano, O., Gruss, P., and Gotz, M. (2003). Increase in reelin-positive cells in the marginal zone of Pax6 mutant mouse cortex. *Cereb. Cortex* 13, 560–571. doi: 10.1093/cercor/13.6.560

Suzuki, A. and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. *J. Cell Sci.* 119, 979–987. doi: 10.1242/jcs.02898

Uemura, M., and Takeichi, M. (2006). Alpha N-catenin deficiency causes defects in axon migration and nuclear organization in restricted regions of the mouse brain. *Dev. Dyn.* 235, 2559–2566. doi: 10.1002/dvdy.20841

Vasioukhin, V., Bauer, C., Degenstein, L., Wise, B., and Fuchs, E. (2001). Hyperproliferation and defects in epithelial polarity upon conditional ablation of alpha-catenin in skin. *Cell* 104, 605–617. doi: 10.1016/S0092-8674(01)00246-X

Vreugdenhil, E., Kolk, S. M., Boekhoorn, K., Fitzsimons, C. P., Schaaf, M., Schouten, T., et al. (2007). Doublecortin-like, a microtubule-associated protein expressed in radial glia, is crucial for neuronal precursor division and radial process stability. *Eur. J. Neurosci.* 25, 635–648. doi: 10.1111/j.1460-9568.2007.05318.x

Weigmann, A., Corbeil, D., Hellwig, A., and Huttner, W. B. (1997). Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 94 12425–12430. doi: 10.1073/pnas.94.23.12425

Wu, S. X., Goebbels, S., Nakamura, K., Nakamura, K., Kometani, K., Minato, N., et al. (2005). Pyramidal neurons of upper cortical layers generated by NEX-positive progenitor cells in the subventricular zone. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17172–17177. doi: 10.1073/pnas.0508560102

Zimmer, C., Tiveron, M. C., Bodmer, R., and Cremer, H. (2004). Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons. *Cereb. Cortex* 14, 1408–1420. doi: 10.1093/cercor/bhh102

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 May 2014; paper pending published: 22 May 2014; accepted: 16 July 2014; published online: 07 August 2014.

Citation: Schmid M-T, Weinandy F, Wilsch-Bräuninger M, Huttner WB, Cappello S and Götz M (2014) The role of **α***-E-catenin in cerebral cortex development: radial glia specific effect on neuronal migration. Front. Cell. Neurosci.* ***8****:215. doi: 10.3389/fncel.2014.00215*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 21 November 2014
doi: 10.3389/fncel.2014.00393

TGF-β1 promotes cerebral cortex radial glia-astrocyte differentiation *in vivo*

Joice Stipursky *, Daniel Francis, Rômulo Sperduto Dezonne, Ana Paula Bérgamo de Araújo, Lays Souza, Carolina A. Moraes and Flávia Carvalho Alcantara Gomes *

Laboratório de Neurobiologia Celular, Programa de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro - Centro de Ciências da Saúde, Rio de Janeiro, RJ, Brazil

Edited by:
Marcos R. Costa, Federal University of Rio Grande do Norte, Brazil

Reviewed by:
Luisa Pinto, University of Minho, Portugal
Silvia Cappello, Ludwig Maximilian University of Munich, Germany

****Correspondence:***
Joice Stipursky and Flávia Carvalho Alcantara Gomes, Laboratório de Neurobiologia Celular, Programa de Biologia Celular e do Desenvolvimento, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro - Centro de Ciências da Saúde, Bloco F, Sala F15, Ilha do Fundão, Rio de Janeiro, RJ 21949-590, Brazil
e-mail: joice@icb.ufrj.br; fgomes@icb.ufrj.br

The major neural stem cell population in the developing cerebral cortex is composed of the radial glial cells, which generate glial cells and neurons. The mechanisms that modulate the maintenance of the radial glia (RG) stem cell phenotype, or its differentiation, are not yet completely understood. We previously demonstrated that the transforming growth factor-β1 (TGF-β1) promotes RG differentiation into astrocytes *in vitro* (Glia 2007; 55:1023-33) through activation of multiple canonical and non-canonical signaling pathways (Dev Neurosci 2012; 34:68-81). However, it remains unknown if TGF-β1 acts in RG-astrocyte differentiation *in vivo*. Here, we addressed the astrogliogenesis induced by TGF-β1 by using the intraventricular *in utero* injection *in vivo* approach. We show that injection of TGF-β1 in the lateral ventricles of E14,5 mice embryos resulted in RG fibers disorganization and premature gliogenesis, evidenced by appearance of GFAP positive cells in the cortical wall. These events were followed by decreased numbers of neurons in the cortical plate (CP). Together, we also described that TGF-β1 actions are region-dependent, once RG cells from dorsal region of the cerebral cortex demonstrated to be more responsive to this cytokine compared with RG from lateral cortex either *in vitro* as well as *in vivo*. Our work demonstrated that TGF-β1 is a critical cytokine that regulates RG fate decision and differentiation into astrocytes *in vitro* and *in vivo*. We also suggest that RG cells are heterogeneous population that acts as distinct targets of TGF-β1 during cerebral cortex development.

Keywords: radial glia, TGF-β, gliogenesis, neurogenesis, cerebral cortex

INTRODUCTION

Radial glia (RG) cells are considered the major progenitor cell population present in the developing cerebral cortex (Kriegstein and Alvarez-Buylla, 2009).These cells have a long radial fiber that elongate from its cell body, in the ventricular zone (VZ), through the entire developing cortical wall. During the initial steps of brain development, RG cells, which are derived from the neuroepithelium, are actively proliferative cells and, by asymmetric divisions, originate neurons that migrate along their radial fibers to their specific layers at the cortical plate (CP). By the end of the neuronal migratory period, RG cells arrest their cycle and differentiate into cortical astrocytes (Munoz-Garcia and Ludwin, 1986; Voigt, 1989; Culican et al., 1990; Bentivoglio and Mazzarello, 1999; Miyata et al., 2001; Noctor et al., 2001, 2002; Götz et al., 2002; Malatesta et al., 2003; Anthony et al., 2004).

Although characteristics of RG cells such as, self-renewal and progenitor capacity, have been assured, it is widely discussed if these features can be attributed to all RG cells of the embryonic brain, or if it is restricted to specific populations of these cells (Pinto and Götz, 2007). Heterogeneity in RG cells has been described along the telencephalon regions, revealed by distinct expression of the transcription factors Pax6, Emx2 and FoxG1, which confers to these cells their neurogenic or gliogenicprogenitor property (Kriegstein and Götz, 2003; Hevner et al., 2006; Pinto and Götz, 2007). These transcription factors have been reported to be under the control of a combination of morphogen gradients along the developing axes, which determines specific telencephalon region territories (O'Leary and Sahara, 2008).

Abbreviations: BLBP, brain lipid binding protein; BMP, bone morphogenetic protein; BrdU, bromodeoxiuridine; BSA, bovine serum albumin; CNS, central nervous system; CP, cortical plate; DAPI, 4_,6-diamidino-2-phenylindole dihydrochloride; DMc, dorsomedial cortex; DMEM/F12, Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12; E14, embryonic day fourteen; E18, embryonic day eighteen; P0, postnatal day 0; ECM, extracellular matrix; EGF, epidermal growth factor; FGFb, basic fibroblast growth factor; GFAP, glial fibrillary acidic protein; IDM, intermediate differentiating morphology; Lc, lateral cortex; LV, lateral ventricle; MAPK, mitogen-activated protein kinase; PBS, phosphate-bufferedsaline; PI3K, phosphatidylinositol-3 kinase; RG, radial glia; SMAD, homologue protein for SMA protein from *C. elegans* and mothers against decapentaplegic (MAD) from *Drosophila*; SVZ, subventricular zone; TBS-T, Tris-bufferedsaline- Tween20; Tc, total cortex; TGFRII, transforming growth factor beta type II receptor; TGF-β1, transforming growth factor beta 1; VZ, ventricular zone.

RG-astrocyte differentiation is a well-recognized event, however the mechanisms and molecules that control generation of different pools of astrocytes and neurons are still elusive. Several lines of evidence suggest that increasing neuronal pools play essential role in the control of RG maintenance and/or differentiation (Hunter and Hatten, 1995; Anton et al., 1997; Nakashima et al., 1999; Mi et al., 2001; Takizawa et al., 2001; Uemura et al., 2002; Patten et al., 2003; Schmid et al., 2003; Nishino et al., 2004; Barnabé-Heider et al., 2005; He et al., 2005; Stipursky and Gomes, 2007; Stipursky et al., 2012a). Although several soluble factors were demonstrated to control astrocytogenesis during CNS development such as leukemia inhibitor factors (LIFs) of the interleukin-6 (IL-6) family, including CNTF, LIF, and Cardiotrophin-1 (CT-1) (for revision see Stipursky et al., 2009), the role of neuronal derived soluble factors on RG-astrocyte transformation is still poorly known.

We previously reported that cerebral cortex neurons induce RG-astrocyte differentiation *in vitro* through secretion of the transforming growth factor-β1 (TGF-β1; Stipursky and Gomes, 2007; Stipursky et al., 2012a).

TGF-β1 is a multifunctional cytokine, present virtually in all tissues, that controls multiple biological and pathological events such as embryogenesis, immune response, extracellular matrix protein (ECM) production, cell differentiation and cell-cycle control in different tissues (Massagué, 1998; Massagué and Gomis, 2006). In the CNS, TGF-β1 has been reported to play key function in neuronal generation, survival and migration (Brionne et al., 2003; Miller, 2003; Espósito et al., 2005), glial differentiation(Sousa Vde et al., 2004; Romão et al., 2008), and synapse formation (Diniz et al., 2012, 2014).

TGF-β1 signaling might be mediated by the canonical pathway that involves SMADs2/3 and SMAD4 transcription factors or non-canonical signaling pathways, that involve the RasGTPAses, mitogen-activated protein kinase (MAPK), or phosphatidylinositol-3 kinase (PI3K) proteins (Javelaud and Mauviel, 2005; Massagué and Gomis, 2006). We previously reported that TGF-β1 controls RG differentiation into neurons and astrocytes by activation of SMADs/PI3K and MAPK, respectively, in distinct RG subpopulations *in vitro* (Stipursky et al., 2012a).

Although the presence of different isoforms of TGF-β molecules have already been described in the proliferative zones of the embryonic cerebral cortex (Mecha et al., 2008), there are few data regarding the expression, modulation and distribution of TGF-β receptors in RG cells *in vivo*. Further, the mechanisms that modulate neurogenesis to gliogenesis switch of RG induced by TGF-β1 are still unknown.

Here, we investigated the role of TGF-β1 on RG-astrocyte switch in the developing cerebral cortex and the implications of RG heterogeneity to this event. We showed that TGF-β1 induces premature gliogenesis and disrupts RG polarity mainly in the dorsomedial area of the cerebral cortex. For the first time, we provide evidence that specific RG subpopulations distinctly respond to TGF-β1 *in vivo*.

METHODS

ETHICAL APPROVAL

All animal protocols were approved by the Animal Research Committee of the Federal University of Rio de Janeiro (DAHEICB024).

RG CELL CULTURES

Gestational day 14 Swiss mice embryos were collected and dissected for cerebral cortex separation. After dissection tissues were dissociated in DMEM/F12 (Invitrogen) medium and after cell counting, 105 cells were plated in 25 cm^2 culture bottles in neurosphere growing media DMEM/F12 containing 1% glutamine, 0.1% de penicillin/streptomycin, 2% B27 (Invitrogen), 20 ng/mL EGF (Epidermal growth factor, Invitrogen) and 20 ng/mL FGFb (basic Fibroblast growth factor, R&D Systems), for 6 days, *in vitro*. The 2/3 of the media was changed every 2 days. After this period, neurospheres were enzymatically dissociated in 0.05% Trypsin/EDTA (Invitrogen), and 105 RG isolated cells were plated in glass coverslips previously coated with 50 μg/mL with poli-L-lisin (Invitrogen) and 10 μg/mL laminin (Invitrogen) in 24 wells culture plates. Cells were kept in DMEM/F12 containing 1% glutamine, 0.1% penicillin/streptomycin, 2% de B27 (Invitrogen), 20 ng/mL EGF (Invitrogen) and 20 ng/mL FGFb (R&D Systems) for 24 h. After this period, cells were treated with 10 ng/mL of TGF-β1 (R&D Systems) or 10 μm of SB431542 (Sigma Aldrich) in medium, without mitogenic factors, for 24 h.

IN UTERO INTRAVENTRICULAR INJECTION

In utero intraventricular injections of E14 mice embryos were performed as described by Walantus et al. (2007). Pregnant Swiss mice in the 14 gestational day were anesthetized with intraperitoneal injection of 2-2-2 Tribromoethanol (Sigma Aldrich) 1 mg/g of body weight. After anesthesia, females were subjected to surgical procedure, in which the uterus was exposed. After visualization of the embryos, they were manually positioned to allow observation of brain hemispheres. Each embryo was subjected to intraventricular injection inside the lateral ventricles of 2 μl of control solution (PBS, 0.05% BSA, 0.025% Fast Green [Sigma Aldrich]), or solution containing 100 ng of TGF-β1 (R&D Systems) or 10 μM of SB431542 (Sigma Aldrich), using glass micropipettes. After injections, the uterus was repositioned inside abdominal cavity and abdominal muscle and skin layers sutured. Bromodeoxiridine (BrdU, Sigma Aldrich) was intraperitoneally injected in the preagnant mouse after 2 and 24 h of surgery, to follow cells generated from RG just after TGF-β1 stimulation and to analyze long lasting effects in RG population. Forty-eight hours after surgery, the female was sacrificed and embryos were perfused with ice cold 4% paraformaldehyde (PFA). Brains were collected and processed for immunohistochemistry and real time RT-PCR.

IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

After culture, cells were fixed in 4% PFA (Vetec) for 15 min. After this period, cells were extensively washed in PBS (phosphate buffered saline) and permeabilized with 0.2% Triton X-100 (Vetec) for 5 min at room temperature. Cells were then incubated with blocking buffer containing 3% serum bovine albumin (BSA),

5% normal goat serum (NGS) (Sigma Aldrich) diluted in PBS for 1 h, followed by 12 h incubation with primary antibodies at 4°C diluted in the same solution. After this period, cells were extensively washed in PBS and incubated with secondary antibodies for 2 h at room temperature. Nuclei were labeled with DAPI (4', 6-Diamidino-2-phenylindole; Sigma Aldrich), or Draq5 (Pierce). Glass coverslips were mounted in glass slides with Faramount mounting media (DakoCytomation), and stained cells were visualized using a fluorescent optical microscope Nikon TE3000. For immunohistochemistry, brain were fixed in 4% PFA for 48 h, and subjected to vibratome sectioning, to obtain 40 μm sections. After sectioning, floating brain slices were incubated with blocking buffer for 1 h under shaking. After incubation with primary antibodies for 12 h at 4°C, followed by extensive washing in PBS, slices were incubated with secondary antibodies for 2 h at room temperature under shaking. Primary antibodies were: mouse anti-Nestin (Chemicon, 1:100), rabbit anti-BLBP (Chemicon, 1:200), rabbit anti-ErbB2 (Santa Cruz Biotechnology, 1:200), rabbit anti-Notch1 (Cell Signaling, 1:500), rabbi anti-TGFRII (Santa Cruz Biotechnology, 1:100), rabbit anti- phophoSmad 2/3 (Santa Cruz Biotechnology, 1:50), rabbit anti-Laminin (Sigma Aldrich, 1:100), rabbit anti-GFAP (Dakocytomation, 1:500), mouse anti-β TubulinIII (Promega, 1:1,000), rabbit anti-Doublecortin (Abcam, 1:200), rati anti-BrdU (Accurate, 1:1,000), rabbit anti-Foxg1 (Santa Cruz Biotechnology, 1:200). Secondary antibodies were conjugated to AlexaFluor 488, AlexaFluor 546, and AlexaFluor 633 (Invitrogen Molecular Probes). Nissl Trace Green (Molecular Probes, 1:1,000) staining was used to label neuronal cell soma. Images of labeled tissue were obtained using a Leica SP5confocal microscope.

WESTERN BLOT

Protein levels were analyzed as previously described (Dezonne et al., 2013). After dissection, cerebral cortex tissues from Swiss mice embryos were lysed in RIPA buffer [20 mMTris-HCl (pH 7.5); 150 mMNaCl; 1 mM Na/EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 2.5 mM sodium pyrophosphate; 1 mMglycerophosphate; 1 mM Na_3VO_4; 1 μg/mLleupeptin]. Cell suspension was homogenized, sonicated, and centrifuged for 10 min at 14,000 rpm in a refrigerated centrifuge. Subsequently, the supernatant was collected and the protein dosage was performed using the BCA Protein Assay kit (Pierce, Rockford, Ill., USA). A total of 20 μg of protein was loaded per lane and submitted to electrophoretic separation in a 10% SDS-PAGE gel. After separation, proteins were electrically transferred onto a nitrocellulose transfer membrane (Protran, Dassel, Germany) for 1 h. The membrane was blocked in 5% BSA in Tris-buffered Tween 20 (TBS-T; Merck, Darmstadt, Germany) and primary antibody incubation was performed overnight at 4°C followed by peroxidase-conjugated secondary antibody incubation for 1 h at room temperature. Proteins were visualized using the enhancing chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, Ill., USA) and nitrocellulose membranes were exposed to autoradiographic films (Kodak, São José dos Campos, Brazil). Primary antibodies were: mouse phosphoSmad2 (Cell Signaling; 1:1,000), rabbit anti-ErbB2 (Santa Cruz Biotechnology 1:200); rabbit anti-TGFRII (Santa Cruz Biotechnology; 1:200); mouse anti-α-tubulin (Sigma Aldrich; 1: 5,000). The secondary peroxidase-conjugated antibodies were: goat anti-rabbit IgG and goat anti-mouse IgG (Amersham Biosciences, Piscataway, N.J., USA; 1: 3,000). After protein detection, densitometric analysis of autoradiographic films was done using Image J 1.48 software. Each experiment was done in triplicate, and proteins were loaded in triplicate in SDS-PAGE gel.

REAL TIME RT-PCR

Total RNA was isolated from embryonic mice cerebral cortex using Direct-zol™ RNA MiniPrep (ZymoReserch, USA) according to the protocol provided by the manufacturer, and quantified using NanoDrop ND-1000 Spectrophotometer ThermoFisherScientific, USA).Two micrograms of total RNA were reverse transcribed with RevertAid first Strand cDNA Synthesis Kit according to the manufacturer (Thermo Fisher Scientific, USA). Sense and antisense specific for FoxG1, and β-actin genes were used. β actin sense: TGG ATC GGT TCC ATC CTG G, anti-sense: GCA GCT CAG TAA CAG TCC GCC TAG A; FoxG1 sense: CGA CAA GAA GAA CGG CAA GTA CGA, anti-sense: AGC ACT TGT TGA GGG ACA GGT TGT. Sequences were verified to be specific using Gen Bank's BLAST (Altschul et al., 1997). Quantitative real-time RT-PCR was performed using Maxima SYBR green qPCR Master Mix (Thermo Scientific, USA). Reactions were per formed on ABI PRISM 7500 Real Time PCR System (Applied Biosystems). The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The amount of target genes expressed in a sample was normalized to the average of thee ndogenous control.

STATISTICAL ANALYSIS

Statistical analyses were done using one-way non-parametric ANOVA coupled with Tukey post-test by GraphPad Prism 4.0 software, and $P < 0.05$ was considered statistically significant. The experiments were performed in triplicate, and each result represents the mean of at least 4–6 animals analyzed.

RESULTS

RG CELLS ARE POTENTIAL TARGETS OF TGF-β1 *IN VIVO*

In order to investigate RG cells responsiveness to TGF-β1, we first identified the TGF-β receptor type II (TGFRII) in RG cells *in vitro* and *in vivo*. To do that, we performed RG isolation from neurospheres derived E14 mice embryos cerebral cortex. Under this culture condition, these cells present a typical radial morphology and label for Nestin, BLPB, Notch1 and ErbB2 (**Figures 1A–C**), attesting their RG cells phenotype. We also detected high staining for TGFRII in their membranes (**Figures 1D–F**). Treatment of RG culture with TGF-β1 induced phosphorylation and nuclear translocation of Smads2/3, a hallmark of TGF-β1 signaling pathway activation (**Figures 1G–J**).

Immunohistochemical assays of the mouse brain revealed that TGFRII is more robustly expressed in the VZ (ventricular zone) and CP (cortical plate) of E14 and in the same layers as well as in SVZ (subventricular zone) of E18 and P0 mice cerebral cortex (**Figures 1K–M, k′–m′**). We identified a punctate TGFRII

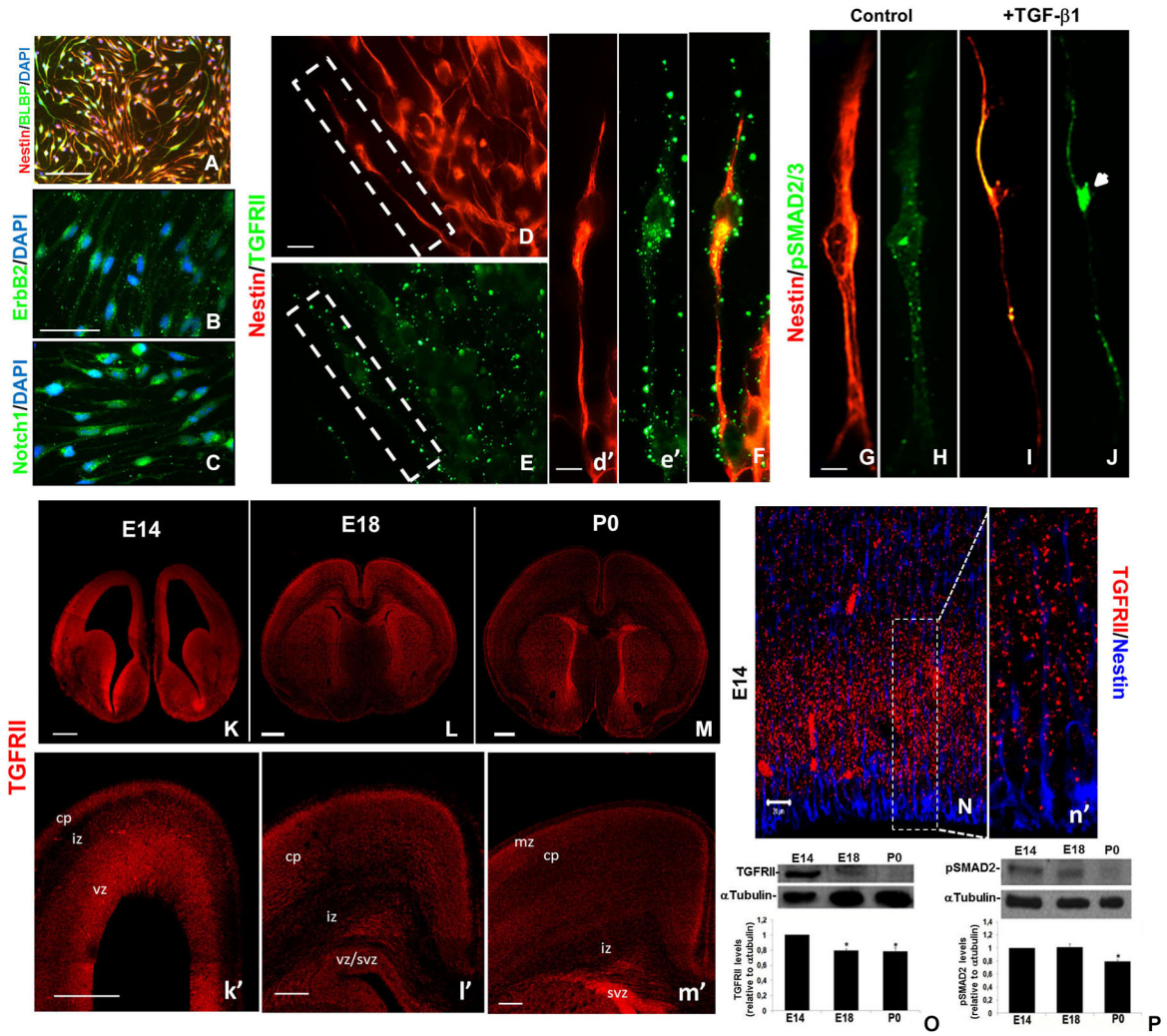


FIGURE 1 | RG cells express the TGF-β1 signaling pathway members. RG enriched cell cultures were isolated from cerebral cortex, grown into neurospheres and analyzed for specific cellular markers. Under these conditions the cells exhibit the typical RG cell elongated morphology and staining for specific markers: nestin/BLBP **(A)**; ErbB2 **(B)**; and Notch1 **(C)**. RG also expresses TGFRII in their membranes **(D–F)**. Treatment of these cells with 10 ng/mL of TGF-β1 induces phosphorylation and nuclear translocation of Smad2/3 **(G–J)**. Note that TGFRII is expressed predominantly in VZ/CP (E14),VZ/SVZ/CP (E18), and SVZ/CP (P0) in mice **(K–M, k'–m')**. TGFRII is distributed as a punctate pattern all over nestin+ RG cell bodies and fibers **(N, n')**. Western blotting assays revealed that either TGFRII **(O)** as phosphorylated Smad2 **(P)** is down regulated during cerebral cortex development. vz:ventricular zone, svz: subventricular zone, cp:cortical plate. $^*P < 0.05$. Scales: 50 μm **(A)**, 20 μm **(B)**, 10 μm **(D, I)** 500 μm **(K–M)**; 200 μm **(k'–m")** 20 μm **(N)**.

staining in RG cell body and processes in the E14 telencephalon (**Figures 1N,n'**). Western blotting analysis revealed that TGFRII is negatively modulated during development, since this protein is present at high levels in E14 telencephalon, is slightly detectable in E18 and tend to disappear in P0 (**Figure 1O**). The down regulation of TGFRII overlaps with the amount of phospho Smad2 at P0 (**Figure 1P**). Together, this data suggest that RG cells might be target of TGF-β1 actions *in vitro*, as well as *in vivo*.

INTRAVENTRICULAR INJECTION OF TGF-β1 DISRUPTS POLARITY OF RG CELLS

RG cell elongated morphology is a critic characteristic that allows neuronal migration and correct positioning in the CP within the different layers of the cerebral cortex (Rakic, 1971, 1995; Hatten, 1999; Yokota et al., 2007; Radakovits et al., 2009). Loss of this typical morphology is a hallmark of RG-astrocyte differentiation.

Intraventricular injection of TGF-β1 resulted in profound morphological alterations especially in the telencephalon, resulting in dilated lateral ventricles, and evident reduction of cortical wall thickness in dorsomedial (DMc) and lateral (Lc) areas of the cortex (**Figures 2A–E**).We also observed reduced VZ thickness in TGF-β-injected brains compared with vehicle solution injected brains (**Figure 2F**).These thickness reduction is observed along rostral to caudal regions of the cerebral cortex (data not shown).Interestingly, these morphological alterations

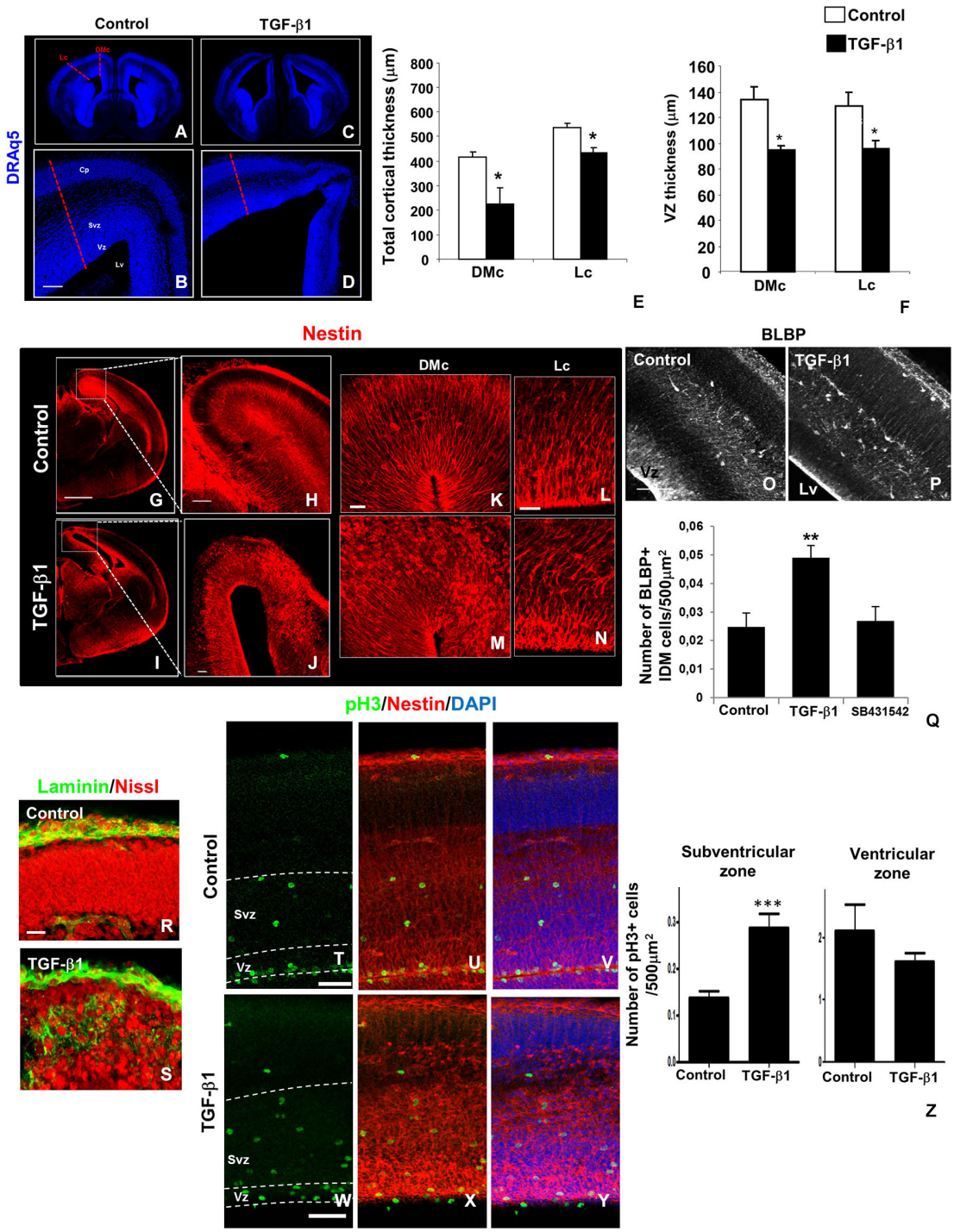


FIGURE 2 | TGF-β1 injection affects cerebral cortex and RG morphology. *In utero* intraventricular injection of TGF-β1 in mouse embryos (injection at E14 and analysis at E16) promoted several morphological alterations in the cerebral cortex wall in lateral (Lc) and dorsomedial (DMc) cortex areas **(A–D)**. TGF-β1 reduces the thickness of total cortical area **(E)** and VZ **(F)** in both regions. Note that TGF-β1 also disrupted nestin+ (red) radial fiber networks **(G–J)**, an event more prominent at DMc than in Lc **(K–N)**. RG loss of polarity induced by TGF-β1 is accompanied by increase in the numbers of BLBP+ cells (white) with intermediate differentiated morphology (IDM) across cortical wall **(O–Q)**. SB431542 injection does not affect BLBP+ IDM cells numbers. These morphological alterations were followed by basal membrane laminin (green) and neuronal cell bodies (Nissl) ectopic distribution **(R,S)**. TGF-β1 also disorganized pH3+ cells (green) distribution across cortical wall, especially VZ pH3+ cells' nucleus alignment **(T–Y)**, without affects its numbers **(Z)**, however increased the numbers of pH3+ cells in the SVZ **(Z)**. $*P < 0.05$, $**P < 0{,}005$, $***P < 0005$. Scales: 500 μm **(G)**, 100 μm **(B,O)**, 50 μm **(H,J,T,W)**, 20 μm **(K,L,R)**. Cp: cortical plate, Vz: ventricular zone, Svz: subventricular zone, Cx: cortex, Lv: lateral ventricle.

were accompanied by severe disorganization of nestin labeled-RG fibers in TGF-β1-injected brains (**Figures 2G,J**). This disorganization characterized loss of polarity of the radial processes and was more prominently observed in DMc rather than in Lc areas of the cortex (**Figures 2K–N**).

In addition to RG fibers displacement, TGF-β1 also promoted an increment in approximately 98% on BLBP-labeled cells witha morphology similar to glial progenitors, in the midway of their differentiation path, which we called RG intermediate differentiation morphology (IDM; **Figures 2O–Q**). Injection with pharmacological inhibitor of TGF-β1 signaling pathway SB431542 did not affect BLBP+ IDM cells generation (**Figure 2Q**). We also observed that TGF-β1 caused ectopic laminin distribution in the pial region of the cortical wall (**Figures 2R,S**).These phenotypes were also associated with increasing numbers of pH3+ cells in SVZ, but not in VZ (**Figures 2T–Z**). In addition RG fibers disorganization were also followed by displacement of pH3+ cells at VZ, leading to ectopic positioning of these proliferative cell's nucleus (**Figures 2T,W**).

These data shows that TGF-β1 regulates cerebral cortex thickness, RG morphology and polarity and progenitor positioning, and suggest that these events might be associated to regulation of basal lamina structure, an issue clearly related to RG cell polarity.

TGF-β1 PROMOTES PREMATURE GLIOGENESIS IN DORSOMEDIAL (DMc) AREA OF THE CEREBRAL CORTEX

We previously demonstrated that TGF-β1 controls RG differentiation into astrocytes and neurons by distinct signaling pathways *in vitro* (Stipursky et al., 2012a). In order to assess the fate of RG under the influence of TGF-β1 *in vivo*, we took the advantage of *in utero* intraventricular injection technique. Injection of TGF-β1 inside the lateral ventricles of mouse embryos also caused robust premature astrocyte generation (**Figure 3**). In the telencephalon TGF-β1 injection caused appearance of GFAP+ cells in distinct regions compared with vehicle injected brains (**Figures 3A,B**), such as the cingulate cortex (**2***) neuroepithelium related to the third ventricle associated with the ventral diencephalic sulcus (**3***), and also at the pial region of the preoptic area (**4***).In the evident hippocampal neuroepithelium there was no difference in GFAP labeling pattern in control and TGF-β1 injected brains (**1***).

Apart from other regions, we observed that in DMc area of the cerebral cortex (cingulate cortex) astrocytogenesis was more evident. The appearance of GFAP+ cells bearing a yet radial-like morphology in this area (**Figures 3C–F**) suggest that TGF-β1 induced RG cells to adopt an astrocyte phenotype.

Astrocyte differentiation was significantly increased by TGF-β1 in the DMc area in comparison to the lateral area of the cerebral cortex (15 X; **Figure 3G**). Injection of a pharmacological inhibitor of TGF-β receptor, SB431542, did not affect the gliogenesis in this area (**Figure 3G**).

In order to confirm the specificity of TGF-β1 actions in different cortical areas, we generated cultures of isolated RG cells from DMc and Lc areas and from total cortex (Tc). We observed that DMc cells were more responsive to TGF-β1 astrocytogenic induction, than Lc cells. The number of GFAP+ cells increased by 5 times in DMc cells treated with TGF-β1 whereas only 3 times in Lc cells. For Tc cells, the increasing in GFAP+ cell numbers was compared to those found in DMc-treated condition (**Figure 3H**).

Thus, RG from different cerebral cortexareas respond to TGF-β1 by acquiring the astrocytic phenotype.

TGF-β1 AFFECTS NEUROGENESIS AND NEURONAL POSITIONING IN CORTICAL PLATE

Neurogenesis and neuronal migration are events that occur during specific time window in the developing cerebral cortex; both events directly dependent of RG cell stem cell and scaffold properties, respectively (Rakic, 1971; Costa et al., 2010; Vogel et al., 2010; Sild and Ruthazer, 2011; Stipursky et al., 2012b). We previously described that as well as astrocytogenesis, neurogenesis can be controlled by TGF-β1 by activation of canonical and non-canonical signaling pathways, respectively (Stipursky et al., 2012a). Although neurogenesis was reported to involve TGF-β1 action *in vitro* (Vogel et al., 2010), it is not known if this factor controls RG neurogenic potential *in vivo*. In order to address this question, we have performed intraventricular injection of TGF-β1.

TGF-β1 also affected neuronal generation and placement in CP of the Lc. Interestingly, numerous βTubulinIII+ cells were present in the VZ of TGF-β1-injected brains, counting for an 66% increment (**Figures 4A–C**), thus suggesting enhanced neurogenesis in this RG cell bodies enriched layer. Pharmacological inhibition of TGF-β1 signaling pathway by SB431542 injection yielded a greater enhancement of βTubulinIII+ cells numbers in VZ, compared to control condition. In order to access if this increment was due to generation of new neurons, we have labeled the cells for BrdU and Doublecortin, which label recent generated neurons from RG cells that migrated through cortical wall and reached their final destination in the CP (Pramparo et al., 2010). We observed a 55% decrease in the number of BrdU+cells in the Lc CP of TGF-β1 injected brains (**Figures 4D–H**), thus demonstrating that both neuronal migration and positioning are modulated by TGF-β1 *in vivo*.

TGF-β1 CONTROLS THE EXPRESSION OF FoxG1 IN DIFFERENT CORTICAL AREAS

Differences between the distinct regions of the brain are mainly generated during developmental controlled axis patterning-related morphogen distribution. Cerebral cortex arealizationor patterning is controlled by the expression of a great repertoire of transcription factors that define neural stem cells and progenitors generation, self-renewal and phenotypes. Those factors, such as FoxG1, are modulated by diverse morphogenetic proteins distinctly distributed in different patterning centers (Takahashi and Liu, 2006; O'Leary and Sahara, 2008).

Quantitative analyses by real time RT-PCR of DMc and Lc tissues revealed that TGF-β1 distinctly modulated the levels of FoxG1 mRNA transcription factors in these regions. Whereas TGF-β1 reduced the expression level of FoxG1 in DMc by 80%, it had no effect in Lc (**Figure 5**). These results suggest that TGF-β1 controls the expression of a transcription factor related to cortical arealization *in vivo*.

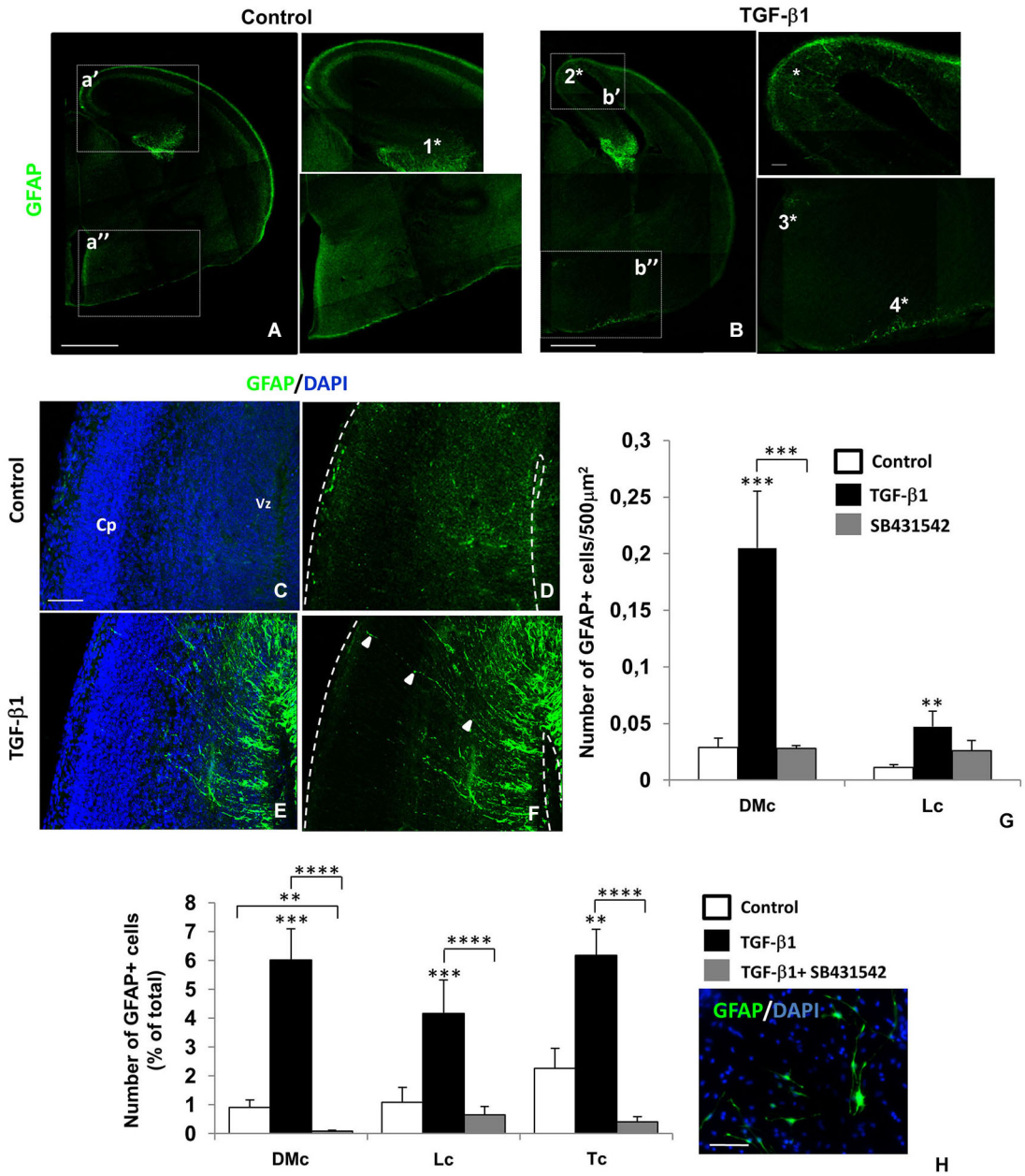


FIGURE 3 | TGF-β1 promotes premature gliogenesis in the cerebral cortex. Intraventricular injection of TGF-β1 in mouse embryos (injection at E14 and analysis at E16) caused premature appearance of GFAP+ cells (green) in different telencephalon regions: dorsomedial cortex/cingulate cortex (2*), neuroepithelium related to the third ventricule (3*) and pial surface of the preoptic area (4*). At the hippocampal formation (1*), GFAP labeling was not affected. TGF-β1 induced gliogenesis was more evident at the dorsomedial area of the cerebral cortex (DMc), than in lateral cortex (Lc) **(C–G)**. Note the GFAP+ (green) radial fibers of differentiating cells (arrows, **F**). In radial glia (RG) isolated cultures, TGF-β1 also promoted appearance of GFAP+ cells in a greater extend in DMc than in Lc and total cortex (Tc) **(H)**. ***$P < 0.0005$, *$P < 0.005$. Scales: 500 μm **(A,B)**, 50 μm **(C,H)**. Cp: cortical plate, Vz: ventricular zone.

DISCUSSION

In this study, we provide evidence for the role of TGF-β1 as a modulator of RG-astrocyte differentiation *in vivo*. Our data is pioneer in two aspects: (1) by demonstration of TGF-β1 action in radial-glial-astrocyte differentiation *in vivo*; (2) by showing distinct effects of TGF-β1 in different subpopulations of RG cells. First, we demonstrated that RG cells express the TGF-β receptor and activate the Smad pathway in response do TGF-β1. Then, we demonstrated that TGF-β1 disrupts RG cells polarized morphology and promotes premature astrocytogenesis and neuronal displacement in specific areas of the cerebral cortex. Our findings show that RG cells are potential targets for TGF-β signaling

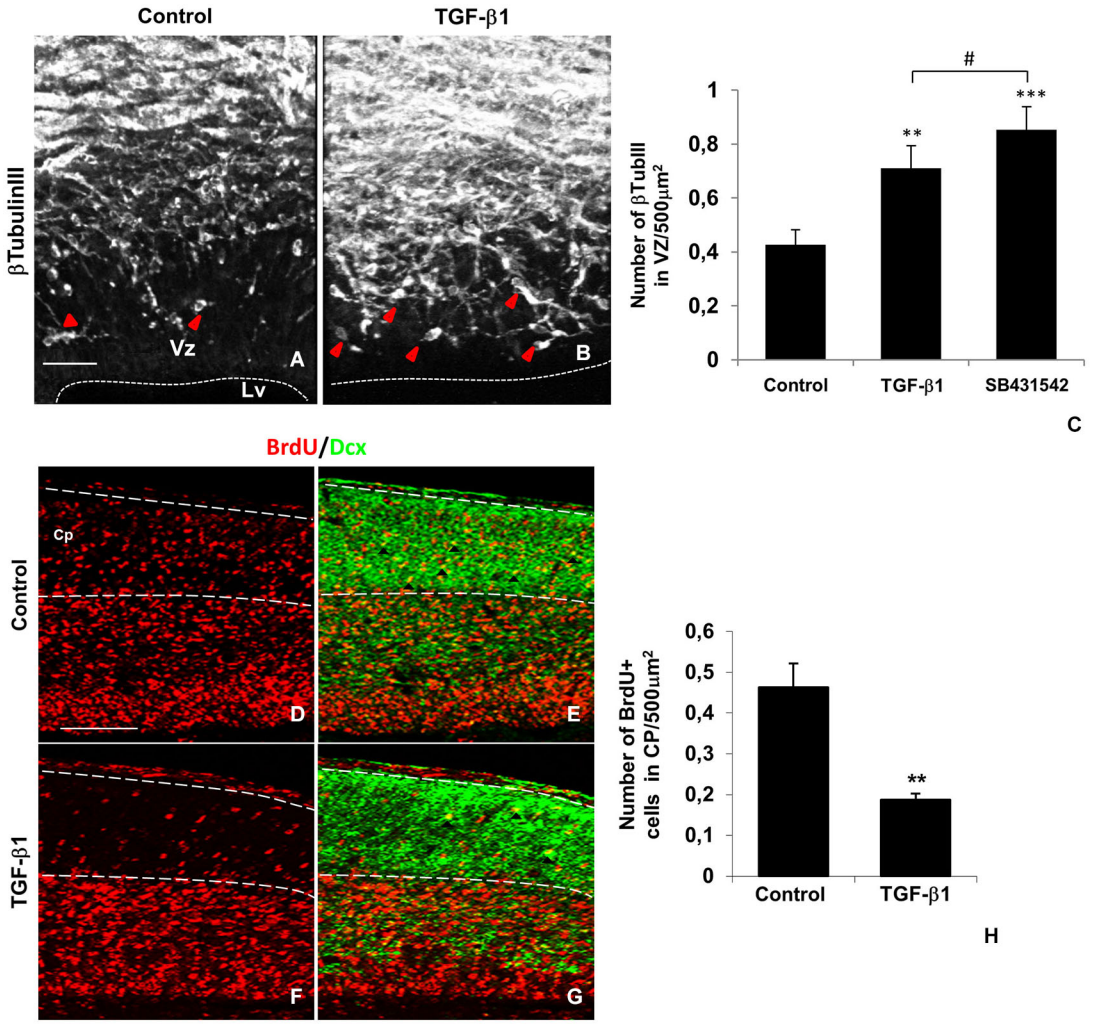


FIGURE 4 | TGF-β1 affects neurogenesis and neuronal positioning in the lateral cortex. TGF-β1 injections (injection at E14 and analysis at E16) increased neurogenesis at the lateral cortex Vz, as shown by the presence of βTubulinIII+ cells (white, arrows) in this layer **(A–C)**. TGF-β1 decreased the number of BrdU+ cells (red) at the cortical plate (Cp) of the lateral cortex **(D,F,H)**. Note that this layer is enriched in Doublecortin+ neurons (green) that were generated at Vz and migrated to the Cp **(E,G)**. $*P < 0.05$, $\#P = 0.063$. Scales: 50 μm **(A,D)**. Vz: ventricularzone, Lv: lateral ventricle.

pathway and suggest that these effects are region dependent. Our data not only contribute to the understanding of the mechanism underlying fate decision and specific phenotype acquisition in the cerebral cortex, but support the hypothesis of the existence of distinct RG subpopulations with different potentials in the cerebral cortex.

RG CELLS AS POTENTIAL TARGETS OF TGF-β1 *IN VIVO*: IMPACT ON RG POLARITY AND ASTROCYTIC DIFFERENTIATION

Evidence suggests that VZ cells are direct targets of different TGF-β family members (Miller, 2003; Mecha et al., 2008), however, the cellular pattern of expression of TGF-β1 signaling pathway members in the developing CNS has not been well characterized. Here, we have shown TGFRII expression in the developing telencephalon, specifically in the VZ/SVZ of the cerebral cortex. Additionally, we precisely identified its distribution in RG soma and fibers, an issue only previously suggested by other authors (Miller, 2003). Moreover, the levels of TGFRII and one of its downstream effectors, phosphorylated Smad2, seems to be negatively modulated through development. These results are corroborated by previous data that showed TGF-β1 and Smad2/3 proteins expression in different CNS regions including cerebral cortex VZ, neurons and progenitor layers *in vivo* (Miller, 2003; Sousa Vde et al., 2004; Mecha et al., 2008; Powrozek and Miller, 2009). In addition, our data is in accordance with previous reports that demonstrated that TGF-β signaling members are expressed in higher levels in early moments of the telencephalon development, and that are determining for the generation of different cell types of the CNS and other regions (Luukko et al., 2001).

RG cell polarity and radial processes extension are essential characteristics that are directly related to RG maintenance of its progenitor potential and scaffold property for neuronal

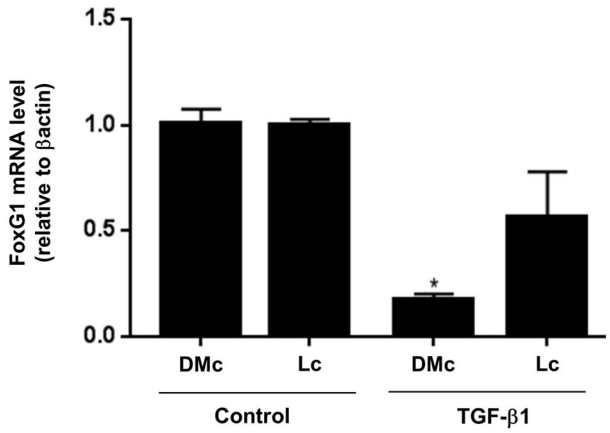


FIGURE 5 | TGF-β1 controls FoxG1 expression in the cerebral cortex. Cerebral cortex tissue from TGF-β1 injected brains (injection at E14 and analysis at E16) were dissected to separate DMc and Lc areas and evaluated for FoxG1 mRNA expression by real time RT-PCR. TGF-β1 injections greatly decreased FoxG1 gene mRNA levels only in DMc area compared to control brains. $*P < 0.01$.

migration (Rakic, 1971). RG differentiation into astrocytes involves disruption of its polarity and gradual acquisition of immature astrocyte morphology (Voigt, 1989; Hartfuss et al., 2001). Here, we have shown that TGF-β1 induces specific disorganization of nestin positive RG fibers and displacement of their cell nucleus labeled for pH3. Moreover, we observed the appearance of BLBP positive cells bearing an intermediate morphology between RG and astrocytes throughout the cortical wall.

Several mechanisms have been proposed to control RG cell polarity and correct positioning of migrating neurons such as modulation of cytoskeleton molecules (Yokota et al., 2007, 2009, 2010; Weimer et al., 2009) and ECM signal transduction (Haubst et al., 2006; Voss et al., 2008). Here we observed that disruption of RG polarity induced by TGF-β1 is followed by impaired organization of the basal membrane that covers pial surface of the telencephalon, where RG cells attach their pial process endfeet (Götz and Huttner, 2005). Laminin labeling revealed an ectopic distribution pattern of this protein in pial region of the cerebral cortex, associated with deficiencies in CP formation and displaced cell bodies. Our data is supported by previous results that TGF-β1 is a potent regulator of the synthesis of laminin, fibronectin, the adhesion protein nCAM and integrins (Brionne et al., 2003; Siegenthaler and Miller, 2004; Gomes et al., 2005). Further, similar phenotypes were found in mutant mice for C3G protein, a guanine nucleotide exchange factor for small GTPases of the Ras family, and also in laminin γ1III4 mutant (Haubst et al., 2006; Voss et al., 2008). In these mice, it is observed a robust loss of radial cell polarity, disruption of basal membrane and neuronal migration and CP deficits. Thus, although we cannot fully rule out additional mechanisms, our data strongly suggested an association between TGF-β1-control of laminin organization and maintenance of RG polarity.

TGF-β1 PROMOTES PREMATURE GLIOGENESIS IN DORSOMEDIAL AREA OF THE CEREBRAL CORTEX: IMPLICATIONS FOR RG HETEROGENEITY

In rodents, by the end of gestation, RG-astrocyte differentiation, is characterized within several molecular mechanisms by replacement of RG markers, such as BLBP and nestin, by astrocytic markers such as GFAP, the glutamate transporter GLAST and the calcium binding protein S100β (Dahl, 1981; Pixley and de Vellis, 1984).The correct timing of RG-astrocyte transformation is a crucial step to ensure correct number of neurons and cerebral cortex lamination. Here, we report that activation of TGF-β1 pathwayled to a premature appearance of GFAP+ cells in different regions of the embryonic telencephalon, mainly, in the cingulate cortex, neuroepithelium related to the third ventricle, and also at the pial region of the preoptic area. Although it has been reported the expression of TGF-β isoforms and also its different roles in these regions (Bouret et al., 2004; Dobolyi and Palkovits, 2008; Srivastava et al., 2014), the role of TGF-β1 in dorsomedial area of the cerebral cortex, cingulate cortex, specifically on astrocyte differentiation, is poorly known.

Here the reported event was region-dependent since in DMc area the appearance of GFAP+ cells and disruption of RG processes were more robust than in Lc area. This observation might be related to 2 alternatives: (1) distinct responsiveness of different brain regions to TGF-β1; (2) heterogeneity of radial glial cells. The first possibility is supported by our previous report that GFAP gene promoter from different brain regions distinctly responds to TGF-β1 (Sousa Vde et al., 2004). It is also possible that TGF-β1 might exert its actions controlling size of a brain area (Falk et al., 2008) by acting into the different subpopulations of RG cells and other progenitors previously described to contribute to cell diversity in CNS (Pinto and Götz, 2007; Stancik et al., 2010), and that this event accounts for diversity in the responsiveness to TGF-β1. Whether this is due to different levels of TGF-β receptor or intracellular signaling molecules, or even, by cell autonomous defined potentials, remains to be determined.

Several molecules have been described to guarantee the maintenance of RG self-renewal, BLBP expression and morphology characteristics, such as the proteins of Neuregulin family and its receptor ErbBs, and Notch1 (Gaiano and Fishell, 2002; Patten et al., 2003; Schmid et al., 2003; Yoon et al., 2004; Anthony et al., 2005; Ghashghaei et al., 2006, 2007). Thus, alterations of ErB2 and Notch1 expression in RG cells could lead to a premature astrocyte differentiation under TGF-β1 influence. This hypothesis is supported by reports that interaction between TGF-β1 signaling pathway proteins and radializing factors such as Notch intracellular cleaved domain (NICD) and ErbB4 is necessary to regulate the expression of target genes in neural precursors (Blokzijl et al., 2003) and the correct time of gliogenesis (Sardi et al., 2006). The exact mechanisms by which TGF-β1 pathway controls RG-astrocyte differentiation in the dorsomedial area of the cerebral cortex will require further investigation.

We reported here that activation of TGF-β1 signaling pathway in the cerebral cortex down regulates the expression of FoxG1 in DMcarea.FoxG1 is a member of the forkhead family

of transcription factors, expressed by cells with high proliferation rates; it controls neurogenesis, by maintaining the undifferentiated state of neural progenitors (Dou et al., 2000; Siegenthaler and Miller, 2008). In addition, FoxG1 is mainly expressed in lateral areas of the mice cerebral cortex (Miller, 2003).Mutant mice models for FoxG1 functions share several similarities with many of the phenotypes described here, including reduction of cortical thickness and layers of the dorsal area. For example, mutant mice for FoxG1, present reduction of dorsal area, and pronounced increase of BMPs, a member of TGF-β family, expression in the telencephalon (Takahashi and Liu, 2006). Further, FoxG1 was described as a potent inhibitor of TGF-β signaling due to its association with Smad proteins (Dou et al., 2000; Siegenthaler and Miller, 2008). Although TGF-β1 affects more robustly DMc area, we also observe the effect of this factor in Lc, such as mild RG fibers morphology and neurogenesis induction, it is possible that other transcription factors responsible for arealization of the cortex might mediate TGF-β1 actions in Lc (O'Leary and Sahara, 2008).

Thus, it is possible that TGF-β1 controls the balance between gliogenesis and neurogenesis by modulating the expression and activation of different transcription factors *in vivo*. Since FoxG1 is a lateral transcription factor, a gliogenic inhibitor, and negatively regulates Smads signaling, it is possible that FoxG1 is a mediator of TGF-β1 signaling in DMc.

Besides the role of TGF-β1 in the modulation of transcription factors at transcriptional level, it is possible that the lateral morphogen gradients might exert an inhibitory action on medial ones. It correlates with our observation that endogenous TGF-β signaling pathway might not be active or engaged in promotion of astrocytogenesis at this developmental stage, since pharmacological inhibition of endogenous TGF-β signaling by SB431542 did not affect RG morphological phenotype, as well as GFAP + cells numbers. Although we have shown that TGF-β1 is a potent inductor of astrocyte differentiation (Stipursky and Gomes, 2007; Stipursky et al., 2012a), this data confirm that RG cells are mainly committed in promoting neurogenesis at this stage (Noctor et al., 2001).

TGF-β1 AFFECTS NEUROGENESIS AND NEURONAL POSITIONING IN THE CORTICAL PLATE

Injection of TGF-β1 decreased the number of BrdU+ cells in the developing CP of the lateral area of the cortex. This effect might be the consequence of neurogenesis and/or migration deficits. The last hypothesis is more likely, since increased number of βTubulinIII+ cells was observed in the VZ, and although in the present work we cannot completely guarantee the identity of the pH3+ cells in the SVZ, it is possible that these cells could also contribute to neurogenic effect promoted by TGF-β1.

The role of TGF-β1 in neurogenesis is controversial; whereas it has been shown as inductor of neurogenesis in the cerebral cortex during embryonic stage and in the adult hippocampus (Vogel et al., 2010; Stipursky et al., 2012a; He et al., 2014); others have reported its action as negative modulator of neurogenesis in the adult SVZ (Roussa et al., 2004; Wachs et al., 2006; Siegenthaler and Miller, 2008). Although TGF-β1 has been shown to induce radial neuronal migration in the cerebral cortex, its effect in RG cell has not been previously addressed (Siegenthaler and Miller, 2004). Here we suggest that although TGF-β1 promotes neuronal generation from RG cells and as we previously demonstrated *in vitro* (Stipursky et al., 2012a), the morphological alterations triggered in radial processes in the lateral area of the cortex, even in a less extension that in DMc area, counteracts its effect and prevent neuronal migration and the accuracy in the establishment of these new generated neurons in the CP.

It is interesting that pharmacological inhibition of TGF-β1 signaling pathway by injection of SB431542 yielded an even greater increase of βTubulinIII+ cells in VZ, when compared with TGF-β1 injected brains. Although apparently contradictory, this result might indicate that endogenous TGF-β signaling pathway might be committed to control neuron generation in cerebral cortex during the neurogenic stage of the CNS development (Vogel et al., 2010). Further, it is possible that different levels of TGF-β signaling activation might be critical to elicit positive or negative responses to this factor. Accordingly, it has been demonstrated that opposite actions of TGF-β1 in neuronal migration is concentration dependent (Siegenthaler and Miller, 2004).

Together our results points to a new feature of TGF-β1 action in patterning the developing telencephalon. By acting in different RG populations, TGF-β1 promotes the generation of astrocytes and/or neurons in a regional dependent manner. Deficits in pathways that operate in RG physiology might generate dysfunctional cells, disorders in neuronal migration and premature astrocytogenesis, leading to diverse types of lamination defects in the developing cortex, such as observed in Lisencephaly and the congenital abnormality cortical dysplasia. Identification and characterization of the mechanisms underlying RG maintenance and differentiation might contribute to generation of therapeutic approaches to cell restocking in CNS parenchyma.

ACKNOWLEDGMENTS

We thank Dr. Eva S. Anton (Associate Professor, Neuroscience Center, University of North Carolina at Chapel Hill) for significant comments and scientific support, and Marcelo Meloni and Adiel Batista do Nascimento for technical assistance. Thisworkwassupportedbygrantsfrom: Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (Flávia Carvalho Alcantara Gomes); Conselho Nacional para o Desenvolvimento Científico e Tecnológico (Daniel Francis; Flávia Carvalho Alcantara Gomes; Joice Stipursky; Lays Souza; Rômulo Sperduto Dezonne); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior(Carolina A. Moraes; Ana Paula Bérgamo de Araújo).

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Anthony, T. E., Klein, C., Fishell, G., and Heintz, N. (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron* 41, 881–890. doi: 10.1016/s0896-6273(04)00140-0

Anthony, T. E., Mason, H. A., Gridley, T., Fishell, G., and Heintz, N. (2005). Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. *Genes Dev.* 19, 1028–1033. doi: 10.1101/gad.1302105

Anton, E. S., Marchionni, M. A., Lee, K. F., and Rakic, P. (1997). Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. *Development* 124, 3501–3510.

Barnabé-Heider, F., Wasylnka, J. A., Fernandes, K. J., Porsche, C., Sendtner, M., Kaplan, D. R., et al. (2005). Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. *Neuron* 48, 253–265. doi: 10.1016/j.neuron.2005.08.037

Bentivoglio, M., and Mazzarello, P. (1999). The history of radial glia. *Brain Res. Bull.* 49, 305–315. doi: 10.1016/s0361-9230(99)00065-9

Blokzijl, A., Dahlqvist, C., Reissmann, E., Falk, A., Moliner, A., Lendahl, U., et al. (2003). Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J. Cell Biol.* 163, 723–728. doi: 10.1083/jcb.200305112

Bouret, S., De Seranno, S., Beauvillain, J. C., and Prevot, V. (2004). Transforming growth factor beta1 may directly influence gonadotropin-releasing hormone gene expression in the rat hypothalamus. *Endocrinology* 145, 1794–1801. doi: 10.1210/en.2003-1468

Brionne, T. C., Tesseur, I., Masliah, E., and Wyss-Coray, T. (2003). Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain. *Neuron* 40, 1133–1145. doi: 10.1016/s0896-6273(03)00766-9

Costa, M. R., Götz, M., and Berninger, B. (2010). What determines neurogenic competence in glia? *Brain Res. Rev.* 63, 47–59. doi: 10.1016/j.brainresrev.2010.01.002

Culican, S. M., Baumrind, N. L., Yamamoto, M., and Pearlman, A. L. (1990). Cortical radial glia: identification in tissue culture and evidence for their transformation to astrocytes. *J. Neurosci.* 10, 684–692.

Dahl, D. (1981). The vimentin-GFA protein transition in rat neuroglia cytoskeleton occurs at the time of myelination. *J. Neurosci. Res.* 6, 741–748. doi: 10.1002/jnr.490060608

Dezonne, R. S., Stipursky, J., Araujo, A. P., Nones, J., Pavão, M. S., Porcionatto, M., et al. (2013). Thyroid hormone treated astrocytes induce maturation of cerebral cortical neurons through modulation of proteoglycan levels. *Front. Cell. Neurosci.* 7:125. doi: 10.3389/fncel.2013.00125

Diniz, L. P., Almeida, J. C., Tortelli, V., Vargas Lopes, C., Setti-Perdigão, P., Stipursky, J., et al. (2012). Astrocyte-induced synaptogenesis is mediated by transforming growth factor beta signaling through modulation of D-serine levels in cerebral cortex neurons. *J. Biol. Chem.* 287, 41432–41445. doi: 10.1074/jbc.m112.380824

Diniz, L. P., Tortelli, V., Garcia, M. N., Araújo, A. P., Melo, H. M., Seixas da Silva, G. S., et al. (2014). Astrocyte transforming growth factor beta 1 promotes inhibitory synapse formation via CaM kinase II signaling. *Glia* 62, 1917–1931. doi: 10.1002/glia.22713

Dobolyi, A., and Palkovits, M. (2008). Expression of latent transforming growth factor beta binding proteins in the rat brain. *J. Comp. Neurol.* 507, 1393–1408. doi: 10.1002/cne.21621

Dou, C., Lee, J., Liu, B., Liu, F., Massague, J., Xuan, S., et al. (2000). BF-1 interferes with transforming growth factor beta signaling by associating with Smad partners. *Mol. Cell. Biol.* 20, 6201–6211. doi: 10.1128/mcb.20.17.6201-6211.2000

Espósito, M. S., Piatti, V. C., Laplagne, D. A., Morgenstern, N. A., Ferrari, C. C., Pitossi, F. J., et al. (2005). Neuronal differentiation in the adult hippocampus recapitulates embryonic development. *J. Neurosci.* 25, 10074–10086. doi: 10.1523/jneurosci.3114-05.2005

Falk, S., Wurdak, H., Ittner, L. M., Ille, F., Sumara, G., Schmid, M. T., et al. (2008). Brain area-specific effect of TGF-beta signaling on Wnt-dependent neural stem cell expansion. *Cell Stem Cell* 2, 472–483. doi: 10.1016/j.stem.2008.03.006

Gaiano, N., and Fishell, G. (2002). The role of notch in promoting glial and neural stem cell fates. *Annu. Rev. Neurosci.* 25, 471–490. doi: 10.1146/annurev.neuro.25.030702.130823

Ghashghaei, H. T., Weber, J., Pevny, L., Schmid, R., Schwab, M. H., Lloyd, K. C., et al. (2006). The role of neuregulin-ErbB4 interactions on the proliferation and organization of cells in the subventricular zone. *Proc. Natl. Acad. Sci. U S A* 103, 1930–1935. doi: 10.1073/pnas.0510410103

Ghashghaei, H. T., Weimer, J. M., Schmid, R. S., Yokota, Y., Mccarthy, K. D., Popko, B., et al. (2007). Reinduction of ErbB2 in astrocytes promotes radial glial progenitor identity in adult cerebral cortex. *Genes Dev.* 21, 3258–3271. doi: 10.1101/gad.1580407

Gomes, F. C., Sousa Vde, O., and Romão, L. (2005). Emerging roles for TGF-beta1 in nervous system development. *Int. J. Dev. Neurosci.* 23, 413–424. doi: 10.1016/j.ijdevneu.2005.04.001

Götz, M., Hartfuss, E., and Malatesta, P. (2002). Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. *Brain Res. Bull.* 57, 777–788. doi: 10.1016/s0361-9230(01)00777-8

Götz, M., and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* 6, 777–788. doi: 10.1038/nrm1739

Hartfuss, E., Galli, R., Heins, N., and Gotz, M. (2001). Characterization of CNS precursor subtypes and radial glia. *Dev. Biol.* 229, 15–30. doi: 10.1006/dbio.2000.9962

Hatten, M. E. (1999). Central nervous system neuronal migration. *Annu. Rev. Neurosci.* 22, 511–539. doi: 10.1146/annurev.neuro.22.1.511

Haubst, N., Georges-Labouesse, E., De Arcangelis, A., Mayer, U., and Gotz, M. (2006). Basement membrane attachment is dispensable for radial glial cell fate and for proliferation, but affects positioning of neuronal subtypes. *Development* 133, 3245–3254. doi: 10.1242/dev.02486

He, F., Ge, W., Martinowich, K., Becker-Catania, S., Coskun, V., Zhu, W., et al. (2005). A positive autoregulatory loop of Jak-STAT signaling controls the onset of astrogliogenesis. *Nat. Neurosci.* 8, 616–625. doi: 10.1038/nn1440

He, Y., Zhang, H., Yung, A., Villeda, S. A., Jaeger, P. A., Olayiwola, O., et al. (2014). ALK5-dependent TGF-beta signaling is a major determinant of late-stage adult neurogenesis. *Nat. Neurosci.* 17, 943–952. doi: 10.1038/nn.3732

Hevner, R. F., Hodge, R. D., Daza, R. A., and Englund, C. (2006). Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum and adult hippocampus. *Neurosci. Res.* 55, 223–233. doi: 10.1016/j.neures.2006.03.004

Hunter, K. E., and Hatten, M. E. (1995). Radial glial cell transformation to astrocytes is bidirectional: regulation by a diffusible factor in embryonic forebrain. *Proc. Natl. Acad. Sci. U S A* 92, 2061–2065. doi: 10.1073/pnas.92.6.2061

Javelaud, D., and Mauviel, A. (2005). Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene* 24, 5742–5750. doi: 10.1038/sj.onc.1208928

Kriegstein, A., and Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. *Annu. Rev. Neurosci.* 32, 149–184. doi: 10.1146/annurev.neuro.051508.135600

Kriegstein, A. R., and Götz, M. (2003). Radial glia diversity: a matter of cell fate. *Glia* 43, 37–43. doi: 10.1002/glia.10250

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Luukko, K., Ylikorkala, A., and Mäkelä, T. P. (2001). Developmentally regulated expression of Smad3, Smad4, Smad6 and Smad7 involved in TGF-beta signaling. *Mech. Dev.* 101, 209–212. doi: 10.1016/s0925-4773(00)00556-6

Malatesta, P., Hack, M. A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F., et al. (2003). Neuronal or glial progeny: regional differences in radial glia fate. *Neuron* 37, 751–764. doi: 10.1016/s0896-6273(03)00116-8

Massagué, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* 67, 753–791. doi: 10.1146/annurev.biochem.67.1.753

Massagué, J., and Gomis, R. R. (2006). The logic of TGFbeta signaling. *FEBS Lett.* 580, 2811–2820. doi: 10.1016/j.febslet.2006.04.033

Mecha, M., Rabadán, M. A., Peña-Melián, A., Valencia, M., Mondéjar, T., and Blanco, M. J. (2008). Expression of TGF-betas in the embryonic nervous system: analysis of interbalance between isoforms. *Dev. Dyn.* 237, 1709–1717. doi: 10.1002/dvdy.21558

Mi, H., Haeberle, H., and Barres, B. A. (2001). Induction of astrocyte differentiation by endothelial cells. *J. Neurosci.* 21, 1538–1547. doi: 10.1046/j.1432-0436.2001.680405.x

Miller, M. W. (2003). Expression of transforming growth factor-beta in developing rat cerebral cortex: effects of prenatal exposure to ethanol. *J. Comp. Neurol.* 460, 410–424. doi: 10.1002/cne.10658

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* 31, 727–741. doi: 10.1016/s0896-6273(01)00420-2

Munoz-Garcia, D., and Ludwin, S. K. (1986). Gliogenesis in organotypic tissue culture of the spinal cord of the embryonic mouse. I. Immunocytochemical and ultrastructural studies. *J. Neurocytol.* 15, 273–290. doi: 10.1007/bf01611431

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., et al. (1999). Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science* 284, 479–482. doi: 10.1126/science.284.5413.479

Nishino, J., Yamashita, K., Hashiguchi, H., Fujii, H., Shimazaki, T., and Hamada, H. (2004). Meteorin: a secreted protein that regulates glial cell differentiation and promotes axonal extension. *EMBO J.* 23, 1998–2008. doi: 10.1038/sj.emboj.7600202

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409, 714–720. doi: 10.1038/35055553

Noctor, S. C., Flint, A. C., Weissman, T. A., Wong, W. S., Clinton, B. K., and Kriegstein, A. R. (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J. Neurosci.* 22, 3161–3173.

O'Leary, D. D., and Sahara, S. (2008). Genetic regulation of arealization of the neocortex. *Curr. Opin. Neurobiol.* 18, 90–100. doi: 10.1016/j.conb.2008.05.011

Patten, B. A., Peyrin, J. M., Weinmaster, G., and Corfas, G. (2003). Sequential signaling through Notch1 and erbB receptors mediates radial glia differentiation. *J. Neurosci.* 23, 6132–6140.

Pinto, L., and Götz, M. (2007). Radial glial cell heterogeneity–the source of diverse progeny in the CNS. *Prog. Neurobiol.* 83, 2–23. doi: 10.1016/j.pneurobio.2007.02.010

Pixley, S. K., and de Vellis, J. (1984). Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. *Brain Res.* 317, 201–209. doi: 10.1016/0165-3806(84)90097-x

Powrozek, T. A., and Miller, M. W. (2009). Ethanol affects transforming growth factor beta1-initiated signals: cross-talking pathways in the developing rat cerebral wall. *J. Neurosci.* 29, 9521–9533. doi: 10.1523/jneurosci.2371-09.2009

Pramparo, T., Youn, Y. H., Yingling, J., Hirotsune, S., and Wynshaw-Boris, A. (2010). Novel embryonic neuronal migration and proliferation defects in Dcx mutant mice are exacerbated by Lis1 reduction. *J. Neurosci.* 30, 3002–3012. doi: 10.1523/jneurosci.4851-09.2010

Radakovits, R., Barros, C. S., Belvindrah, R., Patton, B., and Muller, U. (2009). Regulation of radial glial survival by signals from the meninges. *J. Neurosci.* 29, 7694–7705. doi: 10.1523/jneurosci.5537-08.2009

Rakic, P. (1971). Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res.* 33, 471–476. doi: 10.1016/0006-8993(71)90119-3

Rakic, P. (1995). Radial versus tangential migration of neuronal clones in the developing cerebral cortex. *Proc. Natl. Acad. Sci. U S A* 92, 11323–11327. doi: 10.1073/pnas.92.25.11323

Romão, L. F., Sousa Vde, O., Neto, V. M., and Gomes, F. C. (2008). Glutamate activates GFAP gene promoter from cultured astrocytes through TGF-beta1 pathways. *J. Neurochem.* 106, 746–756. doi: 10.1111/j.1471-4159.2008.05428.x

Roussa, E., Farkas, L. M., and Krieglstein, K. (2004). TGF-beta promotes survival on mesencephalic dopaminergic neurons in cooperation with Shh and FGF-8. *Neurobiol. Dis.* 16, 300–310. doi: 10.1016/j.nbd.2004.03.006

Sardi, S. P., Murtie, J., Koirala, S., Patten, B. A., and Corfas, G. (2006). Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. *Cell* 127, 185–197. doi: 10.1016/j.cell.2006.07.037

Schmid, R. S., Mcgrath, B., Berechid, B. E., Boyles, B., Marchionni, M., Sestan, N., et al. (2003). Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. *Proc. Natl. Acad. Sci. U S A* 100, 4251–4256. doi: 10.1073/pnas.0630496100

Siegenthaler, J. A., and Miller, M. W. (2004). Transforming growth factor beta1 modulates cell migration in rat cortex: effects of ethanol. *Cereb. Cortex* 14, 791–802. doi: 10.1093/cercor/bhh039

Siegenthaler, J. A., and Miller, M. W. (2008). Generation of Cajal-Retzius neurons in mouse forebrain is regulated by transforming growth factor beta-Fox signaling pathways. *Dev. Biol.* 313, 35–46. doi: 10.1016/j.ydbio.2007.09.036

Sild, M., and Ruthazer, E. S. (2011). Radial glia: progenitor, pathway and partner. *Neuroscientist* 17, 288–302. doi: 10.1177/1073858410385870

Sousa Vde, O., Romão, L., Neto, V. M., and Gomes, F. C. (2004). Glial fibrillary acidic protein gene promoter is differently modulated by transforming growth factor-beta 1 in astrocytes from distinct brain regions. *Eur. J. Neurosci.* 19, 1721–1730. doi: 10.1111/j.1460-9568.2004.03249.x

Srivastava, V. K., Hiney, J. K., and Dees, W. L. (2014). Actions and interactions of alcohol and transforming growth factor beta1 on prepubertal hypothalamic gonadotropin-releasing hormone. *Alcohol. Clin. Exp. Res.* 38, 1321–1329. doi: 10.1111/acer.12374

Stancik, E. K., Navarro-Quiroga, I., Sellke, R., and Haydar, T. F. (2010). Heterogeneity in ventricular zone neural precursors contributes to neuronal fate diversity in the postnatal neocortex. *J. Neurosci.* 30, 7028–7036. doi: 10.1523/jneurosci.6131-09.2010

Stipursky, J., de Sampaio e Spohr, T. C. L., Romão, L., and Gomes, F. C. (2009). "Neuron-astrocyte interaction in cell fate commitment in the central nervous system," in *Perspectives of Stem Cells* (Springer), 145–170.

Stipursky, J., Francis, D., and Gomes, F. C. (2012a). Activation of MAPK/PI3K/SMAD pathways by TGF-beta(1) controls differentiation of radial glia into astrocytes in vitro. *Dev. Neurosci.* 34, 68–81. doi: 10.1159/000338108

Stipursky, J., and Gomes, F. C. (2007). TGF-beta1/SMAD signaling induces astrocyte fate commitment in vitro: implications for radial glia development. *Glia* 55, 1023–1033. doi: 10.1002/glia.20522

Stipursky, J., Spohr, T. C., Sousa, V. O., and Gomes, F. C. (2012b). Neuron-astroglial interactions in cell-fate commitment and maturation in the central nervous system. *Neurochem. Res.* 37, 2402–2418. doi: 10.1007/s11064-012-0798-x

Takahashi, H., and Liu, F. C. (2006). Genetic patterning of the mammalian telencephalon by morphogenetic molecules and transcription factors. *Birth Defects Res. C Embryo Today* 78, 256–266. doi: 10.1002/bdrc.20077

Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., et al. (2001). DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. *Dev. Cell* 1, 749–758. doi: 10.1016/s1534-5807(01)00101-0

Uemura, A., Takizawa, T., Ochiai, W., Yanagisawa, M., Nakashima, K., and Taga, T. (2002). Cardiotrophin-like cytokine induces astrocyte differentiation of fetal neuroepithelial cells via activation of STAT3. *Cytokine* 18, 1–7. doi: 10.1006/cyto.2002.1006

Vogel, T., Ahrens, S., Büttner, N., and Krieglstein, K. (2010). Transforming growth factor beta promotes neuronal cell fate of mouse cortical and hippocampal progenitors in vitro and in vivo: identification of Nedd9 as an essential signaling component. *Cereb. Cortex* 20, 661–671. doi: 10.1093/cercor/bhp134

Voigt, T. (1989). Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. *J. Comp. Neurol.* 289, 74–88. doi: 10.1002/cne.902890106

Voss, A. K., Britto, J. M., Dixon, M. P., Sheikh, B. N., Collin, C., Tan, S. S., et al. (2008). C3G regulates cortical neuron migration, preplate splitting and radial glial cell attachment. *Development* 135, 2139–2149. doi: 10.1242/dev.016725

Wachs, F. P., Winner, B., Couillard-Despres, S., Schiller, T., Aigner, R., Winkler, J., et al. (2006). Transforming growth factor-beta1 is a negative modulator of adult neurogenesis. *J. Neuropathol. Exp. Neurol.* 65, 358–370. doi: 10.1097/01.jnen.0000218444.53405.f0

Walantus, W., Castaneda, D., Elias, L., and Kriegstein, A. (2007). In utero intraventricular injection and electroporation of E15 mouse embryos. *J. Vis. Exp.* 239. doi: 10.3791/239

Weimer, J. M., Yokota, Y., Stanco, A., Stumpo, D. J., Blackshear, P. J., and Anton, E. S. (2009). MARCKS modulates radial progenitor placement, proliferation and organization in the developing cerebral cortex. *Development* 136, 2965–2975. doi: 10.1242/dev.036616

Yokota, Y., Eom, T. Y., Stanco, A., Kim, W. Y., Rao, S., Snider, W. D., et al. (2010). Cdc42 and Gsk3 modulate the dynamics of radial glial growth, inter-radial glial interactions and polarity in the developing cerebral cortex. *Development* 137, 4101–4110. doi: 10.1242/dev.048637

Yokota, Y., Gashghaei, H. T., Han, C., Watson, H., Campbell, K. J., and Anton, E. S. (2007). Radial glial dependent and independent dynamics of interneuronal

migration in the developing cerebral cortex. *PLoS One* 2:e794. doi: 10.1371/journal.pone.0000794

Yokota, Y., Kim, W. Y., Chen, Y., Wang, X., Stanco, A., Komuro, Y., et al. (2009). The adenomatous polyposis coli protein is an essential regulator of radial glial polarity and construction of the cerebral cortex. *Neuron* 61, 42–56. doi: 10.1016/j.neuron.2008.10.053

Yoon, K., Nery, S., Rutlin, M. L., Radtke, F., Fishell, G., and Gaiano, N. (2004). Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. *J. Neurosci.* 24, 9497–9506. doi: 10.1523/jneurosci.0993-04.2004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 September 2014; accepted: 03 November 2014; published online: 21 November 2014.

Citation: Stipursky J, Francis D, Dezonne RS, Bérgamo de Araújo AP, Souza L, Moraes CA and Alcantara Gomes FC (2014) TGF-β1 promotes cerebral cortex radial glia-astrocyte differentiation in vivo. Front. Cell. Neurosci. ***8****:393. doi: 10.3389/fncel.2014.00393*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in Cellular Neuroscience

REVIEW
published: 28 April 2015
doi: 10.3389/fncel.2015.00149

Interplay of environmental signals and progenitor diversity on fate specification of cortical GABAergic neurons

*Juliana A. Brandão and Rodrigo N. Romcy-Pereira**

Brain Institute, Federal University of Rio Grande do Norte, Natal, Brazil

Cortical GABAergic interneurons constitute an extremely diverse population of cells organized in a well-defined topology of precisely interconnected cells. They play a crucial role regulating inhibitory-excitatory balance in brain circuits, gating sensory perception, and regulating spike timing to brain oscillations during distinct behaviors. Dysfunctions in the establishment of proper inhibitory circuits have been associated to several brain disorders such as autism, epilepsy, and schizophrenia. In the rodent adult cortex, inhibitory neurons are generated during the second gestational week from distinct progenitor lineages located in restricted domains of the ventral telencephalon. However, only recently, studies have revealed some of the mechanisms generating the heterogeneity of neuronal subtypes and their modes of integration in brain networks. Here we will discuss some the events involved in the production of cortical GABAergic neuron diversity with focus on the interaction between intrinsically driven genetic programs and environmental signals during development.

Keywords: interneuron, cortical development, non-autonomous specification, inhibitory circuit, cell identity

OPEN ACCESS

Edited by:
Cecilia Hedin-Pereira, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Marco Canepari, Institut National de la Santé et de la Recherche Médicale, France
Xin Wang, Stanford University and Howard Hughes Medical Institute, USA

****Correspondence:***
Rodrigo N. Romcy-Pereira, Brain Institute, Federal University of Rio Grande do Norte, Avenida Nascimento de Castro, 2155 Natal, RN 59056-450, Brazil
rnrpereira@neuro.ufrn.br

Received: *11 December 2014*
Accepted: *01 April 2015*
Published: *28 April 2015*

Citation:
Brandão JA and Romcy-Pereira RN (2015) Interplay of environmental signals and progenitor diversity on fate specification of cortical GABAergic neurons. Front. Cell. Neurosci. 9:149. doi: 10.3389/fncel.2015.00149

Introduction

In mammals, the ability to produce their behavioral repertoire relies on a distributed network of neurons that coordinate the action of cortical, subcortical, and spinal cord circuits. From sensorimotor integration to executive functions, all depend on a precise spatio-temporal control of excitation and inhibition in local and long-range networks (Buzsáki, 2010). During brain development, cortical circuits are organized in a well-defined topology of interconnected excitatory and inhibitory neurons whose activities generate coherent behavioral outputs. Inhibitory neurons, in particular, play a crucial role fine-tuning neuronal firing to network oscillations and coordinating the emergence of task-relevant cell assemblies. In addition, GABAergic cells are important to promote the appropriate balance between excitation and inhibition required to prevent overexcitability and excitotoxicity (Somogyi and Klausberger, 2005). Disruption of this inhibitory control has been associated with several neurological disorders including autism, epilepsy, Down syndrome, Fragile X syndrome, X-linked lissencephaly with abnormal genitalia (XLAG), Rett syndrome and schizophrenia (Powell et al., 2003; Levitt et al., 2004; Guidotti et al., 2005; Ramamoorthi and Lin, 2011).

In the adult brain, cortical GABAergic interneurons constitute an extremely diverse population of cells comprising approximately 15–20% of all neurons depending on the cortical region. During

the last 20 years, many studies have characterized and classified interneurons based on morphological and functional properties (DeFelipe, 1993; De Felipe et al., 1997; Kawaguchi and Kubota, 1997; Markram et al., 2004; Ascoli et al., 2008; Klausberger and Somogyi, 2008; Le Magueresse and Monyer, 2013). Although some of these properties are defining features, it is interesting to notice that at least some of them do not remain unchanged during lifetime. Studies have shown that some interneurons are able to change electrophysiological properties or the expression of endogenous molecular markers in response to environmental signals, resulting in functional plasticity in response to particular physiological demands (Steriade et al., 1998; Ascoli et al., 2008). Despite this complexity, accumulating evidence has suggested that none of these features alone can unambiguously define a homogeneous population of inhibitory neurons. In addition to the recent efforts to combine these elements and establish a common criterion to classify interneuron subgroups (Ascoli et al., 2008; DeFelipe et al., 2013), significant progress has been made characterizing the spatial distribution of subtype-specific GABAergic progenitors in the rodent embryo and its molecular determinants. Fate mapping experiments have revealed a group of segregated territories in the embryonic ventral telencephalon (subpallium) capable of generating all major populations of cortical GABAergic interneurons. In these proliferative niches, the patterned expression of transcription factors organize the early commitment of progenitors to specific neuronal fates (Parnavelas et al., 1991; Anderson et al., 1997; Sussel et al., 1999; Nery et al., 2002; Schuurmans and Guillemot, 2002; Xu et al., 2004; Wonders and Anderson, 2006; Flames et al., 2007; Guillemot, 2007; Miyoshi et al., 2010; Gelman et al., 2012). The combination of genetic fate mapping and cell transplantation studies have further revealed the contribution of environmental cues to the post-mitotic stages of interneuron specification thought to occur during migration, lamination, and establishment of synaptic contacts (Magrassi et al., 1998; Wichterle et al., 2001; Nery et al., 2002; Valcanis and Tan, 2003; Butt et al., 2005). In this review, therefore, we will discuss the mechanisms responsible for generating the diversity of GABAergic neurons during development and the interactions between environmental signals (extrinsic cues) and genetic programs (intrinsic factors) required to determine early (molecular) and late (molecular plus functional) cell identity, which will combine a proper laminar integration and functional maturation into specific inhibitory circuits.

Diversity of GABAergic Cortical Interneurons

Cortical GABAergic neurons are aspiny or sparsely spiny non-pyramidal cells that express the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD). They are found across all cortical layers and establish unique connections with excitatory and inhibitory cells in their vicinity. Some of these cells extend long horizontal or vertical axon collaterals within the cortex and have their cell bodies mostly dispersed into layers II–VI. In the adult brain, inhibitory synapses are precisely organized and contact specific sub-cellular compartments (soma, dendritic shafts, dendritic spines, axon initial segment, and pre-synaptic bouton) of particular neuronal types. This synaptic architecture, once formed, establishes the wiring pattern of local inhibitory circuits (Markram et al., 2004; Somogyi and Klausberger, 2005). Understanding cortical circuit function thus, requires a detailed appreciation of the diversity of GABAergic cells wired in the structure and their pattern of neuronal activity.

Some features can be used to define sub-types of GABAergic neurons: the somato-dendritic morphology, axonal arborization arrangement, post-synaptic sub-cellular target, biochemical identity, intrinsic electrophysiological properties, and modes of synaptic and structural plasticity (Gupta et al., 2000; Markram et al., 2004). As for their perisomatic and axonal arborization, cortical GABAergic interneurons are frequently identified as multipolar, bipolar or bitufted, chandelier, basket, or neurogliaform cells with axonal ramifications targeting distinct inhibitory domains on post-synaptic neurons. Basket cells for example show a perisomatic pattern of innervation establishing axo-somatic and axo-dendritic symmetric contacts. In contrast, chandelier cells (or axo-axonic interneurons) contact exclusively the axon initial segment and multipolar Martinotti cells innervate distal dendrites of pyramidal neurons. Such variety of synaptic sites, aiming distinct sub-cellular compartments allows a precise inhibitory control over inputs from different cortical layers.

The molecular composition of GABAergic neurons is also diverse with the expression of calcium-binding proteins parvalbumin (PV), calretinin (CR) and calbindin (CB), and neuropeptides, such as somatostatin (SST), neuropeptide-Y (NPY), cholecystokinin (CCK) and vasoactive intestinal peptide (VIP; Houser et al., 1983; DeFelipe, 1993; Kawaguchi and Kubota, 1997; Gonchar, 2008). Other biochemical markers such as nitric oxide synthase (nNOS), reelin, serotonin receptor 3A (5HTR-3A) are also found in interneuron subpopulations such as ivy cells (neurogliaform), Cajal–Retzius neurons (transient embryonic layer I cells) and superficial layer neurons co-expressing VIP, respectively (Vucurovic et al., 2010; Jaglin et al., 2012). However recent findings have shown that three molecularly distinct non-overlapping populations of neurons can be sorted out according to the expression of PV, SST, and the ionotropic 5HTR-3A (Lee et al., 2010; Rudy et al., 2010). Interestingly, these neurons are born from spatially distinct domains of the embryo.

Interneurons also display a great diversity of intrinsic electrophysiological properties characterized by distinct firing modes in response to input stimuli or step-current injections. They can be classified according to their broad electrophysiological properties as non-accommodating (∼50%), accommodating, (37%) and stuttering cells (∼13%) that can be further sub-grouped into classical spiking (regular fast-spiking and non-fast-spiking), bursting, and delayed onset response cells. Overall, it can be identified at least 14 individual functional sub-types of inhibitory cells (Gupta et al., 2000; Ascoli et al., 2008). Nevertheless, it is important to be cautious when considering this as static properties of a neuronal type. There still little information on inhibitory firing mode plasticity in animals *in vivo*.

Parvalbumin– and somatostatin-expressing interneurons are the two most abundant classes of cortical interneurons with non-overlapping molecular identities and relatively large cell bodies (>20μm; DeFelipe, 1993, 1997; Kawaguchi and Kondo, 2002). GABAergic interneurons expressing PV make up ∼40% of all cortical interneurons of which basket and chandelier cells are typical members. These cells do not express SST, VIP, or CCK, but have some overlap labeling with CR and CB. They emit axonal collaterals to the soma, perisomatic dendrites and axons of post-synaptic targets. Functionally, these neurons show low-input resistance and fast-spiking dynamics of non-accommodating short-duration action potentials that impart a strong inhibitory control over their post-synaptic targets (Kawaguchi and Kubota, 1997; Gupta et al., 2000; Massi et al., 2012). PV-expressing inhibitory neurons represent an important population of cells involved in the modulation of critical period of auditory plasticity and implicated in psychiatric disorders, such as schizophrenia (Beasley and Reynolds, 1997; de Villers-Sidani et al., 2008). SST-expressing neurons, on the other hand, comprise ∼30% of the cortical inhibitory cells with soma preferentially distributed across deep cortical layers V–VI and axons branching into layer I where they exert inhibitory control over distal dendritic processes of pyramidal neurons. Martinotti cells, the most studied sub-population of SST-positive neurons so far, have multipolar somato-dendritic morphology and either regular adapting or intrinsically bursting activity. However, SST is also expressed in three other cell types: the murine X94 transgenic cell line located in layers IV–V with projections to layer IV and either short-duration spikes or stuttering firing pattern (Ma et al., 2006) and, the cells named group 2–3 lines of short asymmetric axons that project to layers II/III. Yet they differ in their electrophysiological properties as one displays strongly adapting regular or stuttering firing modes, whereas the other have regular firing with narrower action potentials (McGarry et al., 2010). SST-positive neurons can also co-express CR (21%), NPY (7%), nNOS, reelin, or CB in various proportions (Pesold et al., 1999; Xu et al., 2010b; Jaglin et al., 2012). The third class of cortical inhibitory neurons with non-overlapping profiles with PV- and SST-positive cells expresses the ionotropic serotonin receptor 5HTR-3A. These cells comprise ∼20–30% of GABAergic neurons in the somatosensory cortex and represent the largest group of superficial neocortical interneurons (Morales and Bloom, 1997; Xu et al., 2010b). Despite the heterogeneity of co-labeling with CCK, NPY, and nNOS, two main subtypes of 5HTR-3A-expressing neurons can be distinguished: cells expressing VIP (∼40% of all 5HTR-3A cells; located in layers II/III) and VIP-negative cells (∼60%), most of them expressing reelin (∼50%; located in layer I). Lee et al. (2010) showed that more than 50% of VIP cells are of bitufted morphology and respond as irregular spiking neurons, while Miyoshi et al. (2010) described four distinct electrophysiological firing modes among VIP cells: burst non-adapting, delayed non-fast spiking, irregular spiking, and fast adapting cells. In contrast, most of VIP-negative GABAergic neurons are neurogliaform cells (reelin-positive) and fire accommodating delayed onset spikes in response to current steps (Lee et al., 2010).

Despite the great heterogeneity, GABAergic interneurons seem to select a group of neurons of the same class and establish synapses with homogenous temporal dynamics. In addition, a study using electrophysiological recordings obtained from pairs of different post-synaptic neurons following stimulation of the same pre-synaptic cell, showed that the same GABAergic axon can establish different types of synapses on distinct classes of target neurons (Gupta et al., 2000). Altogether, it is remarkable but still elusive, how such diversity is generated from a limited number of ventral telencephalic progenitors in the embryo.

Diversity of Cortical Interneuron Progenitors

During embryonic development, excitatory neurons are generated in the ventricular zone (VZ) of the dorsal telencephalon (pallium) and gradually invade the cortical plate by radial migration through specialized glial cells (Rakic, 2009). In the cortex, cell identity and laminar distribution are temporally organized by gene expression patterns and time of cell cycle exit (i.e., birthday), respectively (Angevine and Sidman, 1961; Rakic, 1988). In contrast, most of the brain's GABAergic interneurons derive from progenitors located in four different sub-regions of the subpallium: the medial (MGE), lateral (LGE) and caudal (CGE) ganglionic eminences and, the preoptic area (POA), which are defined by the expression of the homeodomain transcription factor *Dlx1/2* and the absence of *Pax6* gene (**Figure 1**). Neural progenitors localized in the MGE, CGE, and POA give rise to virtually all cortical interneurons, whereas LGE progenitors produce GABAergic cell populations of olfactory bulb, amygdala, and striatum (Van Eden et al., 1989; Nery et al., 2002; Stuhmer et al., 2002; Xu et al., 2004; Flames et al., 2007; Gelman et al., 2011). It is important to mention that excitatory and inhibitory neurons are generated independently, in spatially segregated domains and do not share a common lineage. In the subpallium, regional identity is regulated by a combination of transcription factors with overlapping expression patterns, some of them showing cell fate restriction functions (Anderson et al., 1997; Sussel et al., 1999; Flames and Marin, 2005).

Medial ganglionic eminence progenitors are responsible for generating approximately 60% of all GABAergic neurons in the cortex. Around embryonic day 9 (E9), the action of the signaling molecule *Shh* (*sonic hedgehog*) on MGE cells induces the expression *Nkx2.1* and *Lhx6* transcription factors that orchestrates intracellular cascades required for the specification of SST-expressing and PV-expressing interneurons (Butt et al., 2008; Du et al., 2008). Although *Nkx2.1* and *Lhx6* specifically define the MGE neuroepithelium, *Nkx2.1* is only briefly expressed in MGE progenitors. In a cascade of events, *Lhx6*, a direct target of *Nkx2.1*, is required for the acquisition of molecular identity and laminar positioning (Sussel et al., 1999; Liodis et al., 2007) and *Sox6* and *Satb1* seem to act as downstream effectors of *Lhx6* (Azim et al., 2009; Batista-Brito et al., 2009; Close et al., 2012). Analysis of *Lhx6* mutant mice has shown that even though $Lhx6^{-/-}$ progenitors are still able to migrate to the pallium, most derived interneurons lack PV and SST expression and show abnormal laminar integration (Zhao et al., 2008). Interestingly, Xu et al. (2010a) have recently demonstrated that high levels

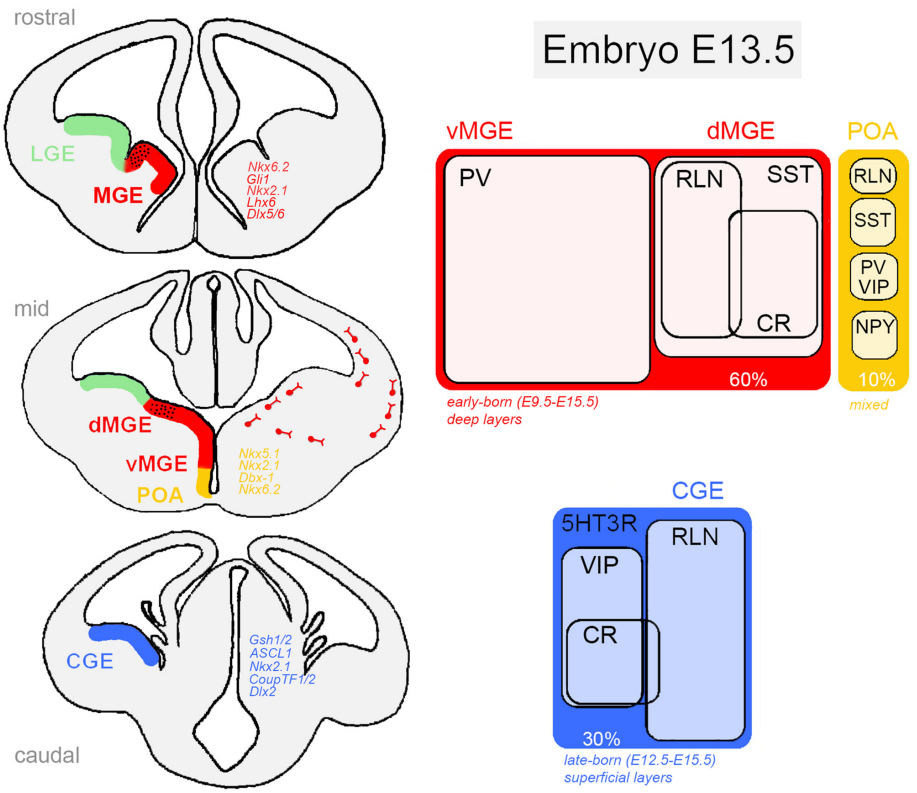


FIGURE 1 | Ventral telencephalic germinative zones of cortical GABAergic neurons in the rodent embryonic brain. The medial ganglionic eminence (dorsal, dMGE; ventral, vMGE), caudal ganglionic eminence (CGE), and the preoptic area (POA) are responsible for generating virtually all cortical interneurons. In each region, neural progenitor classes are defined by a combinatorial action of transcription factors **(Left)** that restrict cells to neurochemically defined fates **(Right)**. Distinct classes of inhibitory cells display characteristic morphology, laminar distribution and electrophysiological properties.

5HT3R, serotonin receptor type 3; CR, calretinin; NPY, neuropeptide Y; PV, parvalbumin; RLN, reelin; SST, somatostatin; VIP, vasointestinal active peptide. Genes: ASCL1, achaete-scute family bHLH transcription factor 1 (Mash1, mammalian homologue); Dbx-1, developing brain homeobox 1; CoupTF1/2, chicken ovalbumin upstream promoter transcription factor; Dlx (Dlx1/2/5/6), distal-less homeobox; Gli1, Gli family zinc finger 1; Gsh1/2, GS homeobox 2; Lhx6, LIM homeobox 6; Nkx (Nkx2.1, Nkx5.1, Nkx6.2), NK homeobox family.

of *Shh* in dorsal MGE compared to ventral MGE is responsible for a dorso-ventral patterning of progenitors in this region, suggesting an important role for soluble factors in early fate determination in the subpallium. As a result, two different territories in the MGE can be identified. The dorsal division (dMGE) is enriched in *Nkx6.2* and *Gli1* genes, and preferentially gives rise to SST-expressing interneurons comprising about 65% of all MGE-derived neurons, including Martinotti cells that co-express CR, NPY-expressing cells and all nNOS-positive neurons. The ventral division (vMGE), in contrast, is enriched in *Dlx5/6* and *Lhx6* genes and generates most of the PV-expressing interneurons of the cortex (∼35%) that includes large basket and chandelier cells (Fogarty et al., 2007; Jaglin et al., 2012).

These two lineages of neurons are born around E12.5-E16.5 (peak E14.5) and migrate tangentially toward the pallium through the subventricular zone (SVZ) and marginal zones (MZ) to be subsequently incorporated into the cortical plate (Butt et al., 2005; Miyoshi et al., 2007). They follow a temporal-positioning code according to their birthdate showing an inside–out neurogenic gradient of lamination. Although the final distribution of MGE-derived neurons encompasses preferentially deep cortical layers (layers IV–VI), some neurons have relatively restricted laminar patterns as seen for chandelier cells that prefer layers II and IV (Taniguchi et al., 2013).

The other major contributing region for the generation of cortical GABAergic neurons is the CGE that can be considered the caudal extension of MGE and LGE in the ventral telencephalon. The CGE is responsible for producing about 30% of all adult cortical interneurons, of which virtually all express the serotonin receptor subtype 3a (5HT3aR; Rudy et al., 2010; Vucurovic et al., 2010). Interneurons from the CGE have bipolar or double-bouquet morphology and display electrophysiological characteristics of irregular firing or fast-adapting cells. Electrophysiological studies have grouped these cells in more than six different subtypes according to their firing patterns and morphology (Lee et al., 2010; Miyoshi et al., 2010). Forty percent of these neurons co-express VIP (some also expressing CR, but negative for SST), whereas about 80% of the remaining VIP-negative cells express the extracellular signaling protein reelin (Nery et al., 2002; Lee et al., 2010; Miyoshi et al., 2010; Jaglin et al., 2012). Overall,

CR-positive/SST-negative cells, as well as the great majority of VIP-, CCK-, and reelin-expressing GABAergic interneurons are derived from 5HT3aR-expressing CGE progenitors.

These progenitors can also be identified by an abundant expression of the transcription factors *CoupTF1/2*, *Gsh1/2* (Sousa et al., 2009; Lee et al., 2010). *Gsh2* is enriched in the CGE and controls the expression of *Mash1*, *ASCL1*, *Dlx2* required for CGE patterning. In fact, there is evidence suggesting an internal regionalization of the CGE, in which the dorsal region expresses *Gsh2* and induces the proneural gene *Mash1* and *Delta* (a Notch ligand), leading to repression of progenitor differentiation, whereas the ventral CGE is enriched in *NKx2.1*. The consequences of this molecular regionalization are still not clear.

Caudal ganglionic eminence-derived neurons are late-born cells with peak production around E16.5, migrating tangentially toward the pallium through the SVZ and MZ (Cavanagh and Parnavelas, 1989). They do not follow a temporal-positioning code, but instead get distributed homogeneously across superficial cortical layers (layers I–III), with ∼40% occupancy in layers II and III. An exception to this rule is the outside-in neurogenic gradient observed in CR-positive interneurons (Lee et al., 2010).

In the ventro-medial region of the subpallium, the POA generates the remaining 10% of cortical interneurons, including multiple classes of GABAergic cells. They consist of a small fraction of PV-, SST-, NPY-, and reelin-expressing cells, all derived from POA progenitors with transcriptional programs distinct from those of the MGE and CGE (Gelman et al., 2011). Interestingly, mice lacking *Lhx6* expression (a specific marker of MGE progenitors) are almost completely depleted of PV-positive and SST-positive neurons, except for some scattered cells possibly derived from POA, seen in deep layers of the cortex (Liodis et al., 2007; Zhao et al., 2008). Fate determination in the POA seems to be orchestrated by the action of several genes under the instruction of *Shh* and *Nkx2.1*. Using genetic fate-mapping and transcription factor expression analysis, it has been shown that POA contains at least two progenitor domains defined by the non-overlapping cellular expression of *Dbx-1*, *Nkx6.2* (ventrally), and *Nkx5.1* (dorsally; Flames et al., 2007; Gelman et al., 2011). About 40% of the POA-derived interneurons are generated by *Dbx-1*-expressing progenitors, most of them born on E12.5, distributed across layers V and VI of the cortex and expressing PV, SST, and reelin. On the other hand, *Nkx5.1*-derived interneurons are mostly found in superficial layers of the cortex and constitute a rather homogeneous population of rapidly adapting interneurons, many of which express NPY. However, it is still not clear so far how such diversity of interneuron subtypes arise from the *Dbx-1*-expressing lineage (Gelman et al., 2009).

Role of Environmental Factors on Cortical Interneuron Differentiation

Cortical interneurons acquire much of their adult identity by the time of birth (i.e., after the last cell division), due to early fate restriction imposed by genetic programs (Xu et al., 2005; Flames et al., 2007; Fogarty et al., 2007; Miyoshi et al., 2007; Butt et al., 2008; Azim et al., 2009; Batista-Brito et al., 2009; Neves et al., 2013; Kessaris et al., 2014; Vogt et al., 2014). However, many of the characteristics used to define interneurons are not evident until late postnatal ages and even adulthood. Therefore, it is reasonable to inquire what are the post-mitotic events necessary to assure proper development of cortical inhibitory circuits. Recent findings have given support to the idea that interactions with environmental signals starting from early post-mitotic stages and all way along their migratory path to final destination are essential for the full expression of mature features (Valcanis and Tan, 2003; Rouaux and Arlotta, 2010; De Marco García et al., 2011; Lodato et al., 2011; Close et al., 2012; Denaxa et al., 2012; Spitzer, 2012). Some of the features that undergo protracted specification include cortical area assignment, laminar positioning, neurochemical identity, dendritic arbor topology, and post-synaptic cell targeting.

Molecular signals in the environment as well as activity-dependent membrane currents seem to be necessary cues to guide the execution and fulfillment of genetic programs set in motion during early stages of interneuron development (**Figure 2**). For instance, evidences show that manipulations of these environmental cues surrounding neural progenitors affect the neurochemical identity of mature interneurons. Resident pyramidal neurons, electrical activity, and local molecular codes are thought to instruct intrinsic programs and promote neuronal migration, layer positioning, cell target selection, and the precise apposition of subcellular synaptic contacts. Here we will discuss some of these studies.

Pyramidal Cells Controls Interneuron Laminar Identity

Laminar positioning of interneurons in the cortex depends both upon their place of origin and date of birth (Miyoshi and Fishell, 2011), however, evidence points to a more interactive and epigenetic perspective on interneuron laminar specification. Valcanis and Tan (2003) have shown that early- and late-born interneuron progenitors have their laminar fate re-specified by extrinsic signals of the new environment. Using transplantation experiments, the authors show that donor progenitor cells can acquire the host laminar distribution once they undergo a last round of cell division in the host tissue (Valcanis and Tan, 2003).

It was also observed that the interaction of migrating interneurons with resident pyramidal cells control the laminar identity of GABAergic cells. Pla et al. (2006) showed that disrupting the normal layering of pyramidal neurons, in *Dab1-/-* mice deficient in reelin signaling, modifies the laminar allocation of MGE-derived cells. Wild-type interneurons transplanted into the cortex of *Dab1* mutants closely matched the layer distribution of host pyramidal neurons, whereas *Dab1-/-* interneurons transplanted in wild-type cortex were able to generate normal interneuron positioning, as if guided by well-positioned local pyramidal cells (Pla et al., 2006). Collectively, these findings support a reelin-independent mechanism where pyramidal neurons are required for proper interneuron integration. The authors also noticed that more than 20% of early born (E12) and ∼60% of late born (E15) wild-type interneurons change their laminar fate upon transplantation into older (E15) developing wild-type cortex.

In agreement with the previous results, Lodato et al. (2011) have recently shown that SST- and PV-expressing interneurons are specifically reduced in layer 5 of *Fezf2*-depleted mice, in which

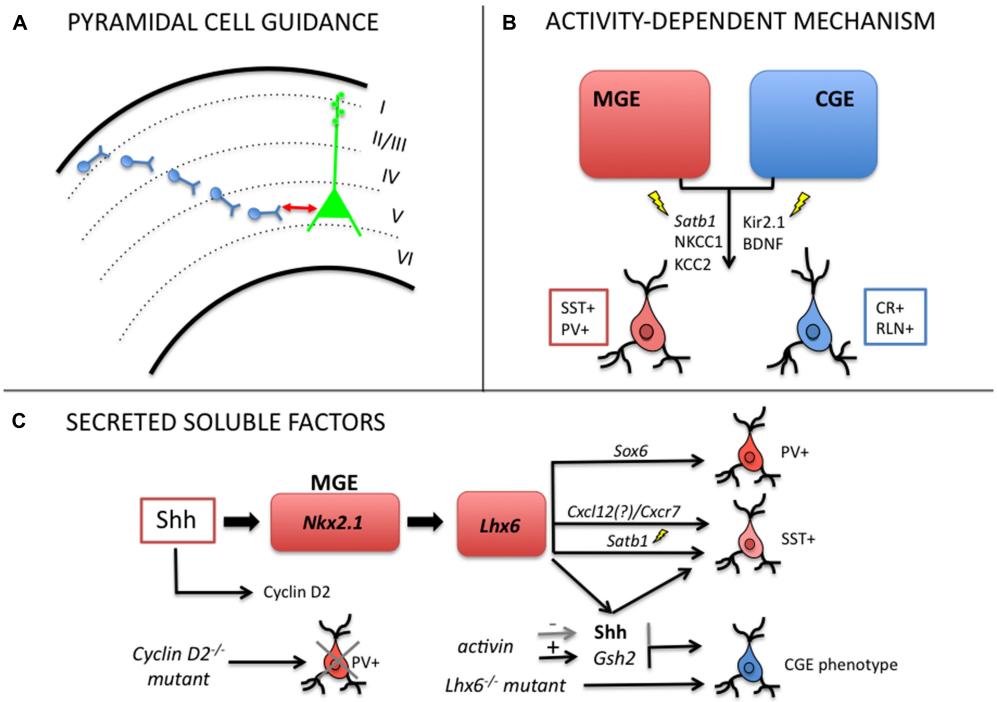


FIGURE 2 | Mechanisms regulating post-mitotic plasticity and late-fate specification of cortical GABAergic interneurons. (A) Local pyramidal cells modulate laminar positioning of inhibitory neurons in the cortex (Pla et al., 2006; Lodato et al., 2011). **(B)** Electrical activity (GABA-mediated membrane depolarization, Ca^{2+} transients) can regulate late acquisition of molecular identity in MGE- and CGE-derived interneurons (Berghuis et al., 2004; Borodinsky et al., 2004; De Marco García et al., 2011; Close et al., 2012; Denaxa et al., 2012). **(C)** Soluble secreted factors can alter the balance of interneuron subtypes through shift in neurochemical identity (Xu et al., 2005; Lopez-Bendito et al., 2008; Cambray et al., 2012; Vogt et al., 2014).

subcerebral pyramidal projection neurons are absent from the cortex. The lack of glutamatergic projection neurons in layer 5 of these animals creates an abnormal distribution of PV and SST neurons, which suggests that these neurons control the laminar positioning of inhibitory migrating cells in the cortex (Lodato et al., 2011).

Electrical Activity Instructs Phenotypic, Laminar and Synaptic Identity

Another environmental signal that regulates late fate specification of cortical interneurons is electrical activity. In CGE-derived neurons, normal excitability is required for proper integration into their final destination. Using *in utero* electroporation with the inward rectifying potassium channel Kir2.1 directed to CGE-derived neurons, De Marco García et al. (2011) showed that depolarization was necessary for the full phenotypic development of CR- and reelin-expressing cells. They observed morphological defects in the axonal arbor and dendritic tree of both neuron subtypes or only in reelin-expressing cells under hyperpolarization, respectively. Although tangential migration was unchanged, it was detected a shift in laminar positioning when potassium currents were induced after post-natal day 5 (P5) in CR+ and Re+ CGE-derived neurons. These results indicate that genetic programs initiated at progenitor stage can be modulated by electrical activity during development.

Furthermore, Berghuis et al. (2004) have shown that neuronal depolarization of PV-expressing neurons maintained in culture enhances the BDNF differentiating effects. While BDNF promotes dendritic branching, somatic differentiation, strengthening of synaptic connections, and frequency modulation of action potentials, the addition of KCl (i.e., depolarization) was required for the establishment of reciprocal inhibitory synapses and significantly accelerated the formation of synaptic contacts (Berghuis et al., 2004). Consistent with this idea, GABA and glutamate play an important role in post-mitotic cortical neuronal motility as it modulates intracellular calcium (Ca^{2+}) transients through GABAA and NMDA/AMPA receptor-mediated depolarization. In contrast, hyperpolarizing GABA decreases the magnitude of Ca^{2+} transients inhibiting motility of migrating cells. After reaching the cortex, migrating interneurons upregulate the potassium/chloride (K^+/Cl^-) exchanger KCC2, which determines the developmental switch from depolarizing to hyperpolarizing action of GABA (Ben-Ari, 2002). KCC2 expression thus can act as a switch to induce a voltage-sensitive, Ca^{2+}-mediated reduction of interneuron motility and function as a migration stop signal (Bortone and Polleux, 2009). Indeed, migrating cortical interneurons may sense and integrate the ambient, local extracellular levels of GABA and glutamate as a way to determine when to stop migration. In the ganglionic eminence, progenitors are the main source of GABA and MGE-derived interneurons begin to express KCC2 a few hours

after reaching the cortex (Hevner et al., 2004; Inamura et al., 2012).

Ca^{2+} spikes also modulate the neurochemical specification of neural progenitors between GABA/glycine and glutamate/acetylcholine in the dorso-ventral axis of neural tube (Borodinsky et al., 2004). Cells exhibiting high frequency of Ca^{2+} spikes express GABA and glycine, whereas cells with a low spike frequency take the glutamatergic and cholinergic phenotype. In the spinal cord, Ca^{2+}-mediated signaling contributes to proliferation, migration, axon pathfinding, dendritic growth, and specification of neurotransmitter subtype (Rosenberg and Spitzer, 2011).

Another particular example concerns the chromatin organizer and transcription factor *Satb1* (special AT-rich binding protein) that is specifically expressed in mature interneurons located in the cortical plate. Two recent studies have demonstrated that *Satb1* is a key molecule acting downstream *Lhx6* to control maturation and late differentiation of cortical interneurons in an activity-dependent manner. Ablation of *Satb1* in mice promotes a dramatic decrease of SST mRNA and protein expression in postnatal cortical interneurons, but no effect on the expression in migrating precursors (Close et al., 2012; Denaxa et al., 2012). Close et al. (2012) showed that embryonic deletion of *Satb1* in MGE-derived interneurons disrupts the migration and synaptic integration of both PV- and SST-expressing neurons into nascent cortical circuits with special effect on the differentiation of SST-expressing cells. Interestingly, *Satb1* is regulated by neuronal activity, which is required for the establishment of mature patterns of inhibition onto cortical pyramidal cells (Close et al., 2012). The second study showed that treatment of dorsal telencephalon cell cultures with KCl induced the expression of *Satb1* and *c-FOS* in cortical GABAergic neurons within 24 h. In addition, *Satb1* was shown to be required for the induction of the neurochemical phenotype typical of mature SST-expressing GABAergic neurons and was regulated by neuronal excitability involving Ca^{2+} influx and GABA receptor activation. Using misexpression experiments in the MGE of E14.5 embryos, the authors observed that *Satb1* could further affect neuronal activity by modulating the expression of *NKCC1* (a Na^{+}–K^{+}–Cl^{-} co-transporter abundantly expressed in immature GABAergic interneurons) and *KCC2* (Denaxa et al., 2012). *KCC2* expression by its turn could set off the termination of cortical interneuron migration in a voltage-sensitive and calcium-dependent manner.

Soluble Factors Shape Cell Identity

Another possible mechanism of post-mitotic plasticity is the interaction of migrating interneurons with soluble molecules in the environment. In an early study, Iacovitti et al. (1987) observed that post-mitotic neurons obtained from E13 embryonic cortical cultures, grown for 1 day, expressed a catecholaminergic phenotype different from what was observed *in vivo* in the cortex. The authors then suggested that external factors might have interfered with neurotransmitter specification *in vitro* that did not occur *in vivo* (Iacovitti et al., 1987).

Other studies further revealed that environmental signals could in fact regulate neurochemical identity of GABAergic neurons. In a first report, dissociated cortical interneurons prepared at birth failed to express VIP at P2, whereas interneurons from neonatal slices of same age did express the neuropeptide (Gotz and Bolz, 1994). Following that Gulacsi and Lillien (2003) showed that dorsal telencephalic progenitors co-cultured with ventral progenitors generated more GABAergic neurons than in single culture. This effect was reduced or enhanced by the addition of cyclopamine, an antagonist of *Shh* or exogenous *Shh*, respectively (Gulacsi and Lillien, 2003). New evidence to *Shh* involvement came from Xu et al. (2005) showing that dissociated interneuron cells treated with cyclopamine expressed reduced PV and SST phenotypes. Moreover, using a conditional mutant mice *NestinCre:Shh(flox/flox)*, in which *Shh* signaling is deficient, they found altered MGE patterning and reduced number of NPY, PV, and SST neurons in the somatosensory cortex (Xu et al., 2005). In fact, *Shh* seems to maintain the identity of cortical interneuron progenitors in the ventral telencephalon through a continuous provision of positional information by regulating *Nkx2.1* expression. In this manner, it plays a critical role in determining the relative composition of cortical excitatory and inhibitory neurons.

It is known that post-mitotic GABAergic neurons are guided toward the cortex along two main migratory streams (MZ and SVZ) by the interaction with soluble chemoattractants and chemorepellents dispersed in the pathway. Among them, Cxcl12 (and its receptors, Cxcr4, Cxcr7), a potent chemoattractant to MGE-derived interneurons is required for normal positioning of interneurons in the cortex (Li et al., 2008; Lopez-Bendito et al., 2008). Recently, it was reported that Arx and Cxcr7 (*Lhx6*-target genes) could rescue the cell identity and laminar phenotype of $Lhx6^{-/-}$ MGE cells. In *Lhx6* mutant mice, PV and SST-expressing neurons are specifically reduced and some cells acquire CGE-like fate. *Lhx6/8* are known to be necessary for *Shh* expression in MGE and to determine cell fate of PV- and SST-neurons. Besides, *Lhx6/8* are also required for repressing *Dlx1* in the pallium, preventing GABAergic fate of local progenitors (Flandin et al., 2011; Vogt et al., 2014).

Another example of a soluble molecule with putative fate specification role is activin. It is a member of the TGF-3 family of neurotrophic factors, produced in LGE/CGE and shown to induce telencephalic precursors to adopt LGE/CGE cell fate. Through the specific induction of *Gsh2* expression and inhibition of *Shh* and *Nkx2.1*, it promotes the acquisition of CGE fate and CR phenotype (Cambray et al., 2012). In fact, neurotrophins were already shown to regulate the relative number of GABAergic and cholinergic neurons in the rodent basal forebrain through their action on p75 receptor during development (Lin et al., 2007). Moreover, some studies show that serotonin depletion transiently delays the incorporation of CGE-derived interneurons into the cortical plate and alters the maturation of CR- and CCK-expressing interneurons in the somatosensory cortex. Such depletion also decreases reelin secretion by Caja–Retzius cells leading to dendritic hyper-complexity in pyramidal neurons (Vitalis et al., 2007, 2013).

More recently, Díaz-Alonso et al. (2012), using *in vitro* and *in vivo* experiments, observed that embryonic endocannabinoids

(eCB) regulate the intrinsic program for layer fate specification of cortical pyramidal neurons through CB1 receptors. The authors showed that CB1 signaling controls the appropriate balance of neuronal differentiation in deep cortical layers by inhibiting *Satb2* and promoting *Ctip2* expression following extracellular levels of eCB. Cortico-spinal and subcerebral projection neurons were particularly affected. Interestingly, CB1 receptor inactivation decreased the expression of deep-layer markers *Fezf2* and *Ctip2* in pyramidal cells, but did not affect *Ctip2* expression in GABAergic neurons (Díaz-Alonso et al., 2012). However, considering that laminar identity of cortical GABAergic interneurons can be regulated by resident glutamatergic cells, these findings also suggest that eCB might indirectly govern the laminar allocation of interneurons.

Another indirect effect on the final organization of cortical interneuron circuits may be exerted by the neurotrophin 3 (Nt3). Through the repressor *Sip1*, Nt3 acts as a feedback signal between post-mitotic and progenitors neurons. It is able to switch the cell fate of apical progenitors and promote overproduction of superficial layer cortical neurons in the developing mouse. Changing the balance of superficial and deep projection neurons, Nt3 might affect interneurons layer positioning. *Sip1* could also switch progenitors fate from neurogenesis to gliogenesis (Seuntjens et al., 2009; Parthasarathy et al., 2014).

Finally, the number of specific interneuronal subtypes in the cortex and their final fate was shown to be critically determined by the regulation of cell cycle in neuronal progenitors. Actually, it was observed that *cyclin D2* delays cell cycle exit of MGE progenitors and its deletion leads to a prominent reduction in PV-positive cells in the mature rodent cortex along with microcephalic phenotype (Glickstein et al., 2007). Interestingly, *cyclin D2* expression seems to be regulated by Nt3 and Shh (Lukaszewicz et al., 2002; Komada et al., 2013).

Conclusion

Cell fate specification of cortical interneurons seems to require the interplay of both intrinsic and extrinsic molecular signals. However, the distinct aspects of such delicate control just began to be unveiled. Time of signaling, cell-type targeting, magnitude of phenotypic effects, and the particular molecular mechanisms involved are still unknown. Future experiments should bring some light on these open questions.

References

Anderson, S. A., Eisenstat, D. D., Shi, L., and Rubenstein, J. L. (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* 278, 474–476.

Angevine, J. B. Jr., and Sidman, R. L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* 192, 766–768.

Ascoli, G. A., Alonso-Nanclares, L., Anderson, S. A., Barrionuevo, G., Benavides-Piccione, R., Burkhalter, A., et al. (2008). Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat. Rev. Neurosci.* 9, 557–568. doi: 10.1038/nrn2402

Azim, E., Jabaudon, D., Fame, R. M., and Macklis, J. D. (2009). SOX6 controls dorsal progenitor identity and interneuron diversity during neocortical development. *Nat. Neurosci.* 12, 1238–1247. doi: 10.1038/nn.2387

Batista-Brito, R., Rossignol, E., Hjerling-Leffler, J., Denaxa, M., Wegner, M., Lefebvre, V., et al. (2009). The cell-intrinsic requirement of Sox6 for cortical interneuron development. *Neuron* 63, 466–481. doi: 10.1016/j.neuron.2009.08.005

Beasley, C. L., and Reynolds, G. P. (1997). Parvalbumin-immunoreactive neurons are reduced in the prefrontal cortex of schizophrenics. *Schizophr. Res.* 24, 349–355.

Ben-Ari, Y. (2002). Excitatory actions of gaba during development: the nature of the nurture. *Nat. Rev. Neurosci.* 3, 728–739. doi: 10.1038/nrn920

Berghuis, P., Dobszay, M. B., Sousa, K. M., Schulte, G., Mager, P. P., Hartig, W., et al. (2004). Brain-derived neurotrophic factor controls functional differentiation and microcircuit formation of selectively isolated fast-spiking GABAergic interneurons. *Eur. J. Neurosci.* 20, 1290–1306. doi: 10.1111/j.1460-9568.2004.03561.x

Borodinsky, L. N., Root, C. M., Cronin, J. A., Sann, S. B., Gu, X., and Spitzer, N. C. (2004). Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. *Nature* 429, 523–530. doi: 10.1038/nature02518

Bortone, D., and Polleux, F. (2009). KCC2 expression promotes the termination of cortical interneuron migration in a voltage-sensitive calcium-dependent manner. *Neuron* 62, 53–71. doi: 10.1016/j.neuron.2009.01.034

Butt, S. J. B., Fuccillo, M., Nery, S., Noctor, S., Kriegstein, A., Corbin, J. G., et al. (2005). The temporal and spatial origins of cortical interneurons predict their physiological subtype. *Neuron* 48, 591–604. doi: 10.1016/j.neuron.2005.09.034

Butt, S. J. B., Sousa, V. H., Fuccillo, M. V., Hjerling-Leffler, J., Miyoshi, G., Kimura, S., et al. (2008). The requirement of Nkx2-1 in the temporal specification of cortical interneuron subtypes. *Neuron* 59, 722–732. doi: 10.1016/j.neuron.2008.07.031

Buzsáki, G. (2010). Neural syntax: cell assemblies, synapsembles, and readers. *Neuron* 68, 362–385. doi: 10.1016/j.neuron.2010.09.023

Cambray, S., Arber, C., Little, G., Dougalis, A. G., De Paola, V., Ungless, M. A., et al. (2012). Activin induces cortical interneuron identity and differentiation in embryonic stem cell-derived telencephalic neural precursors. *Nat. Commun.* 3, 841. doi: 10.1038/ncomms1817

Cavanagh, M. E., and Parnavelas, J. G. (1989). Development of vasoactive-intestinal-polypeptide-immunoreactive neurons in the rat occipital cortex: a combined immunohistochemical-autoradiographic study. *J. Comp. Neurol.* 284, 637–645. doi: 10.1002/cne.902840410

Close, J., Xu, H., De Marco Garcia, N., Batista-Brito, R., Rossignol, E., Rudy, B., et al. (2012). Satb1 is an activity-modulated transcription factor required for the terminal differentiation and connectivity of medial ganglionic eminence-derived cortical interneurons. *J. Neurosci.* 32, 17690–17705. doi: 10.1523/JNEUROSCI.3583-12.2012

DeFelipe, J. (1993). Neocortical neuronal diversity: chemical heterogeneity revealed by colocalization studies of classic neurotransmitters, neuropeptides, calcium-binding proteins, and cell surface molecules. *Cereb. Cortex* 3, 273–289.

DeFelipe, J. (1997). Types of neurons, synaptic connections and chemical characteristics of cells immunoreactive for calbindin-D28K, parvalbumin and calretinin in the neocortex. *J. Chem. Neuroanat.* 14, 1–19.

DeFelipe, J., López-Cruz, P. L., Benavides-Piccione, R., Bielza, C., Larrañaga, P., Anderson, S., et al. (2013). New insights into the classification and nomenclature of cortical GABAergic interneurons. *Nat. Rev. Neurosci.* 14, 202–216. doi: 10.1038/nrn3444

De Felipe, J., Marco, P., Fairén, A., and Jones, E. G. (1997). Inhibitory synaptogenesis in mouse somatosensory cortex. *Cereb. Cortex* 7, 619–634.

De Marco García, N. V., Karayannis, T., and Fishell, G. (2011). Neuronal activity is required for the development of specific cortical interneuron subtypes. *Nature* 472, 351–355. doi: 10.1038/nature09865

de Villers-Sidani, E., Simpson, K. L., Lu, Y. F., Lin, R. C., and Merzenich, M. M. (2008). Manipulating critical period closure across different sectors

of the primary auditory cortex. *Nat. Neurosci.* 11, 957–965. doi: 10.1038/nn.2144

Denaxa, M., Kalaitzidou, M., Garefalaki, A., Achimastou, A., Lasrado, R., Maes, T., et al. (2012). Maturation-promoting activity of SATB1 in MGE-derived cortical interneurons. *Cell Rep.* 2, 1351–1362. doi: 10.1016/j.celrep.2012.10.003

Díaz-Alonso, J., Aguado, T., Wu, C.-S., Palazuelos, J., Hofmann, C., Garcez, P., et al. (2012). The CB(1) cannabinoid receptor drives corticospinal motor neuron differentiation through the Ctip2/Satb2 transcriptional regulation axis. *J. Neurosci.* 32, 16651–16665. doi: 10.1523/JNEUROSCI.0681-12.2012

Du, T., Xu, Q., Ocbina, P. J., and Anderson, S. A. (2008). NKX2.1 specifies cortical interneuron fate by activating Lhx6. *Development* 135, 1559–1567. doi: 10.1242/dev.015123

Flames, N., and Marin, O. (2005). Developmental mechanisms underlying the generation of cortical interneuron diversity. *Neuron* 46, 377–381. doi: 10.1016/j.neuron.2005.04.020

Flames, N., Pla, R., Gelman, D. M., Rubenstein, J. L. R., Puelles, L., and Marín, O. (2007). Delineation of multiple subpallial progenitor domains by the combinatorial expression of transcriptional codes. *J. Neurosci.* 27, 9682–9695. doi: 10.1523/JNEUROSCI.2750-07.2007

Flandin, P., Zhao, Y., Vogt, D., Jeong, J., Long, J., Potter, G., et al. (2011). Lhx6 and Lhx8 coordinately induce neuronal expression of Shh that controls the generation of interneuron progenitors. *Neuron* 70, 939–950. doi: 10.1016/j.neuron.2011.04.020

Fogarty, M., Grist, M., Gelman, D., Marín, O., Pachnis, V., and Kessaris, N. (2007). Spatial genetic patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult cortex. *J. Neurosci.* 27, 10935–10946. doi: 10.1523/JNEUROSCI.1629-07.2007

Gelman, D., Griveau, A., Dehorter, N., Teissier, A., Varela, C., Pla, R., et al. (2011). A wide diversity of cortical gabaergic interneurons derives from the embryonic preoptic area. *J. Neurosci.* 31, 16570–16580. doi: 10.1523/jneurosci.4068-11.2011

Gelman, D. M., Marín, O., and Rubenstein, J. L. R. (2012). "The generation of cortical interneurons," in *Jesper's Basic Mechanisms of the Epilepsies*, 4th Edn, eds J. Noebels, M. Avoli, M. Rogawski, R. Olsen, and A. Delgado-Escueta (New York: Oxford University Press).

Gelman, D. M., Martini, F. J., Nobrega-Pereira, S., Pierani, A., Kessaris, N., and Marin, O. (2009). The embryonic preoptic area is a novel source of cortical GABAergic interneurons. *J. Neurosci.* 29, 9380–9389. doi: 10.1523/JNEUROSCI.0604-09.2009

Glickstein, S. B., Moore, H., Slowinska, B., Racchumi, J., Suh, M., Chuhma, N., et al. (2007). Selective cortical interneuron and GABA deficits in cyclin D2-null mice. *Development* 134, 4083–4093. doi: 10.1242/dev.008524

Gonchar, Y. (2008). Multiple distinct subtypes of GABAergic neurons in mouse visual cortex identified by triple immunostaining. *Front. Neuroan.* 1:3. doi: 10.3389/neuro.05.003.2007

Gotz, M., and Bolz, J. (1994). Differentiation of transmitter phenotypes in rat cerebral cortex. *Eur. J. Neurosci.* 6, 18–32.

Guidotti, A., Auta, J., Davis, J. M., Dong, E., Grayson, D. R., Veldic, M., et al. (2005). GABAergic dysfunction in schizophrenia: new treatment strategies on the horizon. *Psychopharmacology* 180, 191–205. doi: 10.1007/s00213-005-2212-8

Guillemot, F. (2007). Cell fate specification in the mammalian telencephalon. *Progr. Neurobiol.* 83, 37–52. doi: 10.1016/j.pneurobio.2007.02.009

Gulacsi, A., and Lillien, L. (2003). Sonic hedgehog and bone morphogenetic protein regulate interneuron development from dorsal telencephalic progenitors in vitro. *J. Neurosci.* 23, 9862–9872.

Gupta, A., Wang, Y., and Markram, H. (2000). Organizing principles for a diversity of GABAergic interneurons and synapses in the neocortex. *Science* 287, 273–278.

Hevner, R. F., Daza, R. A., Englund, C., Kohtz, J., and Fink, A. (2004). Postnatal shifts of interneuron position in the neocortex of normal and reeler mice: evidence for inward radial migration. *Neuroscience* 124, 605–618. doi: 10.1016/j.neuroscience.2003.11.033

Houser, C. R., Hendry, S. H., Jones, E. G., and Vaughn, J. E. (1983). Morphological diversity of immunocytochemically identified GABA neurons in the monkey sensory-motor cortex. *J. Neurocytol.* 12, 617–638.

Iacovitti, L., Lee, J., Joh, T. H., and Reis, D. J. (1987). Expression of tyrosine hydroxylase in neurons of cultured cerebral cortex: evidence for phenotypic plasticity in neurons of the CNS. *J. Neurosci.* 7, 1264–1270.

Inamura, N., Kimura, T., Tada, S., Kurahashi, T., Yanagida, M., Yanagawa, Y., et al. (2012). Intrinsic and extrinsic mechanisms control the termination of cortical interneuron migration. *J. Neurosci.* 32, 6032–6042. doi: 10.1523/JNEUROSCI.3446-11.2012

Jaglin, X. H., Hjerling-Leffler, J., Fishell, G., and Batista-Brito, R. (2012). The origin of neocortical nitric oxide synthase-expressing inhibitory neurons. *Front. Neural Cir.* 6:44. doi: 10.3389/fncir.2012.00044

Kawaguchi, Y., and Kondo, S. (2002). Parvalbumin, somatostatin and cholecystokinin as chemical markers for specific GABAergic interneuron types in the rat frontal cortex. *J. Neurocytol.* 31, 277–287.

Kawaguchi, Y., and Kubota, Y. (1997). GABAergic cell subtypes and their synaptic connections in rat frontal cortex. *Cereb. Cortex* 7, 476–486.

Kessaris, N., Magno, L., Rubin, A. N., and Oliveira, M. G. (2014). Genetic programs controlling cortical interneuron fate. *Curr. Opin. Neurobiol* 26, 79–87. doi: 10.1016/j.conb.2013.12.012

Klausberger, T., and Somogyi, P. (2008). Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science* 321, 53–57. doi: 10.1126/science.1149381

Komada, M., Iguchi, T., Takeda, T., Ishibashi, M., and Sato, M. (2013). Smoothened controls cyclin D2 expression and regulates the generation of intermediate progenitors in the developing cortex. *Neurosci. Lett.* 547, 87–91. doi: 10.1016/j.neulet.2013.05.006

Lee, S., Hjerling-Leffler, J., Zagha, E., Fishell, G., and Rudy, B. (2010). The largest group of superficial neocortical gabaergic interneurons expresses ionotropic serotonin receptors. *J. Neurosci.* 30, 16796–16808. doi: 10.1523/jneurosci.1869-10.2010

Le Magueresse, C., and Monyer, H. (2013). GABAergic interneurons shape the functional maturation of the cortex. *Neuron* 77, 388–405. doi: 10.1016/j.neuron.2013.01.011

Levitt, P., Eagleson, K. L., and Powell, E. M. (2004). Regulation of neocortical interneuron development and the implications for neurodevelopmental disorders. *Trends Neurosci.* 27, 400–406. doi: 10.1016/j.tins.2004.05.008

Li, G., Adesnik, H., Li, J., Long, J., Nicoll, R. A., Rubenstein, J. L., et al. (2008). Regional distribution of cortical interneurons and development of inhibitory tone are regulated by Cxcl12/Cxcr4 signaling. *J. Neurosci.* 28, 1085–1098. doi: 10.1523/JNEUROSCI.4602-07.2008

Lin, P. Y., Hinterneder, J. M., Rollor, S. R., and Birren, S. J. (2007). Non-cell-autonomous regulation of GABAergic neuron development by neurotrophins and the p75 receptor. *J. Neurosci.* 27, 12787–12796. doi: 10.1523/JNEUROSCI.3302-07.2007

Liodis, P., Denaxa, M., Grigoriou, M., Akufo-Addo, C., Yanagawa, Y., and Pachnis, V. (2007). Lhx6 activity is required for the normal migration and specification of cortical interneuron subtypes. *J. Neurosci.* 27, 3078–3089. doi: 10.1523/JNEUROSCI.3055-06.2007

Lodato, S., Rouaux, C., Quast, K. B., Jantrachotechatchawan, C., Studer, M., Hensch, T. K., et al. (2011). Excitatory projection neuron subtypes control the distribution of local inhibitory interneurons in the cerebral cortex. *Neuron* 69, 763–779. doi: 10.1016/j.neuron.2011.01.015

Lopez-Bendito, G., Sanchez-Alcaniz, J. A., Pla, R., Borrell, V., Pico, E., Valdeolmillos, M., et al. (2008). Chemokine signaling controls intracortical migration and final distribution of GABAergic interneurons. *J. Neurosci.* 28, 1613–1624. doi: 10.1523/JNEUROSCI.4651-07.2008

Lukaszewicz, A., Savatier, P., Cortay, V., Kennedy, H., and Dehay, C. (2002). Contrasting effects of basic fibroblast growth factor and neurotrophin 3 on cell cycle kinetics of mouse cortical stem cells. *J. Neurosci.* 22, 6610–6622.

Ma, Y., Hu, H., Berrebi, A. S., Mathers, P. H., and Agmon, A. (2006). Distinct subtypes of somatostatin-containing neocortical interneurons revealed in transgenic mice. *J. Neurosci.* 26, 5069–5082. doi: 10.1523/JNEUROSCI.0661 - 06.2006

Magrassi, L., Ehrlich, M. E., Butti, G., Pezzotta, S., Govoni, S., and Cattaneo, E. (1998). Basal ganglia precursors found in aggregates following embryonic transplantation adopt a striatal phenotype in heterotopic locations. *Development* 125, 2847–2855.

Markram, H., Toledo-Rodriguez, M., Wang, Y., Gupta, A., Silberberg, G., and Wu, C. (2004). Interneurons of the neocortical inhibitory system. *Nat. Rev. Neurosci.* 5, 793–807. doi: 10.1038/nrn1519

Massi, L., Lagler, M., Hartwich, K., Borhegyi, Z., Somogyi, P., and Klausberger, T. (2012). Temporal dynamics of parvalbumin-expressing axo-axonic and basket cells in the rat medial prefrontal cortex in vivo. *J. Neurosci.* 32, 16496–16502. doi: 10.1523/JNEUROSCI.3475-12.2012

McGarry, L. M., Packer, A. M., Fino, E., Nikolenko, V., Sippy, T., and Yuste, R. (2010). Quantitative classification of somatostatin-positive neocortical interneurons identifies three interneuron subtypes. *Front. Neural. Cir.* 4:12. doi: 10.3389/fncir.2010.00012

Miyoshi, G., Butt, S. J. B., Takebayashi, H., and Fishell, G. (2007). Physiologically distinct temporal cohorts of cortical interneurons arise from telencephalic Olig2-expressing precursors. *J. Neurosci.* 27, 7786–7798. doi: 10.1523/JNEUROSCI.1807-07.2007

Miyoshi, G., and Fishell, G. (2011). GABAergic interneuron lineages selectively sort into specific cortical layers during early postnatal development. *Cereb. Cortex* 21, 845–852. doi: 10.1093/cercor/bhq155

Miyoshi, G., Hjerling-Leffler, J., Karayannis, T., Sousa, V. H., Butt, S. J. B., Battiste, J., et al. (2010). Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons. *J. Neurosci.* 30, 1582–1594. doi: 10.1523/JNEUROSCI.4515-09.2010

Morales, M., and Bloom, F. E. (1997). The 5-HT3 receptor is present in different subpopulations of GABAergic neurons in the rat telencephalon. *J. Neurosci.* 17, 3157–3167.

Nery, S., Fishell, G., and Corbin, J. G. (2002). The caudal ganglionic eminence is a source of distinct cortical and subcortical cell populations. *Nat. Neurosci.* 5, 1279–1287. doi: 10.1038/nn971

Neves, G., Shah, M. M., Liodis, P., Achimastou, A., Denaxa, M., Roalfe, G., et al. (2013). The LIM homeodomain protein Lhx6 regulates maturation of interneurons and network excitability in the mammalian cortex. *Cereb. Cortex* 23, 1811–1823. doi: 10.1093/cercor/bhs159

Parnavelas, J. G., Barfield, J. A., Franke, E., and Luskin, M. B. (1991). Separate progenitor cells give rise to pyramidal and nonpyramidal neurons in the rat telencephalon. *Cereb. Cortex* 1, 463–468.

Parthasarathy, S., Srivatsa, S., Nityanandam, A., and Tarabykin, V. (2014). Ntf3 acts downstream of Sip1 in cortical postmitotic neurons to control progenitor cell fate through feedback signaling. *Development* 141, 3324–3330. doi: 10.1242/dev.114173

Pesold, C., Liu, W. S., Guidotti, A., Costa, E., and Caruncho, H. J. (1999). Cortical bitufted, horizontal, and Martinotti cells preferentially express and secrete reelin into perineuronal nets, nonsynaptically modulating gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3217–3222.

Pla, R., Borrell, V., Flames, N., and Marín, O. (2006). Layer acquisition by cortical GABAergic interneurons is independent of Reelin signaling. *J. Neurosci.* 26, 6924–6934. doi: 10.1523/JNEUROSCI.0245-06.2006

Powell, E. M., Campbell, D. B., Stanwood, G. D., Davis, C., Noebels, J. L., and Levitt, P. (2003). Genetic disruption of cortical interneuron development causes region- and GABA cell type-specific deficits, epilepsy, and behavioral dysfunction. *J. Neurosci.* 23, 622–631.

Rakic, P. (1988). Specification of cerebral cortical areas. *Science* 241, 170–176.

Rakic, P. (2009). Evolution of the neocortex: a perspective from developmental biology. *Nat. Rev. Neurosci.* 10, 724–735. doi: 10.1038/nrn2719

Ramamoorthi, K., and Lin, Y. (2011). The contribution of GABAergic dysfunction to neurodevelopmental disorders. *Trends Mol. Med.* 17, 452–462. doi: 10.1016/j.molmed.2011.03.003

Rosenberg, S. S., and Spitzer, N. C. (2011). Calcium signaling in neuronal development. *Cold Spring Harb. Perspect. Biol.* 3:a004259. doi: 10.1101/cshperspect.a004259

Rouaux, C., and Arlotta, P. (2010). Fezf2 directs the differentiation of corticofugal neurons from striatal progenitors in vivo. *Nat. Neurosci.* 13, 1345–1347. doi: 10.1038/nn.2658

Rudy, B., Fishell, G., Lee, S., and Hjerling-Leffler, J. (2010). Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Dev. Neurobiol.* 71, 45–61. doi: 10.1002/dneu.20853

Schuurmans, C., and Guillemot, F. (2002). Molecular mechanisms underlying cell fate specification in the developing telencephalon. *Curr. Opin. Neurobiol.* 12, 26–34.

Seuntjens, E., Nityanandam, A., Miquelajauregui, A., Debruyn, J., Stryjewska, A., Goebbels, S., et al. (2009). Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. *Nat. Neurosci.* 12, 1373–1380. doi: 10.1038/nn.2409

Somogyi, P., and Klausberger, T. (2005). Defined types of cortical interneurone structure space and spike timing in the hippocampus. *J. Physiol.* 562, 9–26. doi: 10.1113/jphysiol.2004.078915

Sousa, V. H., Miyoshi, G., Hjerling-Leffler, J., Karayannis, T., and Fishell, G. (2009). Characterization of Nkx6-2-derived neocortical interneuron lineages. *Cereb. Cortex* 19(Suppl. 1), i1–i10. doi: 10.1093/cercor/bhp038

Spitzer, N. C. (2012). Activity-dependent neurotransmitter respecification. *Nat. Rev. Neurosci.* 13, 94–106. doi: 10.1038/nrn3154

Steriade, M., Timofeev, I., Durmuller, N., and Grenier, F. (1998). Dynamic properties of corticothalamic neurons and local cortical interneurons generating fast rhythmic (30-40 Hz) spike bursts. *J. Neurophysiol.* 79, 483–490.

Stuhmer, T., Puelles, L., Ekker, M., and Rubenstein, J. L. (2002). Expression from a Dlx gene enhancer marks adult mouse cortical GABAergic neurons. *Cereb. Cortex* 12, 75–85.

Sussel, L., Marin, O., Kimura, S., and Rubenstein, J. L. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* 126, 3359–3370.

Taniguchi, H., Lu, J., and Huang, Z. J. (2013). The spatial and temporal origin of chandelier cells in mouse neocortex. *Science* 339, 70–74. doi: 10.1126/science.1227622

Valcanis, H., and Tan, S.-S. (2003). Layer specification of transplanted interneurons in developing mouse neocortex. *J. Neurosci.* 23, 5113–5122.

Van Eden, C. G., Mrzljak, L., Voorn, P., and Uylings, H. B. (1989). Prenatal development of GABA-ergic neurons in the neocortex of the rat. *J. Comp. Neurol.* 289, 213–227. doi: 10.1002/cne.902890204

Vitalis, T., Ansorge, M. S., and Dayer, A. G. (2013). Serotonin homeostasis and serotonin receptors as actors of cortical construction: special attention to the 5-HT3A and 5-HT6 receptor subtypes. *Front. Cell Neurosci.* 7:93. doi: 10.3389/fncel.2013.00093

Vitalis, T., Cases, O., Passemard, S., Callebert, J., and Parnavelas, J. G. (2007). Embryonic depletion of serotonin affects cortical development. *Eur. J. Neurosci.* 26, 331–344. doi: 10.1111/j.1460-9568.2007.05661.x

Vogt, D., Hunt, R. F., Mandal, S., Sandberg, M., Silberberg, S. N., Nagasawa, T., et al. (2014). Lhx6 directly regulates Arx and CXCR7 to determine cortical interneuron fate and laminar position. *Neuron* 82, 350–364. doi: 10.1016/j.neuron.2014.02.030

Vucurovic, K., Gallopin, T., Ferezou, I., Rancillac, A., Chameau, P., Van Hooft, J. A., et al. (2010). Serotonin 3A receptor subtype as an early and protracted marker of cortical interneuron subpopulations. *Cereb. Cortex* 20, 2333–2347. doi: 10.1093/cercor/bhp310

Wichterle, H., Turnbull, D. H., Nery, S., Fishell, G., and Alvarez-Buylla, A. (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* 128, 3759–3771.

Wonders, C. P., and Anderson, S. A. (2006). The origin and specification of cortical interneurons. *Nat. Rev. Neurosci.* 7, 687–696. doi: 10.1038/nrn1954

Xu, Q., Cobos, I., Cruz, E. D. L., Rubenstein, J. L., Anderson, S. A., and De La Cruz, E. (2004). Origins of Cortical Interneuron Subtypes. *J. Neurosci.* 24, 2612–2622. doi: 10.1523/JNEUROSCI.5667-03.2004

Xu, Q., Guo, L., Moore, H., Waclaw, R. R., Campbell, K., and Anderson, S. A. (2010a). Sonic hedgehog signaling confers ventral telencephalic progenitors with distinct cortical interneuron fates. *Neuron* 65, 328–340. doi: 10.1016/j.neuron.2010.01.004

Xu, X., Roby, K. D., and Callaway, E. M. (2010b). Immunochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells. *J. Comp. Neurol.* 518, 389–404. doi: 10.1002/cne.22229

Xu, Q., Wonders, C. P., and Anderson, S. A. (2005). Sonic hedgehog maintains the identity of cortical interneuron progenitors in the ventral telencephalon. *Development* 132, 4987–4998. doi: 10.1242/dev.02090

Zhao, Y., Flandin, P., Long, J. E., Cuesta, M. D., Westphal, H., and Rubenstein, J. L. (2008). Distinct molecular pathways for development of telencephalic

interneuron subtypes revealed through analysis of Lhx6 mutants. *J. Comp. Neurol.* 510, 79–99. doi: 10.1002/cne.21772

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 20 March 2015
doi: 10.3389/fncel.2015.00076

Astrocyte heterogeneity in the brain: from development to disease

Clarissa Schitine[1], *Luciana Nogaroli*[1], *Marcos R. Costa*[2] *and Cecilia Hedin-Pereira*[1,3]*

[1] *Cellular Neuroanatomy Laboratory, Program in Neurobiology, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil,* [2] *Laboratory of Cellular Neurobiology, Brain Institute, Federal University of Rio Grande do Norte, Natal, Brazil,* [3] *Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil*

In the last decades, astrocytes have risen from passive supporters of neuronal activity to central players in brain function and cognition. Likewise, the heterogeneity of astrocytes starts to become recognized in contrast to the homogeneous population previously predicted. In this review, we focused on astrocyte heterogeneity in terms of their morphological, protein expression and functional aspects, and debate in a historical perspective the diversity encountered in glial progenitors and how they may reflect mature astrocyte heterogeneity. We discussed data that show that different progenitors may have unsuspected roles in developmental processes. We have approached the functions of astrocyte subpopulations on the onset of psychiatric and neurological diseases.

Keywords: astrocyte, heterogeneity, progenitors, cerebral cortex, psychiatric diseases

OPEN ACCESS

Edited by:
Qi Yuan,
Memorial University, Canada

Reviewed by:
Eliana Scemes,
Albert Einstein College of Medicine, USA
Adolfo Toledano,
Instituto Cajal – Consejo Superior de Investigaciones Científicas, Spain

***Correspondence:**
Cecilia Hedin-Pereira,
Cellular Neuroanatomy Laboratory, Program in Neurobiology, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, 21941-902 Rio de Janeiro, Brazil
hedin@biof.ufrj.br

Received: *11 December 2014*
Accepted: *20 February 2015*
Published: *20 March 2015*

Citation:
Schitine C, Nogaroli L, Costa MR and Hedin-Pereira C (2015) Astrocyte heterogeneity in the brain: from development to disease. Front. Cell. Neurosci. 9:76. doi: 10.3389/fncel.2015.00076

Astrocyte Diversity

The human brain is acknowledged as the most complex of all organs, a structure dynamic enough to adapt to dramatic environmental changes through plasticity occurring as a result of normal physiology or during pathological events. Such complexity requires powerful homeostasis processes to assure the perfect functioning of the system (Scuderi et al., 2013). Glial cells are pivotal to maintain the structural integrity and functionality required by the central nervous system (CNS). The so-called macroglia consists of a heterogeneous cell population, which comprises ependymal cells, astrocytes, oligodendrocytes, and NG2 cells. Each of these cell populations is unique, although sharing some properties.

The initial concept of macroglial cells as passive in brain function, working as a supportive element for the maintenance of neurons, has dramatically changed in the last quarter of a century with an increased body of evidence showing active roles of glial cells in the transmission and integration of neural information (Wang and Bordey, 2008). In fact, macroglial cells are directly involved in neuronal function through controlling neurogenesis, synaptogenesis, neurotransmission, synaptic plasticity, neuronal growth, and neuron survival (Scuderi et al., 2013; Zhang and Barres, 2013).

Astrocytes represent the most abundant macroglia and the largest and most heterogeneous group of glial cell types. They participate in a wide variety of complex and essential functions in the brain. For instance, astrocytes are responsible for the reuptake and release of transmitters, distribution of water, pyruvate metabolism, removal of reactive oxygen species, and antioxidant (glutathione) metabolism, organization of the blood brain barrier, ion buffering, synthesis, and secretion of trophic factors and release of gliotransmitters through exocytosis mechanism (Sofroniew and Vinters, 2010; Scuderi et al., 2013).

Albeit the heterogeneity of functions and the prominent roles they exert, astroglial cells have mostly been overlooked in the quest to understand healthy and diseased brain functioning and the classification of astroglial cells still relies greatly on morphological criteria and use of few molecular markers.

Astrocytes are classically divided into two major subpopulations in cerebral cortex: fibrous astrocytes in the white matter and protoplasmic astrocytes in the gray matter (Miller and Raff, 1984). Fibrous astrocytes have long, thin processes, yielding a star-like appearance. Protoplasmic astrocytes have many branching processes, which contact and ensheath synapses, and usually have one or two processes in contact with blood vessels. However, this classification is outdated in light of the great diversity of astrocytes revealed by more detailed morphological and biochemical analyses.

In fact, mature astrocytes can be identified by the expression of glial fibrillary acidic protein (GFAP), calcium-binding protein S100β, glutamate–aspartate transporter and glutamate transporter 1 (GLT-1), and additional markers is recently suggested based on microarray gene expression profiles (Bachoo et al., 2004). Expressions of these markers, as well as astrocyte morphologies, vary considerably amongst cortical regions (Emsley and Macklis, 2006; Regan et al., 2007), suggesting that astrocyte subpopulations could be differentially specified to display distinct biochemical/biophysical properties throughout discrete regions of the cerebral cortex (Emsley and Macklis, 2006).

Astrocyte heterogeneity is also appreciated in other CNS regions. Recent data suggest that the differential expression of ionotropic receptors in thalamic astrocytes could indicate functional heterogeneity. The thalamus, responsible for processing sensory information relayed to the cortex, contains two different astrocyte populations regarding the expression of glutamatergic receptors. Thalamic astrocytes isolated from postnatal transgenic mice expressing human GFAP promoter under the control of enhanced green fluorescent protein (EGFP) were stimulated with kainate, and kainate plus cyclothiazide (CTZ), an AMPA receptor modulator. Only 60% of the cells stimulated showed enhanced inward currents upon kainate and CTZ application (Hoft et al., 2014). In addition, all astrocytes observed in electrophysiological recordings showed K^+ currents upon muscimol stimulus, a selective agonist for the GABA-A receptor (Hoft et al., 2014). These data indicate that astrocyte subpopulations differentially express neurotransmitter receptors, reflecting a putative difference in astrocytic function and physiology.

Using the same transgenic mice model above, two different populations of astrocytes were identified in hippocampal freshly isolated cells or brain slices (Matthias et al., 2003). One population of cells displays weak GFAP fluorescence, thin and short processes, whereas the second group of cells displays intense GFAP-EGFP labeling and more complex process morphology (Matthias et al., 2003). Besides morphology, the two groups of cells differ in their electric properties. In whole cell recordings, the first group of cells shows an outward rectifying K^+ current and the second one an inward K^+ current (Matthias et al., 2003). Application of glutamate or AMPA on weak fluorescent GFAP cells evoke a fast and sensitized current whether D-aspartate does not evoke any current, indicating expression of AMPA receptors and lack of glutamate transporters. On the contrary, the second group of cells has no kainate elicited membrane currents, however, D-aspartate induce inward currents inhibited by the use of glutamate transporter blocker THA (Matthias et al., 2003).

Investigation on the existence of astrocytes with qualitatively different ion current phenotypes and morphology in the hippocampus (Zhou and Kimelberg, 2000, 2001) and thalamus (Hoft et al., 2014) suggest astrocyte heterogeneity by diverse functional properties in different populations of cells, some of which expressing functional AMPA receptors and others glutamate transporters (Matthias et al., 2003). Knowing the importance of neurotransmitters for the regulation of many signaling pathways, further studies are relevant in order to reveal the role of the differential expression of glutamatergic machinery in astrocytes to physiological events such as cell migration and differentiation.

A distinct feature of astrocytes is their extensive gap junctional coupling. Gap junction communication is essential for signaling in neuroglia circuit function in many brain regions. Therefore, coupling differences among those regions could indicate astrocyte heterogeneity with possible functional diversity implications (Froes and Menezes, 2002; Anders et al., 2014). Even more subtle paradigms to measure dye diffusion via gap junction reveals differences in astrocyte performance with temperature variance. Experiments using the fluorescent dye Alexa Fluor 594 to measure those parameters in CA1 and dentate gyrus of the rat hippocampus (Anders et al., 2014) show that astrocyte coupling may differ between these regions in a temperature-dependent manner, probably due to changes in intracellular diffusive properties, rather than measured by the number of astrocytes coupled (Anders et al., 2014).

The Origin of Astrocytes

Gliogenesis generally follows neurogenesis in the developing brain (Miller and Gauthier, 2007; Costa et al., 2009). However, these events partially overlap and their precise temporal relationship *in vivo* and at the individual progenitor level remains largely unexplored. Most macroglial cells in the rodent brain are generated postnatally. In fact, during the first 3 weeks of cerebral cortex postnatal development, the macroglial cell population, which contains predominantly astrocytes, expands six- to eightfold in the rodent brain (Bandeira et al., 2009).

As discussed above, astrocyte diversity in the brain becomes increasingly recognized. Yet, it remains unclear whether astrocyte subtypes are generated from a homogeneous population of progenitors or from separate classes of progenitors previously specified within the germinative niches of the developing telencephalon. Moreover, the developmental sequences undergone by astrocyte precursors are only partially understood. We summarize the main findings related to the generation of differentiated astrocytes in the brain parenchyma.

Five different sources of cortical mature astrocytes were identified to date: (I) radial glia cell (RGC) within ventricular

zone, (II) RGC transformation, (III) glial intermediate progenitors (GP) within subventricular zone, (IV) GPs present in the marginal zone (MZ)/layer 1, (V) superficial layer progenitors. A schematic view of astrogliosis to the cerebral cortex is illustrated in the **Figure 1**.

(I, II and III) Astrocytes Derived from Radial Glia Cell Directly, or by Direct Transformation or by the Generation of Intermediate Progenitors

One of the earliest and most understood source of astrocytes in the cerebral cortex is the direct transformation of RGCs into protoplasmic astrocytes that occurs, at the end of cortical neurogenesis, after birth in different species (Schmechel and Rakic, 1979; Voigt, 1989; Alves et al., 2002; deAzevedo et al., 2003). During this process, RGCs lose their apical process and move toward the pial surface, eventually undergoing mitosis in the subventricular zone (SVZ; Noctor et al., 2004). It is likely that these transforming and proliferating cells represent at least a fraction of the GPs labeled by retrovirus in the SVZ and studied in previous work (Levison et al., 1993), what could help to explain the little dispersion of clonally related astrocytes (Magavi et al., 2012).

Radial glia cells generate intermediate progenitors through asymmetric division within the SVZ. These glial intermediate progenitors also divide and give rise to immature proliferative astrocytes that migrate radially out of the germinative zone and populate the cerebral parenchyma (Levison and Goldman, 1993; Levison et al., 1993; Luskin and McDermott, 1994; Zerlin et al., 1995). When they reach their destination, the immature cells still proliferate and only later fully differentiate into mature astrocytes (Ge et al., 2012).

Interestingly, retroviral-mediated fate mapping of postnatal SVZ progenitors indicate that white and gray matter astrocytes, as well as oligodendrocytes derive from separate macroglial progenitors (Luskin and McDermott, 1994) and that astrocytes generated in the SVZ do not disperse long distances in the rostro-caudal axis (Levison et al., 1993), instead they are regionally restricted. This regionalization may start at earlier time points, as indicated by recent work (Magavi et al., 2012;

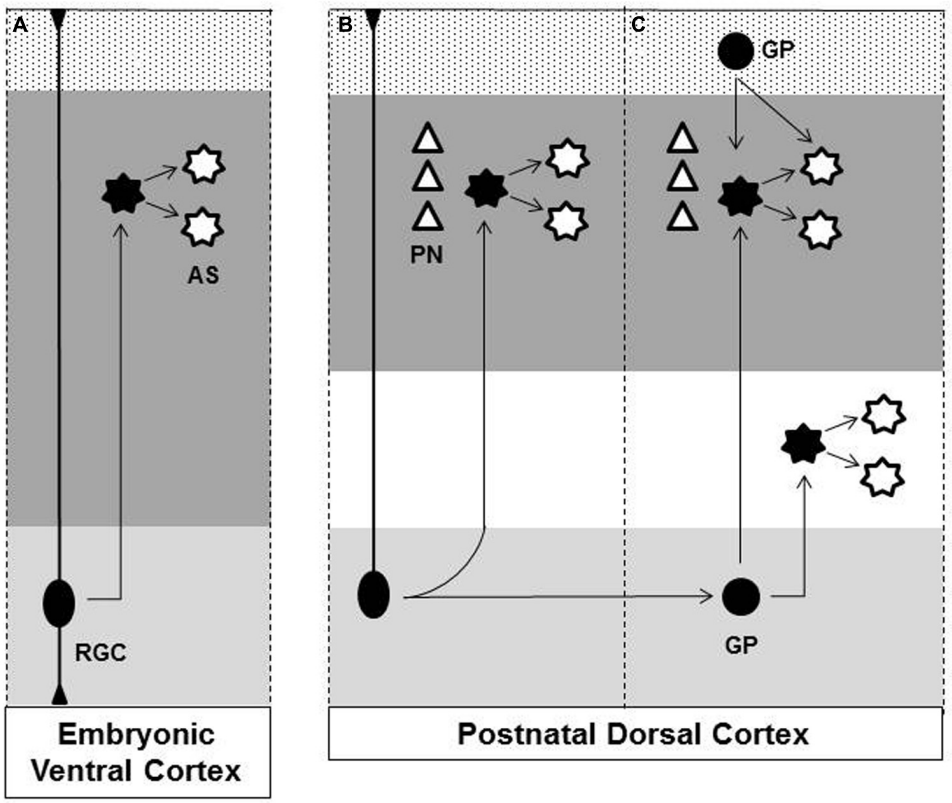


FIGURE 1 | Origins of astrocytes in the cerebral cortex. (A) At later stage of embryonic brain development radial glial cell (RGC), in the ventricular zone (light gray region), gives rise to astrocytes (AS) that disperse throughout the ventral forebrain parenchyma (dark gray region). **(B)** After birth, RGC loses their apical processes and directly transform into cortical astrocytes. **(C)** Glial progenitor (GP) derived from RGC undergoes cell division in the subventricular zone (light gray region) generating astrocytes that disperse radially to the cortical layers (dark gray region) and white matter (white region). Astrocyte proliferates locally amplifying the astrocytic population. GP present in the Marginal Zone (MZ)/Layer 1 (Dotted region) contributes to superficial cortical astrocyte. **(B/C)** During embryonic development dorsal RGC also generates pyramidal neurons (PN) that migrate radially and settle in individual cortical columns in the gray matter. Observe that developing astrocytes maintain the columnar organization with early generated neurons. Dashed lines indicate the boundaries of individual cortical columns. Black filled figures represent mitotic cells. RGC, radial glia cell; AS, astrocyte; GP, glial progenitor; PN, pyramidal neurons.

Garcia-Marques and Lopez-Mascaraque, 2013; Gao et al., 2014). Using a novel genetic tool for tracing GP lineage, the so-called "Star Track," which uses a combinatorial expression of six fluorescent proteins under the control of GFAP promoter, Garcia-Marques and Lopez-Mascaraque (2013) show that clonally related astrocytes disperse radially in the cortex spanning the entire depth of the cortical parenchyma. Similarly, when individual cortical RGCs are labeled at early embryonic stages using genetic strategies, they give rise to discrete columnar structures that contain both projection neurons and protoplasmic astrocytes in the adult animals (Magavi et al., 2012; Gao et al., 2014). Most columns of neurons contained multiple clusters of astrocytes, and the vast majority of labeled astrocytes were found within 50 μm of a labeled neuronal column. The astrocyte to neuron ratio in a single developmental column was similar across the entire neocortex, indicating that column-associated astrocytes account for the majority of protoplasmic astrocytes in the neocortex. Together these data suggest that cortical protoplasmic astrocytes are generated in a spatially restricted manner from progenitors that also give rise to columns of pyramidal neurons (PNs) during embryonic development.

Notably, cortical excitatory neurons are spatially organized into individual cortical columns, which are involved in the processing of similar sensory stimuli (Mountcastle, 1997). It is hypothesized that the generation of neuronal cortical columns could be controlled by transcriptional codes expressed in progenitor cells at early development (Costa and Hedin-Pereira, 2010) and it is tempting to speculate that the columnar organization of astrocytes in the cerebral cortex (Magavi et al., 2012) could also reflect some degree of spatial specification in GPs. Some evidence of this was found in spinal cord where astrocytes have been shown to be regionally specified (Hochstim et al., 2008). In this work, the authors describe three distinct subtypes of astrocytes which could be distinguished by the combinatorial expression of Reelin and Slit1 (Hochstim et al., 2008). These astrocyte subtypes originate from separate progenitor domains expressing the transcription factors Pax6 and Nkx6.1, suggesting that astrocyte diversification could be regulated within progenitors.

Another example of specific progenitors which generate spatially restricted astrocyte subpopulations comes from lineage studies of ventral telencephalon NG2 cells. It is found that ventral embryonic NG2 cells, derived directly from RGCs, generate a subset of protoplasmic astrocytes only to the ventral forebrain, but no astrocytes to the dorsal cortex or cortical white matter, e.g., corpus callosum. (Marshall and Goldman, 2002; Belachew et al., 2003; Zhu et al., 2008, 2012; Huang et al., 2014). Moreover, embryonic NG2 cells only generate astrocytes within the forebrain, since no labeled astrocytes were found in other brain regions (Huang et al., 2014). Thus astrogliogenic potential of NG2 cells seems to be temporal and spatially confined to a subgroup of ventral embryonic progenitors.

On the other hand, dorsal cortical astrocytes are mostly generated from an Emx1-expressing progenitor (Gorski et al., 2002), which is a transcription factor regionally expressed by cortical progenitors in the dorsal VZ/SVZ (Gulisano et al., 1996). Using a conditional double transgenic mouse reporter to trace Emx1-cell lineage, the authors show that the vast majority of, if not all, cortical excitatory neurons and astrocytes generated postnatally is derived from Emx1-expressing cells (Gorski et al., 2002). Thus ventral and dorsal forebrain astrogliogenesis are temporal and spatially confined to a subgroup of GPs. While subpallial astrocytes are dependent on NG2-expressing progenitors, pallial astrocytes rely on Emx1-expressing progenitors.

(IV) From Glial Progenitors Present in the Marginal Zone/Layer 1

In the embryonic and neonatal MZ/layer 1, a separate class of progenitors undergoes cell division and contributes with astrocytes, oligodendrocytes, and neurons to the cerebral cortex (Costa et al., 2007; Breunig et al., 2012) and therefore is described as a novel niche for gliogenesis and neurogenesis in the cerebral cortex. MZ/layer 1 progenitors are derived from ventral (progenitors expressing the transcription factors Nkx2.1 or Gsh2) and dorsal telencephalic ventricular zone (progenitors expressing the transcription factors Emx1), with a predominance of the latter at neonatal stage (Costa et al., 2007). Given the unique composition of the MZ/layer 1, it is possible that local proliferation in this region contributes to the generation of astrocyte diversification in the cerebral cortex (Costa et al., 2007). Actually, evidence from human embryonic cortex shows that different morphologies and developmental stages characterize superficial and deep astrocytes (deAzevedo et al., 2003). Whilst deep astrocytes emerge by the process of RGC transformation in the subplate of human cingulate cortex, superficial astrocytes labeled for GFAP are already present in the MZ and dorsal supragranular layers in the second half of gestation. At this point no GFAP-positive cells can yet be labeled in human cortical plate. The first GFAP-positive cells to be labeled within the human cortical plate appear associated with blood vessels (Marin-Padilla, 1995; deAzevedo et al., 2003) raising the question of whether angiogenesis might have a pivotal role in the appearance of gray matter astrocytes. While in humans GFAP is a conventional marker for RGCs and astrocytes, in rodents this labeling is restricted, bringing our attention to the evolutionary differences in the glial population between rodents and primates, for example (Takahashi et al., 1990; deAzevedo et al., 2003).

(V) Superficial Layer Progenitors

Local proliferation was the main focus on a recently study of Ge et al. (2012). They show that GPs and RGC transformation combined contribute to mature astrocyte differently when observed superficial and deeper cortical layers. While both sources generate most of the astrocytes to deep cortical layers (layers 5–6) and white matter astrocytes, 97% of labeled progenitors, only 3% of these progenitors are dedicated to upper cortical layers (layers 1–4). Using retrovirus injected in the cortex of postnatal mice, the authors labeled dividing cells and provide evidence, at least to superficial cortical layers, that local proliferation of immature astrocytes is responsible to expand the cell population. It is possible that local proliferation in

deeper cortical layers and white matter may also occur. However, more striking is to determine whether this differential astrocyte dispersion through cortical layers confers a distinct glial function within neuronal circuitries. It is also unclear whether these locally proliferating astrocytes are derived from GPs in the MZ/Layer 1.

Future experiments should address more directly the question of whether positionally and functionally distinct subtypes of astrocytes in the forebrain develop from previously specified sets of progenitors and the degree of plasticity of GPs when proliferating in different environments, such as MZ/Layer 1, deep and superficial cortical layers, or white matter. These studies may also shed light on our understanding about the mechanisms subsidizing neurological and psychiatric diseases in which astrocytes are affected at subpopulation distinct manner.

Astrocytes and Disease

Given the diversity of astrocytes and their pleiotropic functions, it is not surprising that their dysfunction is also an important matter to several neurological disorders. However, the involvement of this type of glia in pathological conditions became only recently clear due to a steadily increasing interest in the study of the biology and pathology of astrocytes (Sofroniew and Vinters, 2010). It is tempting to speculate that different subtypes of astrocytes would contribute to the onset or progression of brain diseases. And even more, only a subset of astrocytes would be differentially affected by the microenvironment of the diseased brain.

The response of astroglia in pathological conditions is very heterogeneous. Indeed, in some circumstances, it is possible to observe morphological changes of these cells that become hypertrophic and proliferate, leading to the so-called reactive gliosis state. In these conditions, astrocytes can release different kinds of cytokines with either pro-or anti-inflammatory potential (Sofroniew and Vinters, 2010). Other pathological situations are characterized by astro-degeneration with consequent loss of their physiological supportive functions (Scuderi et al., 2013). Nowadays, there is increasing evidence of astrocytic dysfunction in several brain disorders. The homeostatic failure due to astrocyte degeneration can be fundamental for the initiation and progression of neuropathological diseases. There is an increasing body of evidence showing the contribution of astrocytes in schizophrenia (Kolomeets and Uranova, 2010; Schnieder and Dwork, 2011), autism (Laurence and Fatemi, 2005; Bristot Silvestrin et al., 2013), and drug abuse (Beardsley and Hauser, 2014; Bull et al., 2014; Jackson et al., 2014). Astrocyte dysfunction is also involved in major depression disease and neurodegenerative disorders such as Alexander Disease (AxD), Amyotrophic Lateral Sclerosis (ALS), and Alzheimer's Disease (AD), focused on this review. Although astrocytes present a common reaction to the lesioned nervous system with the upregulation of GFAP for example, we will see in the following session that there is enormous diversity in astrocyte response that may be accounted for by environmental differences or by astrocyte heterogeneity.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis is a neurodegenerative disease that is characterized by the loss of corticospinal and spinal motor neurons. Multiple genes are linked to this disorder, but the discovery of mutations in the Cu-Zn superoxide dismutase (SOD1) led to the generation of transgenic animal models that have contributed to current knowledge on ALS pathology. Evidence support a central role for neuro–glia interactions suggesting that glial cells, and in particular astrocytes, could be a target for novel therapy in ALS (Valori et al., 2014). In fact, massive activation of astrocytes and microglia is associated with motor neuron death in humans, as well as in transgenic animal models (Boillee et al., 2006; Yamanaka et al., 2008). However, selective ablation of proliferating GFAP-expressing astrocytes and microglia fail to modify disease progression (Gowing et al., 2008; Lepore et al., 2008). Using $SOD1^{G93A}$ transgenic (SOD–Tg) rats, Barbeito and collaborators isolated in culture a new type of astrocyte from adult spinal cord of symptomatic animals (Diaz-Amarilla et al., 2011). SOD–Tg-derived astrocytes reached confluence rapidly and could be propagated for 1 year in culture. Because of this feature they were called "aberrant astrocytes" (AbA cells), which are highly proliferative and appear to drive motor neuron death in a cell-type specific fashion *in vitro* (Diaz-Amarilla et al., 2011).

Aberrant astrocytes cells are almost undistinguishable morphologically from primary neonatal astrocytes. They express a set of distinctive antigenic markers of undifferentiated astrocytes including intense staining for S100β in the cell nucleus and cytoplasm, high connexin 43 expression and low levels of diffuse perinuclear labeling of non-filamentous GFAP (Diaz-Amarilla et al., 2011). This low expression of GFAP could be partially responsible for failure observed in earlier studies, which aimed to control proliferation of GFAP-expressing astrocytes in ALS lesion.

Interestingly, AbA cells do not express detectable GLT-1, a specific glial glutamate transporter, what could explain further excitotoxic damage to motor neurons *in vivo*. Moreover, the neurotoxicity of AbA cells conditioned medium is specific to motor neurons (Diaz-Amarilla et al., 2011). This cell-type specific interaction has been already reported for mutant SOD1 expressing primary astrocytes, which reduce viability of both primary and embryonic stem cell-derived motor neurons in co-culture, but not interneurons or dorsal root ganglion neurons (Nagai et al., 2007).

In culture, AbA cells also show increased proliferation and lack of replicative senescence, suggesting there is a defect in contact inhibition, but they do not appear to be fully transformed cells (Diaz-Amarilla et al., 2011). Additionally S100β expression did not co-localize with NG2-labeling cells, which proliferate in the ALS spinal cord (Kang et al., 2010), suggesting that the dividing S100β-positive cells constitute a different cell population that is not derived from NG2 cells. Recently, it was found that AbA cells are most likely derived from activated microglia present in the spinal cord lesion (Trias et al., 2013). After two weeks in culture, SOD-Tg-derived microglia cells start their transformation into astrocytes. Thus, a subpopulation of microglia in culture loses its markers, such as Iba1 and CD163, and increases the expression of the astrocyte markers GFAP and S100β. Changes in the protein

profile are also followed by phenotypical alterations (Trias et al., 2013). This finding raises a novel perspective on astrocyte studies in ALS disease, which should then consider impeding the transformation of microglia into astrocyte-like cells as a therapeutic strategy.

For all these evidence, authors suggest that AbA cells may be considered a distinct subpopulation of highly toxic astrocytes generated during recruitment and phenotypic transition of glial cells in an inflammatory microenvironment. And since astrocytes are involved in the rapid progression of paralysis characteristic of the ALS animal model, AbA cells could represent an additional cellular target for future treatment of ALS (Diaz-Amarilla et al., 2011; Trias et al., 2013).

Alexander Disease

Alexander disease is an autosomal dominant leukodystrophy, which predominantly affects infants and children (Goyal et al., 2014; Verkhratsky and Parpura, 2014). It is an inherited gliopathology, associated with sporadic mutations in the GFAP encoding gene that was first described in 1949 by Stewart Alexander (Alexander, 1949; Brenner et al., 2001; Verkhratsky and Parpura, 2014). Those pathogenic mutations are thought to confer cytotoxicity through gain-of-function mechanisms (Brenner et al., 2001; Prust et al., 2011). The impaired function of astroglia affects brain development and results in progressive failure of central myelination, developmental delay, seizures, megalencephaly, and progressive deterioration, with increased severity in neonatal patients (Prust et al., 2011; Verkhratsky and Parpura, 2014). Histopathological analysis shows that AxD is associated with the appearance of cytoplasmic inclusions in astroglial cells known as the Rosenthal fibers (Verkhratsky and Parpura, 2014). These are protein aggregates containing GFAP, ubiquitin, heat shock protein hsp-27, and B-crystallin and they are expressed in astrocytes adjacent to areas of demyelination (Goyal et al., 2014).

Observation that Rosenthal fiber formation can be induced by the overexpression of human GFAP in transgenic mice in a dose dependent manner (Messing et al., 1998; Quinlan et al., 2007) lead to the search for mutations in the GFAP gene. *Gfap* encodes for an intermediate filament protein that can be alternatively spliced (Quinlan et al., 2007). Relative abundance of GFAP transcripts is often low, dependent on astrocyte location, and induced by disorders (Roelofs et al., 2005; Kamphuis et al., 2012, 2014), suggesting an interesting source for astrocyte heterogeneity. Brenner et al. (2001) found that the genetic mechanism of AxD is based in the *de novo* mutations in four different GFAP residues, R79, R239, R258, and R416 observed in 12 unrelated individuals. In addition, other investigators found several spots for mutations in GFAP gene in early and later onset, indicating dominant missense GFAP mutations for nearly all forms of this disorder (Li et al., 2005; Quinlan et al., 2007). The genetic basis for AxD is now very well established, however, little is known about the mechanisms by which GFAP mutations lead to disease. To understand how the pathology progresses, transgenic mice overexpressing wild type GFAP that develop an encephalopathy with identical aggregates present in AxD were analyzed in different phases by microarray assays (Hagemann et al., 2005; Quinlan et al., 2007). Transcription profiles reveals alteration in genes involved in stress and immune responses (Hagemann et al., 2005). At 3 months age, transgenic mice show stress responses including increase in genes expression involved in glutathione metabolism, peroxide detoxification, and iron homeostasis (Hagemann et al., 2005). GFAP overexpression in those mice also induces an increase in activation of cytokine, cytokine receptor genes, and complement components. These transcripts are further elevated with age, with additional induction of macrophage-specific markers, suggesting activation of microglia (Hagemann et al., 2005; Quinlan et al., 2007). At 4 months, in contrast to those genes showing increased expression at 3 months, there is a decrease in expression of microtubule-associated proteins (Hagemann et al., 2005). Numerous genes involved in neurotransmission and vesicular transport are also downregulated including both GABA and glutamate receptors (Hagemann et al., 2005; Quinlan et al., 2007). The transcriptional profiles from olfactory bulb also show a decrease in transcriptional factors and signaling molecules involved in neurogenesis such as Dlx family genes (Hagemann et al., 2005). Therefore, this completely remodeled scenario affects neuron-glia signaling, leading to neuronal dysfunction in advanced stages of pathology.

Alzheimer's Disease

Neurodegenerative disorders such as AD are the most common diseases of modern society. The gradual and irreversible disturbances in homeostasis, leading to synaptic dysfunction and cognition impairment are characteristic features of the disease. Symptomatically, AD is characterized by marked deficiencies in episodic memory, attention, perception, reasoning, and language as well as altered mood (Mesulam, 1999; Hancock et al., 2014). Pathologically, it is defined by the accumulation of intracellular neurofibrillary tangles, comprised of abnormally phosphorylated tau protein and extracellular plaques, including misfolded forms of the amyloid-β (Aβ) peptide within the brain (Mesulam, 1999; Hancock et al., 2014).

The overall assumption in AD is that astrocyte response involves a generalized overexpression of GFAP and an increase in proliferation. However, a growing body of evidence shows that there are two different populations of astrocytes in AD, revealing the heterogeneity in cell response upon different stimuli and environment.

Studies from a triple transgenic mouse model of AD, which mimics the progression of the disease in humans, show that the patterns of GFAP expression differ among brain areas and during the different phases of the neurodegenerative process (Olabarria et al., 2010; Yeh et al., 2011). The number of GFAP-positive astrocytes does not change with the age of the transgenic animals, however, in mice from 6, 12, and 18 months there is a reduction in volume and area of GFAP-expressing cells in the dentate gyrus, indicating astrocyte atrophy (Olabarria et al., 2010). However, in Aβ plaque surrounding areas, there is an opposite profile of GFAP expression, observed by an increase in GFAP volume and superficial expression pattern in the dentate gyrus and CA1 regions, demonstrating a hypertrophy of

astrocytes (Olabarria et al., 2010). Therefore, two different populations of astrocytes are revealed in AD. This concomitant astrocyte atrophy and astrogliosis in AD does not seem to occur in all brain regions. Analyses of the entorhinal cortex, a fundamental structure for cognitive and memory processes, show that in the triple transgenic mouse model of AD, there is a reduction in primary and secondary branches accompanied by a decrease in volume and area of GFAP expression. These morphological changes are observed in 1 month old animals and persist up to 12 months (Yeh et al., 2011). The progression of the disease established by the accumulation of Aβ deposits does not trigger a reactive gliosis, indicating an absence of astrocytic hypertrophy during AD in the entorhinal cortex (Yeh et al., 2011).

Another brain area involved in the symptomatology of AD is the medial prefrontal cortex, which is associated with cognitive, memory, and emotional processes. In this brain area, the number of GFAP-positive cells does not change significantly with age as compared to wild type mice (Kulijewicz-Nawrot et al., 2012). However, at 3 months, transgenic mice show astroglial cytoskeletal atrophy that remains throughout the disease progression (Kulijewicz-Nawrot et al., 2012). Reduction in volume and area of GFAP-positive profiles in the medial prefrontal cortex show a clear layer-specific pattern, with layers 1–2 being strongly affected and similar changes being found in the deep layers 4 and 5, while layer 3 is only affected from intermediary phases of the disease progression (Kulijewicz-Nawrot et al., 2012). In contrast to other brain regions affected in AD, such as the hippocampus (Olabarria et al., 2010; Yeh et al., 2011), no plaque formation is observed in medial prefrontal cortex (Kulijewicz-Nawrot et al., 2012). However, the Aβ aggregates are present, especially in the deeper layers. Those findings show that astrocytic atrophy occurs in early stages of the disease in specific brain areas. This alteration of astrocytes may represent a very relevant aspect for the progression of the disease. Astrocytic dysfunction compromises brain homoeostasis on many levels, reducing brain energy and neurotransmitter homoeostasis, increasing excitotoxicity. In addition, atrophied astrocytes can reduce synaptic coverage, leading to a decrease in number and functional synapses, decreased connectivity, imbalanced neurotransmission, synaptic strength, and synaptic maintenance. These data indicate that astrocytes within distinct brain regions may respond in a very peculiar manner to similar stimuli, supporting the view that astrocytes are heterogeneous and play different roles in disease progression.

At later stages of the AD, the astrocytic morphology is complex. There is formation of senile plaques resulting in astrogliosis revealed by astrocytic hypertrophy, thicker processes, increased volume and area of GFAP-positive profiles surrounding Aβ deposits (Olabarria et al., 2010).

Neuropathology data using human brains show that astrocytes activated by Aβ (Scuderi et al., 2013) secrete pro-inflammatory signals and toxic cytokines that lead to further damage, increasing nitric oxide radicals and TNF-α levels, which in turn triggers a neurodegenerative cascade (Zhang et al., 2010). The inflammation itself can lead to neuronal dysfunction, independently of cell death. The parallel pro-inflammatory cytokine network induces dysfunction in astrocytes in their effort to maintain environment homeostasis, which in turn increases neuronal vulnerability. Thus, astrocyte impairment can occur during early and late stages of the disease depending on the brain region and how astrocytes modulate GFAP expression and the secretion of cytokines or trophic factors in response to stimuli.

Glial fibrillary acidic protein gene can be alternatively spliced and the canonical isoform GFAP-α expressed in astrocytes contains nine exons (Kamphuis et al., 2012, 2014). So far, nine isoforms are described in different species (Kamphuis et al., 2012). Three splice variants GFAPDEx6, GFAPD164, and GFAPD lacking sequences in exons 6–7 are found in AD (Hol et al., 2003). GFAP transcripts from alternative splicing have variable alterations in their C-terminal region. The C-terminal region is important to direct the assembly of GFAP filaments and their interaction to other proteins (Kamphuis et al., 2014). Thus, different C-terminals lead to different GFAP expression patterns and cellular functions. Evidence that GFAP can be translated from different transcripts corroborates the idea that astrocytes are distinct cells populations with a specific transcriptional regulation repertoire leading to putative differences in their function.

Major Depression Disorder

Major depressive disorder (MDD) is one of the most prevalent mood disorders, affecting millions of people worldwide. MDD is a chronic, recurrent and debilitating mental illness, characterized by core symptoms such as depressed mood, loss of interest or pleasure, changes in weight and in sleep, fatigue or loss of energy, feeling of worthlessness, concentration difficulties, and thoughts of death or suicide (Rajkowska and Stockmeier, 2013). Several hypotheses including chronic stress, failure of hippocampal neurogenesis in the adult, altered neuroplasticity, dysfunction of monoaminergic systems and genetic factors have been studied to elucidate depressive-related behaviors (Smialowska et al., 2013). The general knowledge about depression was originally taken from studies showing impairment in the monoamine system and is supported by the understanding of both the pathophysiology of depression and the action of pharmacological treatments. However, in the past few years, this concept related to monoamines has shifted to a putative deficit in glutamatergic signaling contributing to depressive symptoms (Catena-Dell'Osso et al., 2013; Smialowska et al., 2013). Indeed, the use of ketamine, a *N*-methyl-D-aspartate (NMDA) receptor antagonist, provided the most promising results in preclinical studies and produced a consistent and rapid, although transient, antidepressant effect with a good tolerability profile in humans (Catena-Dell'Osso et al., 2013).

Astrocytes play a remarkable range of roles to maintain homeostasis and optimum neuronal function. As mentioned before, astrocytes can remove the excess of K^+ and water, reuptake and release neurotransmitters, secrete trophic factors, and regulate metabolic pathways. Astroglial cells are the most important cells in the balance of glutamate and GABA signaling due to their ability to uptake those transmitters, control their release and to provide glutamine for glutamate and GABA synthesis. Therefore, astroglial homeostatic cascades are neuroprotective and can

prevent neuronal damage by maintaining brain metabolism and attenuating excitotoxicity through removal of glutamate excess (Verkhratsky et al., 2014).

Histopathological studies from *postmortem* brain tissue reveal prominent glial pathology in MDD. Astroglial changes are represented by a decrease in density of astrocytes stained by Nissl technique as well as a decrease in the number of GFAP-positive astrocytes (Rajkowska and Stockmeier, 2013). These alterations in astrocyte number and morphology are observed in many brain regions from MDD subjects. Cortical layers from prefrontal cortex (Cotter et al., 2002), orbitofrontal cortex, subgenual cortex (Ongur et al., 1998), anterior cingulate cortex (Cotter et al., 2001) the hippocampus, and the amygdala (Ongur et al., 1998; Bowley et al., 2002; Rajkowska and Stockmeier, 2013; Verkhratsky et al., 2014) were analyzed with different methodologies and display glia reduction and or morphology alteration (Verkhratsky et al., 2014). Increase in the levels of S100β in blood serum of MDD patients, which is attenuated by antidepressant treatment is another evidence of astrocyte degeneration in the disease (Schroeter et al., 2002; Smialowska et al., 2013).

In contrast to these data, MDD elderly subjects show an increase in GFAP density in cortical layers 3, 4, and 5 of dorsal prefrontal cortex compared to younger MDD patients (Miguel-Hidalgo et al., 2000; Verkhratsky et al., 2014). Indeed, cingulate cortex and orbitofrontal from MDD elderly patients did not show any reductions in GFAP expression (Khundakar et al., 2011a,b; Rajkowska and Stockmeier, 2013), indicating a possible difference in GFAP expression upon aging. There is some evidence that do not confirm GFAP reduction in MDD. Results from entorhinal cortex show no alterations in density and GFAP morphology in MDD subjects (Damadzic et al., 2001). Torres-Platas et al. (2011) demonstrates a hypertrophy in white matter astrocytes in studies from post mortem samples of anterior cingulate cortex of suicide subjects. This observation corroborates data showing inflammatory processes underlying MDD pathology (Maes et al., 2009; Rajkowska and Stockmeier, 2013). In general, apart from data associated with aging or inflammation, there is a decrease in GFAP expression and other related glial markers in brain areas associated with mood disorders (Smialowska et al., 2013).

Reduction of the number of GFAP-positive cells in MDD patients is accompanied by a reduction in the expression of several genes involved with glutamate signaling, mainly expressed in astrocytes in the locus coeruleus (Bernard et al., 2011). *In situ* hybridization data from animal models of MDD also show a reduction in the expression of the GLT-1 in the hippocampus and cerebral cortex (Zink et al., 2010), suggesting a dysfunction in glutamate reuptake, glutamine synthesis and in the glutamate-GABA shunt, possibly underlying the pathology of MDD (Rajkowska and Stockmeier, 2013; Verkhratsky et al., 2014).

Expression of aquaporin 4 and connexins (Cx30 and Cx43) is also reduced in cortical and subcortical astrocytes in both MDD and in an experimental stress model (Rajkowska and Stockmeier, 2013; Verkhratsky et al., 2014). Accordingly, work by Sun et al. (2012) shows that animals submitted to unpredictable stress, a rodent model of depression, exhibited significant decrease in diffusion of gap junction channel permeable dye and expression of Cx43. Furthermore, injections of carbenoxolone, a blocker of gap junctions, into the prelimbic cortex induce anhedonia and anxiety in mice submitted to different behavioral tests (Sun et al., 2012). These data suggest that alteration in mice behavior related to MDD pathophysiology may involve astrocytic communication failure, at least in part, since CBX is an unspecific blocker. This altered scenario can be crucial for the information processing and the establishment of MDD pathophysiology (Verkhratsky et al., 2014).

Conclusion

Taken together, many of the cellular and molecular markers for astrocyte heterogeneity were shown to be key players in astrocyte mediated disease processes. However, the precise role of different astrocyte populations in disease onset and progression still remains to be addressed.

References

Alexander, W. S. (1949). Progressive fibrinoid degeneration of fibrillary astrocytes associated with mental retardation in a hydrocephalic infant. *Brain* 72, 373–381. doi: 10.1093/brain/72.3.373

Alves, J. A., Barone, P., Engelender, S., Froes, M. M., and Menezes, J. R. (2002). Initial stages of radial glia astrocytic transformation in the early postnatal anterior subventricular zone. *J. Neurobiol.* 52, 251–265. doi: 10.1002/neu.10087

Anders, S., Minge, D., Griemsmann, S., Herde, M. K., Steinhauser, C., and Henneberger, C. (2014). Spatial properties of astrocyte gap junction coupling in the rat hippocampus. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130600. doi: 10.1098/rstb.2013.0600

Bachoo, R. M., Kim, R. S., Ligon, K. L., Maher, E. A., Brennan, C., Billings, N., et al. (2004). Molecular diversity of astrocytes with implications for neurological disorders. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8384–8389. doi: 10.1073/pnas.0402140101 0402140101

Bandeira, F., Lent, R., and Herculano-Houzel, S. (2009). Changing numbers of neuronal and non-neuronal cells underlie postnatal brain growth in the rat. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14108–14113. doi: 10.1073/pnas.0804650106

Beardsley, P. M., and Hauser, K. F. (2014). Glial modulators as potential treatments of psychostimulant abuse. *Adv. Pharmacol.* 69, 1–69. doi: 10.1016/B978-0-12-420118-7.00001-9

Belachew, S., Chittajallu, R., Aguirre, A. A., Yuan, X., Kirby, M., Anderson, S., et al. (2003). Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. *J. Cell Biol.* 161, 169–186. doi: 10.1083/jcb.200210110

Bernard, R., Kerman, I. A., Thompson, R. C., Jones, E. G., Bunney, W. E., Barchas, J. D., et al. (2011). Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Mol. Psychiatry* 16, 634–646. doi: 10.1038/mp.2010.44

Boillee, S., Yamanaka, K., Lobsiger, C. S., Copeland, N. G., Jenkins, N. A., Kassiotis, G., et al. (2006). Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 312, 1389–1392. doi: 10.1126/science.1123511

Bowley, M. P., Drevets, W. C., Ongur, D., and Price, J. L. (2002). Low glial numbers in the amygdala in major depressive disorder. *Biol. Psychiatry* 52, 404–412. doi: 10.1016/S0006-3223(02)01404-X

Brenner, M., Johnson, A. B., Boespflug-Tanguy, O., Rodriguez, D., Goldman, J. E., and Messing, A. (2001). Mutations in GFAP, encoding glial fibrillary acidic

protein, are associated with Alexander disease. *Nat. Genet.* 27, 117–120. doi: 10.1038/83679

Breunig, J. J., Gate, D., Levy, R., Rodriguez, J. Jr., Kim, G. B., Danielpour, M., et al. (2012). Rapid genetic targeting of pial surface neural progenitors and immature neurons by neonatal electroporation. *Neural Dev.* 7:26. doi: 10.1186/1749-8104-7-26

Bristot Silvestrin, R., Bambini-Junior, V., Galland, F., Daniele Bobermim, L., Quincozes-Santos, A., Torres Abib, R., et al. (2013). Animal model of autism induced by prenatal exposure to valproate: altered glutamate metabolism in the hippocampus. *Brain Res.* 1495, 52–60. doi: 10.1016/j.brainres.2012.11.048

Bull, C., Freitas, K. C., Zou, S., Poland, R. S., Syed, W. A., Urban, D. J., et al. (2014). Rat nucleus accumbens core astrocytes modulate reward and the motivation to self-administer ethanol after abstinence. *Neuropsychopharmacology* 39, 2835–2845. doi: 10.1038/npp.2014.135

Catena-Dell'Osso, M., Fagiolini, A., Rotella, F., Baroni, S., and Marazziti, D. (2013). Glutamate system as target for development of novel antidepressants. *CNS Spectr.* 18, 188–198. doi: 10.1017/S1092852912000971

Costa, M. R., Bucholz, O., Schroeder, T., and Gotz, M. (2009). Late origin of glia-restricted progenitors in the developing mouse cerebral cortex. *Cereb. Cortex* 19(Suppl. 1), i135–i143. doi: 10.1093/cercor/bhp046

Costa, M. R., and Hedin-Pereira, C. (2010). Does cell lineage in the developing cerebral cortex contribute to its columnar organization? *Front. Neuroanat.* 4:26. doi: 10.3389/fnana.2010.00026

Costa, M. R., Kessaris, N., Richardson, W. D., Gotz, M., and Hedin-Pereira, C. (2007). The marginal zone/layer I as a novel niche for neurogenesis and gliogenesis in developing cerebral cortex. *J. Neurosci.* 27, 11376–11388. doi: 10.1523/JNEUROSCI.2418-07.2007

Cotter, D., Mackay, D., Chana, G., Beasley, C., Landau, S., and Everall, I. P. (2002). Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder. *Cereb. Cortex* 12, 386–394. doi: 10.1093/cercor/12.4.386

Cotter, D., Mackay, D., Landau, S., Kerwin, R., and Everall, I. (2001). Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. *Arch. Gen. Psychiatry* 58, 545–553. doi: 10.1001/archpsyc.58.6.545

Damadzic, R., Bigelow, L. B., Krimer, L. S., Goldenson, D. A., Saunders, R. C., Kleinman, J. E., et al. (2001). A quantitative immunohistochemical study of astrocytes in the entorhinal cortex in schizophrenia, bipolar disorder and major depression: absence of significant astrocytosis. *Brain Res. Bull.* 55, 611–618. doi: 10.1016/S0361-9230(01)00529-9

deAzevedo, L. C., Fallet, C., Moura-Neto, V., Daumas-Duport, C., Hedin-Pereira, C., and Lent, R. (2003). Cortical radial glial cells in human fetuses: depth-correlated transformation into astrocytes. *J. Neurobiol.* 55, 288–298. doi: 10.1002/neu.10205

Diaz-Amarilla, P., Olivera-Bravo, S., Trias, E., Cragnolini, A., Martinez-Palma, L., Cassina, P., et al. (2011). Phenotypically aberrant astrocytes that promote motoneuron damage in a model of inherited amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18126–18131. doi: 10.1073/pnas.1110689108

Emsley, J. G., and Macklis, J. D. (2006). Astroglial heterogeneity closely reflects the neuronal-defined anatomy of the adult murine CNS. *Neuron Glia Biol.* 2, 175–186. doi: 10.1017/S1740925X06000202

Froes, M. M., and Menezes, J. R. (2002). Coupled heterocellular arrays in the brain. *Neurochem. Int.* 41, 367–375. doi: 10.1016/S0197-0186(02)00016-5

Gao, P., Postiglione, M. P., Krieger, T. G., Hernandez, L., Wang, C., Han, Z., et al. (2014). Deterministic progenitor behavior and unitary production of neurons in the neocortex. *Cell* 159, 775–788. doi: 10.1016/j.cell.2014.10.027

Garcia-Marques, J., and Lopez-Mascaraque, L. (2013). Clonal identity determines astrocyte cortical heterogeneity. *Cereb. Cortex* 23, 1463–1472. doi: 10.1093/cercor/bhs134

Ge, W. P., Miyawaki, A., Gage, F. H., Jan, Y. N., and Jan, L. Y. (2012). Local generation of glia is a major astrocyte source in postnatal cortex. *Nature* 484, 376–380. doi: 10.1038/nature10959

Gorski, J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L., and Jones, K. R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J. Neurosci.* 22, 6309–6314.

Gowing, G., Philips, T., Van Wijmeersch, B., Audet, J. N., Dewil, M., Van Den Bosch, L., et al. (2008). Ablation of proliferating microglia does not affect motor neuron degeneration in amyotrophic lateral sclerosis caused by mutant superoxide dismutase. *J. Neurosci.* 28, 10234–10244. doi: 10.1523/JNEUROSCI.3494-08.2008

Goyal, M., Mehndiratta, S., Faruq, M., Dwivedi, M. K., and Kapoor, S. (2014). Infantile onset alexander disease with normal head circumference: a genetically proven case report. *J. Clin. Diagn. Res.* 8, PD03–PD04. doi: 10.7860/JCDR/2014/10211.5200

Gulisano, M., Broccoli, V., Pardini, C., and Boncinelli, E. (1996). Emx1 and Emx2 show different patterns of expression during proliferation and differentiation of the developing cerebral cortex in the mouse. *Eur. J. Neurosci.* 8, 1037–1050. doi: 10.1111/j.1460-9568.1996.tb01590.x

Hagemann, T. L., Gaeta, S. A., Smith, M. A., Johnson, D. A., Johnson, J. A., and Messing, A. (2005). Gene expression analysis in mice with elevated glial fibrillary acidic protein and Rosenthal fibers reveals a stress response followed by glial activation and neuronal dysfunction. *Hum. Mol. Genet.* 14, 2443–2458. doi: 10.1093/hmg/ddi248

Hancock, S. M., Finkelstein, D. I., and Adlard, P. A. (2014). Glia and zinc in ageing and Alzheimer's disease: a mechanism for cognitive decline? *Front. Aging Neurosci.* 6:137. doi: 10.3389/fnagi.2014.00137

Hochstim, C., Deneen, B., Lukaszewicz, A., Zhou, Q., and Anderson, D. J. (2008). Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. *Cell* 133, 510–522. doi: 10.1016/j.cell.2008.02.046

Hoft, S., Griemsmann, S., Seifert, G., and Steinhauser, C. (2014). Heterogeneity in expression of functional ionotropic glutamate and GABA receptors in astrocytes across brain regions: insights from the thalamus. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130602. doi: 10.1098/rstb.2013.0602

Hol, E. M., Roelofs, R. F., Moraal, E., Sonnemans, M. A., Sluijs, J. A., Proper, E. A., et al. (2003). Neuronal expression of GFAP in patients with Alzheimer pathology and identification of novel GFAP splice forms. *Mol. Psychiatry* 8, 786–796. doi: 10.1038/sj.mp.4001379

Huang, W., Zhao, N., Bai, X., Karram, K., Trotter, J., Goebbels, S., et al. (2014). Novel NG2-CreERT2 knock-in mice demonstrate heterogeneous differentiation potential of NG2 glia during development. *Glia* 62, 896–913. doi: 10.1002/glia.22648

Jackson, A. R., Shah, A., and Kumar, A. (2014). Methamphetamine alters the normal progression by inducing cell cycle arrest in astrocytes. *PLoS ONE* 9:e109603. doi: 10.1371/journal.pone.0109603

Kamphuis, W., Mamber, C., Moeton, M., Kooijman, L., Sluijs, J. A., Jansen, A. H., et al. (2012). GFAP isoforms in adult mouse brain with a focus on neurogenic astrocytes and reactive astrogliosis in mouse models of Alzheimer disease. *PLoS ONE* 7:e42823. doi: 10.1371/journal.pone.0042823

Kamphuis, W., Middeldorp, J., Kooijman, L., Sluijs, J. A., Kooi, E. J., Moeton, M., et al. (2014). Glial fibrillary acidic protein isoform expression in plaque related astrogliosis in Alzheimer's disease. *Neurobiol. Aging* 35, 492–510. doi: 10.1016/j.neurobiolaging.2013.09.035

Kang, S. H., Fukaya, M., Yang, J. K., Rothstein, J. D., and Bergles, D. E. (2010). $NG2^+$ CNS glial progenitors remain committed to the oligodendrocyte lineage in postnatal life and following neurodegeneration. *Neuron* 68, 668–681. doi: 10.1016/j.neuron.2010.09.009

Khundakar, A. A., Morris, C. M., Oakley, A. E., and Thomas, A. J. (2011a). Cellular pathology within the anterior cingulate cortex of patients with late-life depression: a morphometric study. *Psychiatry Res.* 194, 184–189. doi: 10.1016/j.pscychresns.2011.04.008

Khundakar, A., Morris, C., Oakley, A., and Thomas, A. J. (2011b). A morphometric examination of neuronal and glial cell pathology in the orbitofrontal cortex in late-life depression. *Int. Psychogeriatr.* 23, 132–140. doi: 10.1017/S1041610210000700

Kolomeets, N. S., and Uranova, N. (2010). Ultrastructural abnormalities of astrocytes in the hippocampus in schizophrenia and duration of illness: a post-tortem morphometric study. *World J. Biol. Psychiatry* 11(2 Pt 2), 282–292. doi: 10.1080/15622970902806124

Kulijewicz-Nawrot, M., Verkhratsky, A., Chvatal, A., Sykova, E., and Rodriguez, J. J. (2012). Astrocytic cytoskeletal atrophy in the medial prefrontal cortex of a triple transgenic mouse model of Alzheimer's disease. *J. Anat.* 221, 252–262. doi: 10.1111/j.1469-7580.2012.01536.x

Laurence, J. A., and Fatemi, S. H. (2005). Glial fibrillary acidic protein is elevated in superior frontal, parietal and cerebellar cortices of autistic subjects. *Cerebellum* 4, 206–210. doi: 10.1080/14734220500208846

Lepore, A. C., Dejea, C., Carmen, J., Rauck, B., Kerr, D. A., Sofroniew, M. V., et al. (2008). Selective ablation of proliferating astrocytes does not affect disease outcome in either acute or chronic models of motor neuron degeneration. *Exp. Neurol.* 211, 423–432. doi: 10.1016/j.expneurol.2008.02.020

Levison, S. W., Chuang, C., Abramson, B. J., and Goldman, J. E. (1993) The migrational patterns and developmental fates of glial precursors in the rat subventricular zone are temporally regulated. *Development* 119, 611–622.

Levison, S. W., and Goldman, J. E. (1993). Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. *Neuron* 10, 201–212. doi: 10.1016/0896-6273(93)90311-E

Li, R., Johnson, A. B., Salomons, G., Goldman, J. E., Naidu, S., Quinlan, R., et al. (2005). Glial fibrillary acidic protein mutations in infantile, juvenile, and adult forms of Alexander disease. *Ann. Neurol.* 57, 310–326. doi: 10.1002/ana.20406

Luskin, M. B., and McDermott, K. (1994). Divergent lineages for oligodendrocytes and astrocytes originating in the neonatal forebrain subventricular zone. *Glia* 11, 211–226. doi: 10.1002/glia.440110302

Maes, M., Yirmyia, R., Noraberg, J., Brene, S., Hibbeln, J., Perini, G., et al. (2009). The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. *Metab. Brain Dis.* 24, 27–53. doi: 10.1007/s11011-008-9118-1

Magavi, S., Friedmann, D., Banks, G., Stolfi, A., and Lois, C. (2012). Coincident generation of pyramidal neurons and protoplasmic astrocytes in neocortical columns. *J. Neurosci.* 32, 4762–4772. doi: 10.1523/JNEUROSCI.3560-11.2012

Marin-Padilla, M. (1995). Prenatal development of fibrous (white matter), protoplasmic (gray matter), and layer I astrocytes in the human cerebral cortex: a Golgi study. *J. Comp. Neurol.* 357, 554–572. doi: 10.1002/cne.903570407

Marshall, C. A., and Goldman, J. E. (2002). Subpallial dlx2-expressing cells give rise to astrocytes and oligodendrocytes in the cerebral cortex and white matter. *J. Neurosci.* 22, 9821–9830.

Matthias, K., Kirchhoff, F., Seifert, G., Huttmann, K., Matyash, M., Kettenmann, H., et al. (2003). Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. *J. Neurosci.* 23, 1750–1758.

Messing, A., Head, M. W., Galles, K., Galbreath, E. J., Goldman, J. E., and Brenner, M. (1998). Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice. *Am. J. Pathol.* 152, 391–398.

Mesulam, M. M. (1999). Neuroplasticity failure in Alzheimer's disease: bridging the gap between plaques and tangles. *Neuron* 24, 521–529. doi: 10.1016/S0896-6273(00)81109-5

Miguel-Hidalgo, J. J., Baucom, C., Dilley, G., Overholser, J. C., Meltzer, H. Y., Stockmeier, C. A., et al. (2000). Glial fibrillary acidic protein immunoreactivity in the prefrontal cortex distinguishes younger from older adults in major depressive disorder. *Biol. Psychiatry* 48, 861–873. doi: 10.1016/S0006-3223(00)00999-9

Miller, F. D., and Gauthier, A. S. (2007). Timing is everything: making neurons versus glia in the developing cortex. *Neuron* 54, 357–369. doi: 10.1016/j.neuron.2007.04.019

Miller, R. H., and Raff, M. C. (1984). Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. *J. Neurosci.* 4, 585–592.

Mountcastle, V. B. (1997). The columnar organization of the neocortex. *Brain* 120(Pt 4), 701–722. doi: 10.1093/brain/120.4.701

Nagai, M., Re, D. B., Nagata, T., Chalazonitis, A., Jessell, T. M., Wichterle, H., et al. (2007). Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat. Neurosci.* 10, 615–622. doi: 10.1038/nn1876

Noctor, S. C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.* 7, 136–144. doi: 10.1038/nn1172.nn1172

Olabarria, M., Noristani, H. N., Verkhratsky, A., and Rodriguez, J. J. (2010). Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia* 58, 831–838. doi: 10.1002/glia.20967

Ongur, D., Drevets, W. C., and Price, J. L. (1998). Glial reduction in the subgenual prefrontal cortex in mood disorders. *Proc. Natl. Acad. Sci. U.S.A.* 95, 13290–13295. doi: 10.1073/pnas.95.22.13290

Prust, M., Wang, J., Morizono, H., Messing, A., Brenner, M., Gordon, E., et al. (2011). GFAP mutations, age at onset, and clinical subtypes in Alexander disease. *Neurology* 77, 1287–1294. doi: 10.1212/WNL.0b013e3182309f72

Quinlan, R. A., Brenner, M., Goldman, J. E., and Messing, A. (2007). GFAP and its role in Alexander disease. *Exp. Cell Res.* 313, 2077–2087. doi: 10.1016/j.yexcr.2007.04.004

Rajkowska, G., and Stockmeier, C. A. (2013). Astrocyte pathology in major depressive disorder: insights from human postmortem brain tissue. *Curr. Drug Targets* 14, 1225–1236. doi: 10.2174/13894501113149990156

Regan, M. R., Huang, Y. H., Kim, Y. S., Dykes-Hoberg, M. I., Jin, L., Watkins, A. M., et al. (2007). Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. *J. Neurosci.* 27, 6607–6619. doi: 10.1523/JNEUROSCI.0790-07.2007

Roelofs, R. F., Fischer, D. F., Houtman, S. H., Sluijs, J. A., Van Haren, W., Van Leeuwen, F. W., et al. (2005). Adult human subventricular, subgranular, and subpial zones contain astrocytes with a specialized intermediate filament cytoskeleton. *Glia* 52, 289–300. doi: 10.1002/glia.20243

Schmechel, D. E., and Rakic, P. (1979). A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anat. Embryol. (Berl.)* 156, 115–152. doi: 10.1007/BF00300010

Schnieder, T. P., and Dwork, A. J. (2011). Searching for neuropathology: gliosis in schizophrenia. *Biol. Psychiatry* 69, 134–139. doi: 10.1016/j.biopsych.2010.08.027

Schroeter, M. L., Abdul-Khaliq, H., Diefenbacher, A., and Blasig, I. E. (2002). S100B is increased in mood disorders and may be reduced by antidepressive treatment. *Neuroreport* 13, 1675–1678. doi: 10.1097/00001756-200209160-00021

Scuderi, C., Stecca, C., Iacomino, A., and Steardo, L. (2013). Role of astrocytes in major neurological disorders: the evidence and implications. *IUBMB Life* 65, 957–961. doi: 10.1002/iub.1223

Smialowska, M., Szewczyk, B., Wozniak, M., Wawrzak-Wlecial, A., and Domin, H. (2013). Glial degeneration as a model of depression. *Pharmacol. Rep.* 65, 1572–1579. doi: 10.1016/S1734-1140(13)71518-4

Sofroniew, M. V., and Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathol.* 119, 7–35. doi: 10.1007/s00401-009-0619-8

Sun, J. D., Liu, Y., Yuan, Y. H., Li, J., and Chen, N. H. (2012). Gap junction dysfunction in the prefrontal cortex induces depressive-like behaviors in rats. *Neuropsychopharmacology* 37, 1305–1320. doi: 10.1038/npp.2011.319

Takahashi, T., Misson, J. P., and Caviness, V. S. Jr. (1990). Glial process elongation and branching in the developing murine neocortex: a qualitative and quantitative immunohistochemical analysis. *J. Comp. Neurol.* 302, 15–28. doi: 10.1002/cne.903020103

Torres-Platas, S. G., Hercher, C., Davoli, M. A., Maussion, G., Labonte, B., Turecki, G., et al. (2011). Astrocytic hypertrophy in anterior cingulate white matter of depressed suicides. *Neuropsychopharmacology* 36, 2650–2658. doi: 10.1038/npp.2011.154

Trias, E., Díaz-Amarilla, P., Olivera-Bravo, S., Isasi, E., Drechsel, D. A., Lopez, N., et al. (2013). Phenotypic transition of microglia into astrocyte-like cells associated with disease onset in a model of inherited ALS. *Front. Cell Neurosci.* 7:274. doi: 10.3389/fncel.2013.00274

Valori, C. F., Brambilla, L., Martorana, F., and Rossi, D. (2014). The multifaceted role of glial cells in amyotrophic lateral sclerosis. *Cell. Mol. Life Sci.* 71, 287–297. doi: 10.1007/s00018-013-1429-7

Verkhratsky, A., and Parpura, V. (2014). Neurological and psychiatric disorders as a neuroglial failure. *Period Biol.* 116, 115–124.

Verkhratsky, A., Rodriguez, J. J., and Steardo, L. (2014). Astrogliopathology: a central element of neuropsychiatric diseases? *Neuroscientist* 20, 576–588. doi: 10.1177/1073858413510208

Voigt, T. (1989). Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. *J. Comp. Neurol.* 289, 74–88. doi: 10.1002/cne.902890106

Wang, D. D., and Bordey, A. (2008). The astrocyte odyssey. *Prog. Neurobiol.* 86, 342–367. doi: 10.1016/j.pneurobio.2008.09.015

Yamanaka, K., Chun, S. J., Boillee, S., Fujimori-Tonou, N., Yamashita, H., Gutmann, D. H., et al. (2008). Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat. Neurosci.* 11, 251–253. doi: 10.1038/nn2047

Yeh, C. Y., Vadhwana, B., Verkhratsky, A., and Rodriguez, J. J. (2011). Early astrocytic atrophy in the entorhinal cortex of a triple transgenic animal model of Alzheimer's disease. *ASN Neuro.* 3, 271–279. doi: 10.1042/AN20110025

Zerlin, M., Levison, S. W., and Goldman, J. E. (1995). Early patterns of migration, morphogenesis, and intermediate filament expression of subventricular zone cells in the postnatal rat forebrain. *J. Neurosci.* 15, 7238–7249.

Zhang, D., Hu, X., Qian, L., O'Callaghan, J. P., and Hong, J. S. (2010). Astrogliosis in CNS pathologies: is there a role for microglia? *Mol. Neurobiol.* 41, 232–241. doi: 10.1007/s12035-010-8098-4

Zhang, Y., and Barres, B. A. (2013). A smarter mouse with human astrocytes. *Bioessays* 35, 876–880. doi: 10.1002/bies.201300070

Zhou, M., and Kimelberg, H. K. (2000). Freshly isolated astrocytes from rat hippocampus show two distinct current patterns and different [K(+)](o) uptake capabilities. *J. Neurophysiol.* 84, 2746–2757.

Zhou, M., and Kimelberg, H. K. (2001). Freshly isolated hippocampal CA1 astrocytes comprise two populations differing in glutamate transporter and AMPA receptor expression. *J. Neurosci.* 21, 7901–7908.

Zhu, X., Bergles, D. E., and Nishiyama, A. (2008). NG2 cells generate both oligodendrocytes and gray matter astrocytes. *Development* 135, 145–157. doi: 10.1242/dev.004895

Zhu, X., Zuo, H., Maher, B. J., Serwanski, D. R., LoTurco, J. J., Lu, Q. R., et al. (2012). Olig2-dependent developmental fate switch of NG2 cells. *Development* 139, 2299–2307. doi: 10.1242/dev.078873

Zink, M., Vollmayr, B., Gebicke-Haerter, P. J., and Henn, F. A. (2010). Reduced expression of glutamate transporters vGluT1, EAAT2 and EAAT4 in learned helpless rats, an animal model of depression. *Neuropharmacology* 58, 465–473. doi: 10.1016/j.neuropharm.2009.09.005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

REVIEW ARTICLE
published: 28 July 2014
doi: 10.3389/fncel.2014.00201

How big is the myelinating orchestra? Cellular diversity within the oligodendrocyte lineage: facts and hypotheses

Giulio Srubek Tomassy[1]* and Valentina Fossati[2]

[1] *Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA, USA*
[2] *The New York Stem Cell Foundation, New York, NY, USA*

Edited by:
Marcos R. Costa, Federal University of Rio Grande do Norte, Brazil

Reviewed by:
Robert Weissert, University of Regensburg, Germany
Babette Fuss, Virginia Commonwealth University, USA

****Correspondence:***
Giulio Srubek Tomassy, Department of Stem Cell and Regenerative Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA
e-mail: giulio_tomassy@harvard.edu

Since monumental studies from scientists like His, Ramón y Cajal, Lorente de Nó and many others have put down roots for modern neuroscience, the scientific community has spent a considerable amount of time, and money, investigating any possible aspect of the evolution, development and function of neurons. Today, the complexity and diversity of myriads of neuronal populations, and their progenitors, is still focus of extensive studies in hundreds of laboratories around the world. However, our prevalent neuron-centric perspective has dampened the efforts in understanding glial cells, even though their active participation in the brain physiology and pathophysiology has been increasingly recognized over the years. Among all glial cells of the central nervous system (CNS), oligodendrocytes (OLs) are a particularly specialized type of cells that provide fundamental support to neuronal activity by producing the myelin sheath. Despite their functional relevance, the developmental mechanisms regulating the generation of OLs are still poorly understood. In particular, it is still not known whether these cells share the same degree of heterogeneity of their neuronal companions and whether multiple subtypes exist within the lineage. Here, we will review and discuss current knowledge about OL development and function in the brain and spinal cord. We will try to address some specific questions: do multiple OL subtypes exist in the CNS? What is the evidence for their existence and those against them? What are the functional features that define an oligodendrocyte? We will end our journey by reviewing recent advances in human pluripotent stem cell differentiation towards OLs. This exciting field is still at its earliest days, but it is quickly evolving with improved protocols to generate functional OLs from different spatial origins. As stem cells constitute now an unprecedented source of human OLs, we believe that they will become an increasingly valuable tool for deciphering the complexity of human OL identity.

Keywords: oligodendrocytes, cellular diversity, myelin sheath, CNS development, oligodendrocyte progenitors, cellular identity

INTRODUCTION

One of the main challenges of modern neurobiology is discovering how cellular diversity in the brain emerges during development. Together with synaptic plasticity, neuronal diversity is the *mantra* that supports a functional complexity that would otherwise be difficult to explain. In the last decade we can find many examples that have taught us about the importance of dissecting the nervous system into small groups of neuronal subtypes, and, sometimes, even subtypes of subtypes. Years of studies on the generation and specification of neuronal identity in the spinal cord, retina and cerebral cortex have revealed common and divergent paths that lead to the establishment of extremely intricate networks (Livesey and Cepko, 2001; Arlotta et al., 2005; Migliore and Shepherd, 2005; Tomassy et al., 2010; Belgard et al., 2011). Understanding how millions of different neurons develop, integrate and eventually function as a whole is not just a mere intellectual exercise aimed at satisfying our *Faustian* aspirations, but it may also have direct consequences for our clinical approach to disease. A very recent example of this comes from studies on Rett syndrome, a neurodevelopmental disorder caused by mutations in the gene Mecp2 (Bienvenu and Chelly, 2006; Chahrour and Zoghbi, 2007). Although Mecp2 is expressed by many different types of neuronal and non neuronal cells (Kishi and Macklis, 2004; Caballero and Hendrich, 2005), its loss apparently does not affect all brain areas in a similar way (Tudor et al., 2002; Chahrour and Zoghbi, 2007).

However, neurons are not the only citizen in the nervous system where, although the proportion is still controversial, a significant fraction is represented by glial cells (Pakkenberg and Gundersen, 1988; Azevedo et al., 2009; Kandel et al., 2012). In the central nervous system (CNS), glial cells come in three flavors: astrocytes, oligodendrocytes (OLs) and microglia (Verkhratsky and Butt, 2007; Stern, 2010). The many roles for this "adhesive" type of cells have recently begun to attract a well-deserved

attention (the number of glia-centered articles tripled in the last 30 years according to NCBI). Despite the fact that neurons are critically supported by glial cells in the generation of active synapses and without glia would not communicate in a timely manner, just to name some of the glial contribution to neuronal function (Baumann and Pham-Dinh, 2001; Clarke and Barres, 2013), neuroscience, as its name suggests, is still strongly neuron-centric. However, neuronal diversity would mean nothing without the 24/7 support of their glial partners. In light of all this, one obvious question stands up for an answer: are glial cells as diverse as neurons? In other words how many types of astrocytes exist? How many OLs? And if multiple types exist, how much diversity exists among the progenitors that give rise to glial cells? Astrocytes seem to constitute a fairly heterogeneous population of cells (Zhang and Barres, 2010), but our knowledge about OL still lags far behind. Here we will focus on this type of glial cells, and their progenitors oligodendrocyte progenitor cells (OPCs), that are in charge of making the myelin sheath. Currently, there is much debate ongoing on whether OPCs constitute a homogeneous population of cells or whether different subtypes exist, as would be suggested based on different molecular and functional parameters (Gensert and Goldman, 2001; Dimou et al., 2008; Rivers et al., 2008; Lin et al., 2009; Psachoulia et al., 2009). Here, we will first review the different developmental origins of OPCs and the multiple lineages to which they belong. We will then review what is known about OL ability to produce the myelin sheath. We will then consider the evidences about some of the "other" functions of OL and how they may suggest the existence of multiple cell types, or subtypes. As stated before, these are not trivial issues, whose implications may also have clinical relevance, considering how many developmental and degenerative diseases affect the maturation and survival of OLs and their fatty membrane (Di Rocco et al., 2004; Trapp and Nave, 2008). We will end our article by reviewing current technical advances in generating OPCs and OLs in a dish, for therapeutic purposes, and we will speculate on the implications of a yet unexplored heterogeneity on such studies and on our understanding of human myelin pathologies.

SPACE ODDITY. DIFFERENT SITES OF ORIGIN FOR THE MYELINATING CELLS

Although the spatial origin of OLs has long been debated (Richardson et al., 2006), a consensus has now been reached about the dual nature of these cells as deriving from both ventral and dorsal domains of the neuraxis. Here, we will briefly review our current knowledge about four regions of the murine CNS, namely the neocortex, the olfactory bulbs (OB), the cerebellum and the spinal cord, and we will try to summarize and highlight common themes and divergent paths.

NEOCORTEX

The migratory fluxes that contribute to the formation of the neocortical wall have been matter of extensive research over the last decades. During development, two major streams contribute to the building of the cortical structure: a radial one, perpendicular to the pia, that starts in the proximity of the ependymal surface and a tangential one, that runs over long distances starting in the germinal zones of the ventral telencephalon. Classically, cortical projection neurons, born in the VZ and subventricular zone (SVZ) of the cortical primordium, migrate radially, while GABAergic cortical interneurons migrate tangentially from the medial and caudal ganglionic eminences (MGE, CGE) of the ventral telencephalon and enter the cortical primordium through two main tangential streams (Marín and Rubenstein, 2001, 2003). Although it was thought for a long time that cortical OLs originated only in the ventral telencephalon (Tekki-Kessaris et al., 2001), Kessaris et al. (2005) subsequently demonstrated the existence of three different migratory paths, each belonging to a different cell lineage (**Figure 1**). Two of these migratory paths, corresponding to $Gsx2^+$ and $Nkx2.1^+$ cell lineages, originate in the lateral ganglionic eminence (LGE) and in the MGE, respectively, while a third one, $Emx1^+$ lineage, originate within the cortical anlage itself. In the adult cortex, the Gsx2 and the Emx1 waves are the only two lineages contributing to myelination, as the Nkx2.1-derived OPCs apparently do not give rise to any myelinating OL, and they disappear after the first week of postnatal life (Kessaris et al., 2005).

OLFACTORY BULBS

Very little is known about the origin of OLs of the OB. Experimental evidences including heterotopic, heterocronic transplantations as well as genetic fate mapping point at an intrinsic source of these cells, although additional and more conclusive analysis has not been conducted yet (Spassky et al., 2001). In the postnatal mouse brain, $NG2^+$ progenitor cells in the SVZ migrate through the rostral migratory stream (RMS) and give rise to OB oligodendrocytes as well as neurons (Aguirre and Gallo, 2004). In addition, it has been shown that these SVZ-derived OPCs belong to the $Mash1^+$ lineage (**Figure 1**). Indeed, Mash1 mutant mice showed a dramatic decrease of OPCs in the OBs (Parras et al., 2004).

CEREBELLUM

In the chicken brain, cerebellar OLs originate in the parabasal bands of the ventral mesencephalum, from which they migrate tangentially and enter the developing cerebellum through the velum medullare (Mecklenburg et al., 2011). In the murine cerebellum, Ascl1-expressing OLs originate outside the cerebellar germinal epithelium, although the exact region still needs to be identified (Buffo and Rossi, 2013; **Figure 1**). As in the neocortex and in the spinal cord (see below), a dorsal, intracerebellar source of OLs cannot be ruled out and some evidence suggest that at least some OLs may indeed originate within the local germinal zones: by injecting a beta-galactosidase expressing retrovirus into post-natal deep germinal zone of the rat cerebellum, Zhang and Goldman showed that many labeled cells gave rise to OLs. However, this endogenous production of cerebellar OLs ceases by the third week of post-natal life (Zhang and Goldman, 1996; Grimaldi et al., 2009; Sudarov et al., 2011; Zhang et al., 2011).

SPINAL CORD

In the spinal cord two regions have been recognized as sources of OLs. The first one, lying within the expression territory of the transcription factors Nkx6.1 and Nkx6.2 (Cai et al., 2005;

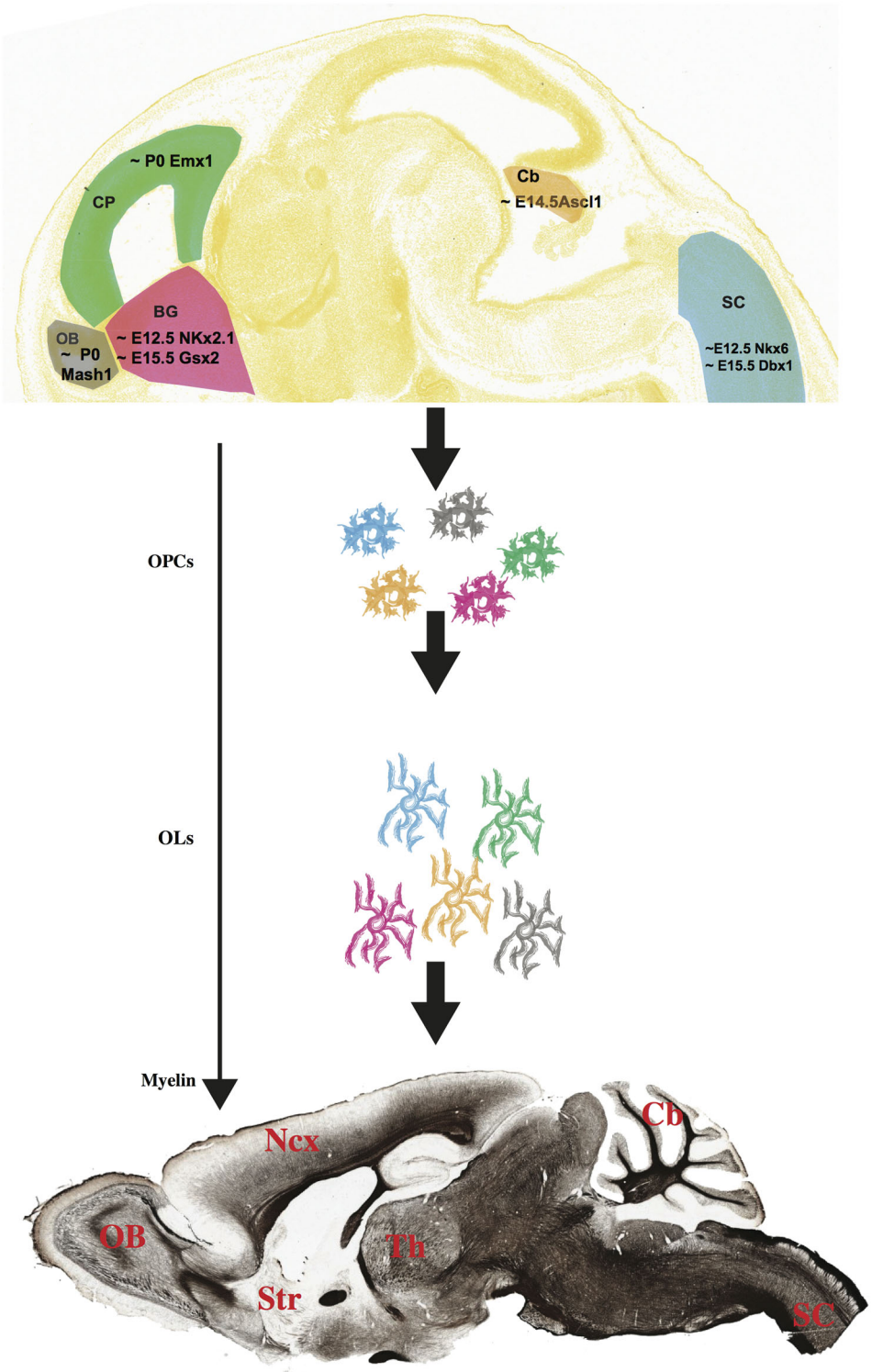


FIGURE 1 | Multiple progenitors, one myelin? (Top) OPCs have different birthdates and spatial origins in the embryonic and postnatal mouse brain and spinal cord (Image credit: Allen Institute for Brain Science, www.alleninstitute.org). How diverse are these multi-lineage OPC cells, and do they give rise to different groups of myelinating OLs? The myelin stained sagittal section on the bottom has been downloaded from the Brain Architecture Project (http://mouse.brainarchitecture.org) and licensed under a Creative Commons (CC) Attribution-ShareAlike 3.0 Unported License (http://creativecommons.org/licenses/by-sa/3.0/). OB, olfactory bulb; CP, cortical plate; BG, basal ganglia; Cb, cerebellum; SC, spinal cord; Ncx, neocortex; Str, striatum; Th, thalamus. Scale bar, 1 mm.

Fogarty et al., 2005; Vallstedt et al., 2005) is located in the pMN domain of the embryonic cord, where the transcription factor Olig2 regulates the generation of both OLs and motor neurons (Rowitch et al., 2002) and accounts for 85% of all spinal cord OLs (Richardson et al., 2006). However, OLs are still generated in the Nkx6 knockout spinal cord, suggesting that other sources exist in the developing cord (Cai et al., 2005; Vallstedt et al., 2005). Indeed, fate mapping experiments using a Dbx1-driven Cre reporter line confirmed the existence of a smaller but consistent group of cells that are born in the dP3 to dP5 dorsal domains of the cord (Fogarty et al., 2005; **Figure 1**).

TIME IS ON MY SIDE. MULTIPLE DEVELOPMENTAL STAGES OF OLIGODENDROGENESIS

The emerging picture shows that OLs originate in many different regions along the rostro-caudal and dorso-ventral axes of both brain and spinal cord. Furthermore, these cells are generated at different times during both embryonic and post-natal development. In the spinal cord, generation of ventral progenitors starts around embryonic (E) day 12.5, while the first dorsal progenitors are generated at approximately E14.5 (Cai et al., 2005). By then, the only OPCs that can be found in the neocortex belong to the Nkx2.1 lineage, while $Gsx2^+$ OPCs have not reached the cortical plate yet (Kessaris et al., 2005), and $Olig2^+$ cells can be already detected in the developing cerebellum (Buffo and Rossi, 2013). Meanwhile, $Emx1^+$ progenitors in the developing neocortical wall are still producing projection neurons of the upper layers, with their gliogenic potential still on standby (Angevine and Sidman, 1961; Gorski et al., 2002) and waiting to give rise to OLs only at birth (around P0) (Kessaris et al., 2005; **Figure 1**). Aside from the $Emx1^+$ lineage, that produces neocortical OLs postnatally, all other OPCs are born embryonically, and their "myelination potential" (the actual formation of a compact myelin envelope) is "released" gradually, following a spatio-temporal sequence that is a developmental signature for any given species (Baumann and Pham-Dinh, 2001). In mice, this sequence starts in the spinal cord at birth and follows a caudorostral direction toward the brain, with the neocortex being the last region to be myelinated. In humans, myelination begins at midgestation in the spinal cord, and continues for at least the first two decades of life (Baumann and Pham-Dinh, 2001).

THE SONG REMAINS THE SAME. MANY OLIGODENDROCYTES, ONE MYELIN?

Does this spatial and temporal heterogeneity produce a heterogeneous population of OLs? If we look at neurogenesis, the birthdate and place of origin of a neuron are typically linked to its identity and function. A classical example comes from the neocortex. Here, glutamatergic projection neurons and GABAergic interneurons are born in the germinal zones of two distant regions of the developing telencephalon, the dorsal neocortical epithelium and the MGE and CGE of the ventral telencephalon, respectively (Marín and Rubenstein, 2003; Xu et al., 2004; Butt et al., 2005; Molyneaux et al., 2007). MGE-derived interneurons comprise two types of cells with very distinct molecular and functional features, i.e., $Nkx2.1^+$/$Parvalbumin^+$ and $Nkx2.1^+$/$Somatostatin^+$ interneurons (Butt et al., 2008), that in turn can be further subdivided into different subtypes, mainly based on their electrophysiological properties (Ma et al., 2006; Runyan et al., 2010). Since a cohort of neocortical OPCs also belongs to the $Nkx2.1^+$ lineage (Corbin et al., 2001; Marín and Rubenstein, 2001; Kessaris et al., 2005), it is tempting to hypothesize that these cells might also share some level of heterogeneity, like their neuronal counterparts in the lineage. Remaining within the borders of this audacious analogy, $Emx1^+$ glutamatergic neuron identity is strongly correlated to their birthdate, such that neurons born first, between E12.5 and E13.5 become corticofugal projection neurons of the deep layers of the neocortex, while late born cells become commissural projection neurons of the superficial layers (Molyneaux et al., 2007). Thus, an obvious question is whether $Emx1^+$ neocortical OLs born at different times also have different cellular identities and/or functions. Also, how different are the $Emx1^+$ OLs from the $Gsx2^+$ OLs? Nicoletta Kessaris and William Richardson crossed a Sox10-lox-GFP-poly(A)-lox-DTA mouse line with either a Gsx2-Cre or an Emx1-Cre effector line. The conditional excision of the GFP activated the DTA in selected cells and killed them (Kessaris et al., 2005). With this elegant approach, the authors showed that ablation of one type of OLs, e.g., Gsx2-derived or Emx1-derived, does not affect the final number of $Sox10^+$ cells (i.e., all OLs) nor the level of myelination in every region analyzed, including the neocortex. Thus, when one precursor pool of OLs is lost, the other one may compensate for its absence, implying that these cells are fully interchangeable and functionally equivalent. On the other hand, however, the same group has recently demonstrated that, in the spinal cord, dorsally and ventrally-derived OLs are not equally able to myelinate the dorsal corticospinal tract (CST), which runs in the dorsal funiculus of the cord. The authors showed that the CST is mainly myelinated by dorsally-derived OLs, that within the first 2 months of post-natal life outnumber and almost completely replace their ventrally-derived partners (Tripathi et al., 2011). Thus, in the spinal cord, OLs with different spatial and temporal origins may be differentially able to myelinate neighboring axons, suggesting that at least part of their identity must be affected by their developmental history. Although the reasons for this have not been investigated, one possibility is that dorsal and ventral OLs may express different "codes" of molecules (e.g., membrane receptors) that may govern interactions with specific neuronal subtypes (e.g., corticospinal motor neurons). Indeed, it is widely accepted that OL development and myelin biogenesis are strongly influenced by neuron-derived signals (Barres and Raff, 1999; Stevens et al., 2002; Nave and Salzer, 2006; Taveggia et al., 2010; Wake et al., 2011). However, one may speculate that for the same reasons, different OLs myelinating sequential segments of one single CST axon, must share some common traits, regardless of their origin or birthdate. This is puzzling, considering the remarkable length of the CST and the different regions of the brain and spinal cord that it runs through (Arlotta et al., 2005; Martin, 2005). Further research on the interactions between multiple OLs lineages and long axons like the CST are certainly desirable, and will further expand our understanding of neuron-oligodendrocyte biology.

Within the neocortical gray matter, the scenario is even more compelling, as suggested by a very recent paper from the laboratory of Paola Arlotta, in which we have shown that while Pdgfrα$^+$ OPCs populate all layers of the neocortex, their ability to generate mature APC$^+$/Plp1$^+$ OLs is dependent on their laminar position within the neocortex; accordingly, the amount of myelin found in the superficial layers is dramatically lower as compared to the deep layers (Tomassy et al., 2014; **Figure 2**). What are the reasons for this uneven distribution of myelinating cells in the neocortical wall? Superficial layers (II-IV) mostly contain commissural pyramidal neurons (CPN), that connect the two hemispheres of the brain as well as different cortical areas within the same hemisphere. Deep layer V and VI, instead, contain corticofugal pyramidal neurons (CFuPN), connecting the cortex with subcerebral and subcortical targets (Molyneaux et al., 2007, 2009). We showed that the layer-specific ability of neocortical OPCs to give rise to myelinating OLs is affected by the neuronal subtype present in their immediate proximity. Specifically, by changing the position of deep layer pyramidal neurons within the cortical wall, OLs redistribute and the myelination profile of the cortex changes accordingly; for example, in the Dab1$^{-/-}$ neocortex, where layers are nearly inverted, (i.e., deep layer neurons are located in the upper part of the cortex, while upper layer neurons are located in the deep layers) (Sweet et al., 1996; Ware et al., 1997), both OLs and myelin lose their gradient profile and instead cover the full extent of the cortex (**Figure 2**; Tomassy et al., 2014). Thus, our study suggests that different combinations of neuron-oligodendrocyte interactions may exist in different layers of the cortex; however, also layer-specific cell-autonomous differences among neocortical OPCs and/or OLs may not be ruled out. As a matter of fact, regional differences in OPCs behavior have been previously reported by several groups. Marsupials are a great model to study oligodendrogenesis, because of the extended development of their CNS, and a temporal analysis of CNPase expression on glia revealed a heterogeneous distribution of CNPase$^+$ cells over time, with only a transient expression in certain areas such as the optic pathway (Barradas et al., 1998). Magdalena Götz and Leda Dimou used genetic fate mapping in mice to follow the fate of Olig2$^+$ cells in the adult brain and showed that these cells generate myelinating OLs in the white matter, but remain as NG2$^+$ postmitotic cells in the gray matter (Dimou et al., 2008). They later went on and performed homo and heterotopic transplantation of traceable cells from adult gray and white matter to demonstrate that there are intrinsic differences between the progenitors residing in these two different environments; more specifically, only white matter-derived cells can efficiently generate myelinating OLs in both white and gray matter, while cells from the gray matter have a lower differentiation potential and fail to differentiate in a non-supportive environment such as the gray matter (Viganò et al., 2013). A plausible way to explain those regional differences is to assume that the OL population is heterogeneous. The existence of different types of OLs has actually been suggested from the very first work of Del Rio Ortega, which described four

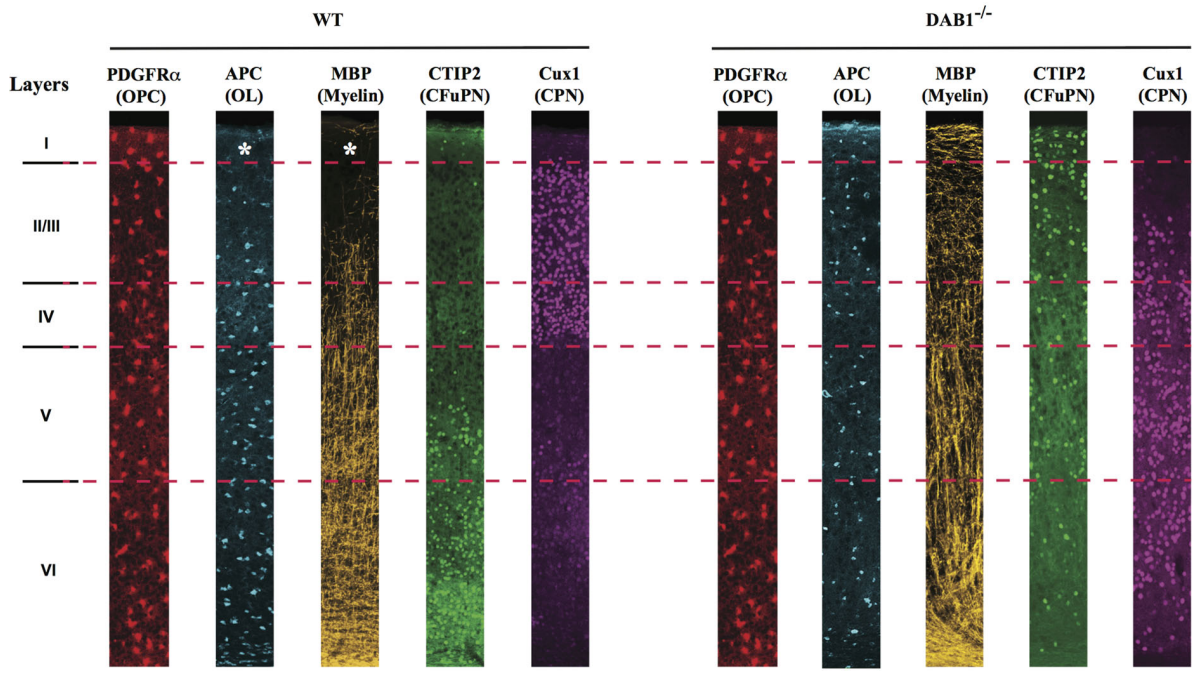


FIGURE 2 | Neocortical OPCs of different layers are not equally able to give rise to myelinating OLs. Schematic representation of laminar distribution of PDGFRα$^+$ OPCs, APC$^+$ OLs, MBP$^+$ myelin, CTIP2$^+$ corticofugal (CFuPN) and Cux1$^+$ commisural (CPN) projection neurons in the neocortex of (left) WT and (right) Dab1$^{-/-}$ mice. OPCs are uniformly distributed in all layers of the cortex, but only in layer IV to VI they generate mature, myelin-forming OLs. In the nearly inverted cortex of the DAB1$^{-/-}$ mice, both mature OLs and myelin are found in the most superficial part of the cortex, suggesting that repositioning of deep layer CFuPN has shaped the myelin profile of the neocortex.

types of OLs, identified by morphology and number of processes (del Rio Hortega, 1928). Later studies highlighted a correlation between the different types of OLs and the size of the axons myelinated by them, showing that small/highly ramified type I-II OLs, expressing carbonic anhydrase II (CAII), preferentially myelinate small caliber axons, while type III-IV larger OLs, with only one or two processes and negative for CAII myelinate large fibers (Butt et al., 1995). In addition, OLs have been divided into subsets according to the distribution of myelin basic proteins within their cytoplasm, nucleus and plasmalemma (Hardy et al., 1996). More recently, the development of sophisticated imaging techniques has been helpful to dissect OL heterogeneity; for example, using a computerized cell tracing system, Murtie et al. (2007) identified in the mouse frontal cortex an unknown subpopulation of OLs with numerous processes and short myelin internodes.

All together, these studies strongly support the existence of at least some level of heterogeneity within the oligodendrocyte lineage.

Understanding such diversity is crucial, giving the direct clinical implications that this might have: for example, it is well known that in multiple sclerosis (MS) there are two types of lesions, depending on the stage of disease, those with depletion of OPCs and those with normal number of OPCs that fail to differentiate and myelinate (Chang et al., 2002; Franklin, 2002). Although multiple reasons may account for these differences, one possibility is that different types of OPCs behave differently upon demyelinating conditions; this has been also suggested by studies showing that, upon demyelination of the rat spinal cord, two different populations exist, dividing and non-dividing OPCs, that respond differently to the demyelinating event (Keirstead et al., 1998). Also, the analysis of periventricular white matter injury (PWMI) in the ovine model brought to light the existence of areas more vulnerable to ischemia, due to a lower degree of maturity of the residing glia cells, suggesting that a more extensive maturation of OLs could protect infants from PWMI (Riddle et al., 2006). To note, an interesting study emphasizes the concept of regional differences in relation to cell replacement therapies, since different areas of the brain show specific sensitivity to irradiation, resulting in different levels of OPCs depletion and consequently in a variable degree of repopulation of the irradiated areas (Irvine and Blakemore, 2007).

HEY HEY, WHAT CAN I DO? WHAT DO OLIGODENDROCYTES (AND THEIR PROGENITORS) DO, APART FROM MAKING MYELIN?

Apart from their neurotransmitter system of choice and the networks where they integrate, all neurons have the same basic constituents: a cell body, an axon and multiple dendrites, and more importantly an excitable membrane, which is probably the most peculiar feature of this cell type. If we look at OLs, the most peculiar feature of these cells is their ability to wrap their plasma membrane around axons and form the myelin sheath. However, what else do we know about them? Are there properties by which one could distinguish cell subtypes or functional specializations?

OPCs have been described to share some "neuron-like" electrical features, such as expression of ion channels and ability to fire single action potentials, *in vitro* and *in vivo* (Kettenmann et al., 1984; Barres et al., 1990a,b; Bergles et al., 2010; Almeida and Lyons, 2013; Sun and Dietrich, 2013). In the white and gray matter of the mouse forebrain, OPCs have distinct physiological properties and express different profiles of Na^+ and K^+ channels. More importantly, a group of cells in the gray, but not white matter, responds with single, TTX-sensitive spikes upon depolarizing current injections. This spiking population of cortical $NG2^+$ cells also expresses functional AMPA receptors (Chittajallu et al., 2004). This is similar to what has been found in the rat hippocampus, where OPCs receive both glutamatergic as well as GABAergic synaptic inputs (Bergles et al., 2000; Lin and Bergles, 2004). These data seem to suggest that these physiological properties are not a common trait of the OL lineage, but rather a specialization of a subtype of cells of the gray matter, that may distinguish them from OPCs located in the white matter. However, in the white matter of the early postnatal rat cerebellum, two distinct populations of morphologically identical OPCs were found: again, one population expressed Na^+ and K^+ channels, received both inhibitory as well as excitatory synaptic inputs and, more importantly, was able to fire action potentials upon stimulation. The other population instead was not able to generate action potentials and did not receive any synaptic input (Káradóttir et al., 2008). Thus, the distinction between spiking and non-spiking cells may not be relevant in distinguishing white versus gray matter OPCs, but rather a specific feature that can be utilized to identify two functionally different subtypes, regardless of their location within the CNS. Interestingly, although physiologically active, spiking OPCs maintain their mitotic status, suggesting that neuronal inputs may be required to control or modulate their proliferative activity (Ge et al., 2009). Indeed, it has been demonstrated that neuronal activity influences oligodendrogenesis and myelination both *in vitro* and *in vivo* (Demerens et al., 1996; Wake et al., 2011; Gibson et al., 2014). All together, these data suggest that two subtypes of OPCs may exist, both in the gray as well as in the white matter, that can be distinguished based on their membrane properties and ability to spike action potentials. Interestingly, one of the symptoms of human oligodendrogliomas, a primary tumor of the brain (Harvey and Cushing, 1926; Russell and Rubinstein, 1959; Canoll and Goldman, 2008), is the frequent occurrence of epileptic seizures, likely due to the ability of these tumor cells to generate action potentials (Patt et al., 1996). Although one possibility is that these cells infiltrate the tissue and produce seizures by changing the microenvironment around neighboring neurons, another intriguing possibility is that this type of tumor exclusively may originate from the spiking, but not from the non-spiking subtype of OPCs.

TOMORROW NEVER KNOWS. UNDERSTANDING HUMAN OLs THROUGH PLURIPOTENT STEM CELLS DIFFERENTIATION

The ultimate goal of basic research, apart from pure scientific curiosity, is to translate what we learn into practical tools that we can use to treat and possibly cure human diseases. Since one of the current main challenges of myelin research is to being able to produce functional OPCs and OLs *in vitro*, for therapeutic

use (Goldman et al., 2012), understanding what are the common features that univocally identify these cells, as well as those that may distinguish distinct subtypes, is compelling and worth a concerted effort of the whole scientific community.

During the last two decades, the stem cell field has developed at a remarkable pace. Through the discovery of human embryonic stem cells (ESC) first (Thomson et al., 1998), and the generation of human induced pluripotent stem (iPS) cells via genetic reprogramming of somatic cells, less than 10 years later (Takahashi et al., 2007), we now own an unprecedented tool for studying human embryonic development and for generating all types of cells of the body. Pioneering studies on ESC differentiation clearly showed that embryonic development can be successfully recapitulated in a step-wise manner *in vitro* and that the fundamental pathways of lineage commitment are largely conserved from mouse to human (D'Amour et al., 2005; Kennedy et al., 2007). Those principles have been applied to oligodendrocyte development and resulted in several differentiation protocols, in which human pluripotent stem cells (hPSC, encompassing both ESC and iPSC) could be efficiently committed to an oligodendrocyte fate by patterning with critical molecules identified through studies on rodents (Nistor et al., 2005; Izrael et al., 2007; Hu et al., 2009; **Figure 3**).

A combination of retinoic acid (RA), insulin, insulin-like growth factor (IGF-1), triiodothhyroidin (T3) hormone, epidermal growth factor (EGF) and basic fibroblast growth (bFGF) was required to achieve the first oligodendrocyte differentiation from hESCs (Nistor et al., 2005). Platelet derived growth factor (PDGF-AA) and neurotrophin3 (NT3) were also added later on to drive maturation of OPCs to OLs (Hu et al., 2009). RA has been extensively used *in vitro* to mimic caudalization of neural tissues (Wichterle et al., 2002). Insulin and IGF-1 act as survival factors for oligodendrocyte progenitors and mature OLs (Barres et al., 1992). T3 plays a critical role in various stages of oligodendrocyte development, by promoting generation and expansion of early progenitors, differentiation to mature oligodendrocyte, and myelination (Rodríguez-Peña, 1999). EGF and FGF promote OPC generation and proliferation (McKinnon et al., 1990; Gonzalez-Perez and Alvarez-Buylla, 2011). Further attempts at obtaining an efficient *in vitro* differentiation protocol focused on spinal cord development. The progression toward mature OLs was followed through the sequential upregulation of OLIG2, NKX2.2, SOX10, PDGFRα, O4, and MBP (Izrael et al., 2007). Induction through RA and sonic hedgehog (SHH) signaling recapitulated *in vitro* the patterning of neuroepithelial cells to OLIG2$^+$ progenitors of the pMN domain (Hu et al., 2009). Interestingly, while this strategy confirmed that the transcriptional network regulating oligodendrocyte development is largely conserved among mammals, the study uncovered differences between human and mouse. First, NKX2.2 is expressed in human cells *in vitro* immediately after OLIG2 and before PDGFRα, as it occurs in the chick (Xu et al., 2000) and in the mouse hindbrain development (Vallstedt et al., 2005), but not in the mouse spinal cord (Qi et al., 2001). Second, bFGF in human cultures plays two distinct roles at different stages of the differentiation process, increasing the number of OLIG2$^+$ progenitors by preventing

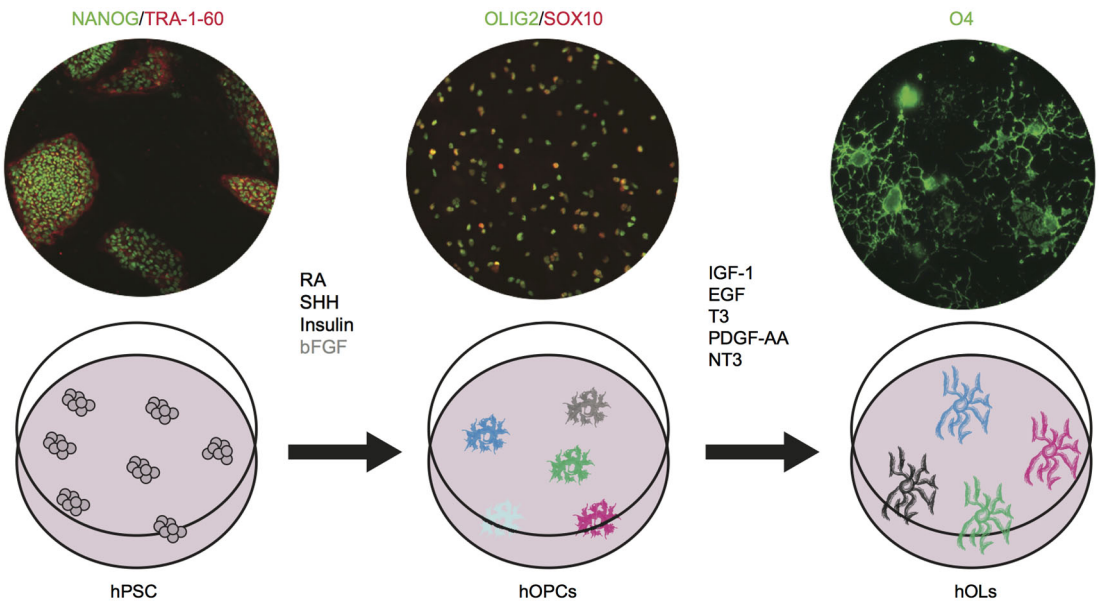


FIGURE 3 | Human OPCs and OLs can be differentiated *in vitro* from pluripotent stem cells. hPSC are shown as NANOG$^+$ (green)/TRA-1-60$^+$(red); hOPCs as OLIG2$^+$(green)/SOX10$^+$(red); and hOL as O4$^+$ cells (green). Images are based on results in press in Stem Cell Reports (Douvaras et al., 2014). The critical agents that promote oligodendrocyte differentiation have been identified. We showed that bFGF (shown in gray) is dispensable. Future studies will investigate whether the cells generated in the dish constitute one homogenous population or different subtypes. RA: retinoic acid, SHH: sonic hedgehog, bFGF: basic fibroblast growth factor, IGF-1: insulin like growth factor-1, EGF: epidermal growth factor, T3: triiodothyronine, PDGF-AA: platelet derived growth factor, NT3: neurotrophin 3.

motor neuron differentiation and subsequently inhibiting SHH signaling and the differentiation from OLIG2$^+$/NKX2.2$^+$ pre-OPCs to SOX10$^+$ OPCs. Finally, a long transition time (around 9 weeks) from pre-OPCs to OPCs appears to be a distinctive feature of human cultures, while it is not seen during mouse ESCs differentiation (Najm et al., 2011). This could reflect the slower temporal progression in human fetal development compared to mouse (Jakovcevski et al., 2009), but it could also be—at least in part—due to suboptimal culture conditions, as shown by our more recent protocol, in which the transition phase from pre-OPCs to OPCs is significantly shortened. Interestingly, we have also found that exogenous FGF signaling was dispensable in our cultures (Douvaras et al., 2014). With the discovery of iPS cells, *in vitro* differentiation studies largely moved to the optimization of the available protocols to extend the reproducibility to hiPSC lines (Pouya et al., 2011; Wang et al., 2013; Douvaras et al., 2014). Studies with iPS cells have confirmed that patterning with RA and SHH are an effective strategy to recapitulate oligodendrogenesis of the spinal cord, but to date the characterization of OLs differentiation has been purely restricted to well established markers such as SOX10, PDGFRα, NG2, O4, O1, MBP. One may wonder whether, in reality, we are generating a mixed population of OLs, and whether multiple subtypes of OPCs and OLs exist and can be recognized *in vitro*. Following the example of neuronal studies, single cell gene expression profiling could help addressing this question (Citri et al., 2012).

A recent study attempted for the first time to generate and characterize OPCs and OLs from both spinal cord and forebrain. Once more, the lesson was learned from mouse embryonic development, where FGF signaling, via *Fgfr1* and *Fgfr2* was identified as critical to generate *Pdgfrα*$^+$, *Olig2*$^+$ OPCs from ventral forebrain. The translation of those findings to human differentiation *in vitro* allowed the successful generation of human forebrain GSX2$^+$/NXX2.1$^+$ OPCs through induction with bFGF and SHH (Stacpoole et al., 2013). The next step will be to investigate whether forebrain OLs exhibit functional features that are distinct from their spinal cord counterparts. Interestingly, the same study provided the first evidence that hOPCs, similarly to mouse and rat OPCs, can be divided into spiking and nonspiking, and future studies will possibly identify molecular and functional differences associated with these subtypes. While our understanding of oligodendrogenesis improves by means of embryological studies in animal models, we will soon develop increasingly optimized protocols for differentiating hPSC towards OPCs from different developmental origins and with different identities. As a result, these *in vitro* systems will provide a novel compelling platform to thoroughly dissect human OL diversity. We envision that forthcoming studies of human OLs in a dish will lead the search for the answer. As stem cells constitute an unprecedented source of human OLs, they will become a fundamental tool for deciphering the complexity of oligodendroglia identity. The more we will learn from basic developmental biology and physiological studies, the more we will be able to "make" cells *in vitro* that will closely resemble their *in vivo* counterpart.

CONCLUSIONS

How much diversity exists within the oligodendrocyte lineage? Once more, the question is more practical than it may sound, with profound, direct implications into our clinical approach. From a "myelination perspective", OPCs from one region of the CNS can substitute for populations derived from other regions (e.g., neonatal forebrain SVZ could generate OLs when injected into the neonatal cerebellum), suggesting that these cells have a "default" myelinogenic potential that doesn't change with the environment (Milosevic et al., 2008). One-sixty years after the term "nervenkitt" was coined (Virchow, 1859), we have just started to turn our magnifying glasses toward the right direction and recognize these cells as something more than just cerebral glue. Today's technology will certainly help us accelerating this process and we can envision that the immediate future will bring us new knowledge as well as new concepts and ideas. Modern high-throughput genome and transcriptional profiling techniques (Cahoy et al., 2008; Wang et al., 2009; Shapiro et al., 2013) combined with the latest imaging (Lichtman and Denk, 2011) and electrophysiology tools (Fenno et al., 2011) will certainly boost our understanding of the diversity of OPCs and OLs in the CNS. Thus, it doesn't matter whether OLs perform as a symphony orchestra, a solo or a rock band, relax and enjoy the music: their show has just started.

ACKNOWLEDGMENTS

We are extremely grateful to Paola Arlotta (Harvard University), for her support. We thank James Goldman (Columbia University) for his extremely useful comments on the manuscript and for the fruitful scientific discussion we had together. We thank Marcos Costa (UFRN, Brasil) and Caroline Rouaux (INSERM, France) who gave us the chance to contribute with the present manuscript to this Research Topic issue of Frontiers in Cellular Neuroscience.

REFERENCES

Aguirre, A., and Gallo, V. (2004). Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. *J. Neurosci.* 24, 10530–10541. doi: 10.1523/jneurosci.3572-04.2004

Almeida, R. G., and Lyons, D. A. (2013). On the resemblance of synapse formation and CNS myelination. *Neuroscience* doi: 10.1016/j.neuroscience.2013.08.062. [Epub ahead of print].

Angevine, J. B., and Sidman, R. L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* 192, 766–768. doi: 10.1038/192766b0

Arlotta, P., Molyneaux, B. J., Chen, J., Inoue, J., Kominami, R., and Macklis, J. D. (2005). Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron* 45, 207–221. doi: 10.1016/j.neuron.2004.12.036

Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., et al. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *J. Comp. Neurol.* 513, 532–541. doi: 10.1002/cne.21974

Barradas, P. C., Gomes, S. S., and Cavalcante, L. A. (1998). Heterogeneous patterns of oligodendroglial differentiation in the forebrain of the opossum Didelphis marsupialis. *J. Neurocytol.* 27, 15–25. doi: 10.1023/A:1006930818708

Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D., et al. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70, 31–46. doi: 10.1016/0092-8674(92)90531-g

Barres, B. A., Koroshetz, W. J., Chun, L. L., and Corey, D. P. (1990a). Ion channel expression by white matter glia: the type-1 astrocyte. *Neuron* 5, 527–544. doi: 10.1016/0896-6273(90)90091-s

Barres, B. A., Koroshetz, W. J., Swartz, K. J., Chun, L. L., and Corey, D. P. (1990b). Ion channel expression by white matter glia: the O-2A glial progenitor cell. *Neuron* 4, 507–524. doi: 10.1016/0896-6273(90)90109-s

Barres, B. A., and Raff, M. C. (1999). Axonal control of oligodendrocyte development. *J. Cell Biol.* 147, 1123–1128. doi: 10.1083/jcb.147.6.1123

Baumann, N., and Pham-Dinh, D. (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol. Rev.* 81, 871–927.

Belgard, T. G., Marques, A. C., Oliver, P. L., Abaan, H. O., Sirey, T. M., Hoerder-Suabedissen, A., et al. (2011). A transcriptomic atlas of mouse neocortical layers. *Neuron* 71, 605–616. doi: 10.1016/j.neuron.2011.06.039

Bergles, D. E., Jabs, R., and Steinhäuser, C. (2010). Neuron-glia synapses in the brain. *Brain Res. Rev.* 63, 130–137. doi: 10.1016/j.brainresrev.2009.12.003

Bergles, D. E., Roberts, J. D., Somogyi, P., and Jahr, C. E. (2000). Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. *Nature* 405, 187–191. doi: 10.1038/35012083

Bienvenu, T., and Chelly, J. (2006). Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. *Nat. Rev. Genet.* 7, 415–426. doi: 10.1038/nrg1878

Buffo, A., and Rossi, F. (2013). Origin, lineage and function of cerebellar glia. *Prog. Neurobiol.* 109, 42–63. doi: 10.1016/j.pneurobio.2013.08.001

Butt, S. J. B., Fuccillo, M., Nery, S., Noctor, S., Kriegstein, A., Corbin, J. G., et al. (2005). The temporal and spatial origins of cortical interneurons predict their physiological subtype. *Neuron* 48, 591–604. doi: 10.1016/j.neuron.2005.09.034

Butt, A. M., Ibrahim, M., Ruge, F. M., and Berry, M. (1995). Biochemical subtypes of oligodendrocyte in the anterior medullary velum of the rat as revealed by the monoclonal antibody Rip. *Glia* 14, 185–197. doi: 10.1002/glia.440140304

Butt, S. J. B., Sousa, V. H., Fuccillo, M. V., Hjerling-Leffler, J., Miyoshi, G., Kimura, S., et al. (2008). The requirement of Nkx2–1 in the temporal specification of cortical interneuron subtypes. *Neuron* 59, 722–732. doi: 10.1016/j.neuron.2008.07.031

Caballero, I. M., and Hendrich, B. (2005). MeCP2 in neurons: closing in on the causes of Rett syndrome. *Hum. Mol. Genet.* 14 Spec No 1, R19–R26. doi: 10.1093/hmg/ddi213

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., et al. (2008). A transcriptome database for astrocytes, neurons and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* 28, 264–278. doi: 10.1523/JNEUROSCI.4178-07.2008

Cai, J., Qi, Y., Hu, X., Tan, M., Liu, Z., Zhang, J., et al. (2005). Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and shh signaling. *Neuron* 45, 41–53. doi: 10.1016/j.neuron.2004.12.028

Canoll, P., and Goldman, J. E. (2008). The interface between glial progenitors and gliomas. *Acta Neuropathol.* 116, 465–477. doi: 10.1007/s00401-008-0432-9

Chahrour, M., and Zoghbi, H. Y. (2007). The story of Rett syndrome: from clinic to neurobiology. *Neuron* 56, 422–437. doi: 10.1016/j.neuron.2007.10.001

Chang, A., Tourtellotte, W. W., Rudick, R., and Trapp, B. D. (2002). Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *N. Engl. J. Med.* 346, 165–173. doi: 10.1056/nejmoa010994

Chittajallu, R., Aguirre, A., and Gallo, V. (2004). NG2-positive cells in the mouse white and grey matter display distinct physiological properties. *J. Physiol.* 561, 109–122. doi: 10.1113/jphysiol.2004.074252

Citri, A., Pang, Z. P., Südhof, T. C., Wernig, M., and Malenka, R. C. (2012). Comprehensive qPCR profiling of gene expression in single neuronal cells. *Nat. Protoc.* 7, 118–127. doi: 10.1038/nprot.2011.430

Clarke, L. E., and Barres, B. A. (2013). Emerging roles of astrocytes in neural circuit development. *Nat. Rev. Neurosci.* 14, 311–321. doi: 10.1038/nrn3484

Corbin, J. G., Nery, S., and Fishell, G. (2001). Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. *Nat. Neurosci.* 4, 1177–1182. doi: 10.1038/nn749

D'Amour, K. A., Agulnick, A. D., Eliazer, S., Kelly, O. G., Kroon, E., and Baetge, E. E. (2005). Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat. Biotechnol.* 23, 1534–1541. doi: 10.1038/nbt1163

del Rio Hortega, P. (1928). *Tercera Aportación al Conocimiento Morfológico e Interpretación Funcional de la Oligodendrología.* Madrid: Real Sociedad Española de Historia Natural.

Demerens, C., Stankoff, B., Logak, M., Anglade, P., Allinquant, B., Couraud, F., et al. (1996). Induction of myelination in the central nervous system by electrical activity. *Proc. Natl. Acad. Sci. U S A* 93, 9887–9892. doi: 10.1073/pnas.93.18.9887

Di Rocco, M., Biancheri, R., Rossi, A., Filocamo, M., and Tortori-Donati, P. (2004). Genetic disorders affecting white matter in the pediatric age. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 129B, 85–93. doi: 10.1002/ajmg.b.30029

Dimou, L., Simon, C., Kirchhoff, F., Takebayashi, H., and Götz, M. (2008). Progeny of Olig2-expressing progenitors in the gray and white matter of the adult mouse cerebral cortex. *J. Neurosci.* 28, 10434–10442. doi: 10.1523/JNEUROSCI.2831-08.2008

Douvaras, P., Wang, J., Zimmer, M., Hanchuk, S., O'Bara, M. A., Sadiq, S., et al. (2014). Efficient generation of myelinating oligodendrocytes from primary progressive multiple sclerosis patients by induced pluripotent stem cells. *Stem Cell Reports.* In press.

Fenno, L., Yizhar, O., and Deisseroth, K. (2011). The development and application of optogenetics. *Annu. Rev. Neurosci.* 34, 389–412. doi: 10.1146/annurev-neuro-061010-113817

Fogarty, M., Richardson, W. D., and Kessaris, N. (2005). A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. *Development* 132, 1951–1959. doi: 10.1242/dev.01777

Franklin, R. J. M. (2002). Why does remyelination fail in multiple sclerosis? *Nat. Rev. Neurosci.* 3, 705–714. doi: 10.1038/nrn917

Ge, W.-P., Zhou, W., Luo, Q., Jan, L. Y., and Jan, Y. N. (2009). Dividing glial cells maintain differentiated properties including complex morphology and functional synapses. *Proc. Natl. Acad. Sci. U S A* 106, 328–333. doi: 10.1073/pnas.0811353106

Gensert, J. M., and Goldman, J. E. (2001). Heterogeneity of cycling glial progenitors in the adult mammalian cortex and white matter. *J. Neurobiol.* 48, 75–86. doi: 10.1002/neu.1043.abs

Gibson, E. M., Purger, D., Mount, C. W., Goldstein, A. K., Lin, G. L., Wood, L. S., et al. (2014). Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. *Science* 344:1252304. doi: 10.1126/science.1252304

Goldman, S. A., Nedergaard, M., and Windrem, M. S. (2012). Glial progenitor cell-based treatment and modeling of neurological disease. *Science* 338, 491–495. doi: 10.1126/science.1218071

Gonzalez-Perez, O., and Alvarez-Buylla, A. (2011). Oligodendrogenesis in the subventricular zone and the role of epidermal growth factor. *Brain Res. Rev.* 67, 147–156. doi: 10.1016/j.brainresrev.2011.01.001

Gorski, J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L. R., and Jones, K. R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J. Neurosci.* 22, 6309–6314.

Grimaldi, P., Parras, C., Guillemot, F., Rossi, F., and Wassef, M. (2009). Origins and control of the differentiation of inhibitory interneurons and glia in the cerebellum. *Dev. Biol.* 328, 422–433. doi: 10.1016/j.ydbio.2009.02.008

Hardy, R. J., Lazzarini, R. A., Colman, D. R., and Friedrich, V. L. (1996). Cytoplasmic and nuclear localization of myelin basic proteins reveals heterogeneity among oligodendrocytes. *J. Neurosci. Res.* 46, 246–257. doi: 10.1002/(sici)1097-4547(19961015)46:2<246::aid-jnr13>3.0.co;2-0

Harvey, P. B., and Cushing, H. W. (1926). *A Classification of the Tumors of the Glioma Group on a Histo-Genetic Basis, with a Correlated Study of Prognosis . . . with 108 Illustrations*. Philadelphia: J. B. Lippincott Company.

Hu, B.-Y., Du, Z.-W., Li, X.-J., Ayala, M., and Zhang, S.-C. (2009). Human oligodendrocytes from embryonic stem cells: conserved SHH signaling networks and divergent FGF effects. *Development* 136, 1443–1452. doi: 10.1242/dev.029447

Irvine, K.-A., and Blakemore, W. F. (2007). A different regional response by mouse oligodendrocyte progenitor cells (OPCs) to high-dose X-irradiation has consequences for repopulating OPC-depleted normal tissue. *Eur. J. Neurosci.* 25, 417–424. doi: 10.1111/j.1460-9568.2007.05313.x

Izrael, M., Zhang, P., Kaufman, R., Shinder, V., Ella, R., Amit, M., et al. (2007). Human oligodendrocytes derived from embryonic stem cells: effect of noggin on phenotypic differentiation in vitro and on myelination in vivo. *Mol. Cell. Neurosci.* 34, 310–323. doi: 10.1016/j.mcn.2006.11.008

Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., and Zecevic, N. (2009). Oligodendrocyte development and the onset of myelination in the human fetal brain. *Front. Neuroanat.* 3:5. doi: 10.3389/neuro.05.005.2009

Kandel, E., Schwartz, J., Jessell, T., Siegelbaum, S., and Hudspeth, A. J. (2012). *Principles of Neural Science.* 5th Edn. New York: McGraw-Hill Professional.

Káradóttir, R., Hamilton, N. B., Bakiri, Y., and Attwell, D. (2008). Spiking and nonspiking classes of oligodendrocyte precursor glia in CNS white matter. *Nat. Neurosci.* 11, 450–456. doi: 10.1038/nn2060

Keirstead, H. S., Levine, J. M., and Blakemore, W. F. (1998). Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. *Glia* 22, 161–170. doi: 10.1002/(sici)1098-1136(199802)22:2<161::aid-glia7>3.0.co;2-a

Kennedy, M., D'Souza, S. L., Lynch-Kattman, M., Schwantz, S., and Keller, G. (2007). Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. *Blood* 109, 2679–2687.

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., and Richardson, W. D. (2005). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat. Neurosci.* 9, 173–179. doi: 10.1038/nn1620

Kettenmann, H., Orkand, R. K., and Lux, H. D. (1984). Some properties of single potassium channels in cultured oligodendrocytes. *Pflugers Arch.* 400, 215–221. doi: 10.1007/bf00581550

Kishi, N., and Macklis, J. D. (2004). MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions. *Mol. Cell. Neurosci.* 27, 306–321. doi: 10.1016/j.mcn.2004.07.006

Lichtman, J. W., and Denk, W. (2011). The big and the small: challenges of imaging the brain's circuits. *Science* 334, 618–623. doi: 10.1126/science.1209168

Lin, S.-C., and Bergles, D. E. (2004). Synaptic signaling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus. *Nat. Neurosci.* 7, 24–32. doi: 10.1038/nn1162

Lin, G., Mela, A., Guilfoyle, E. M., and Goldman, J. E. (2009). Neonatal and adult O4(+) oligodendrocyte lineage cells display different growth factor responses and different gene expression patterns. *J. Neurosci. Res.* 87, 3390–3402. doi: 10.1002/jnr.22065

Livesey, F. J., and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. *Nat. Rev. Neurosci.* 2, 109–118. doi: 10.1038/35053522

Ma, Y., Hu, H., Berrebi, A. S., Mathers, P. H., and Agmon, A. (2006). Distinct subtypes of somatostatin-containing neocortical interneurons revealed in transgenic mice. *J. Neurosci.* 26, 5069–5082. doi: 10.1523/JNEUROSCI.0661-06.2006

Marín, O., and Rubenstein, J. L. (2001). A long, remarkable journey: tangential migration in the telencephalon. *Nat. Rev. Neurosci.* 2, 780–790. doi: 10.1038/35097509

Marín, O., and Rubenstein, J. L. R. (2003). Cell migration in the forebrain. *Annu. Rev. Neurosci.* 26, 441–483. doi: 10.1146/annurev.neuro.26.041002.131058

Martin, J. H. (2005). The corticospinal system: from development to motor control. *Neuroscientist* 11, 161–173. doi: 10.1177/1073858404270843

McKinnon, R. D., Matsui, T., Dubois-Dalcq, M., and Aaronson, S. A. (1990). FGF modulates the PDGF-driven pathway of oligodendrocyte development. *Neuron* 5, 603–614. doi: 10.1016/0896-6273(90)90215-2

Mecklenburg, N., Garcia-López, R., Puelles, E., Sotelo, C., and Martinez, S. (2011). Cerebellar oligodendroglial cells have a mesencephalic origin. *Glia* 59, 1946–1957. doi: 10.1002/glia.21236

Migliore, M., and Shepherd, G. M. (2005). Opinion: an integrated approach to classifying neuronal phenotypes. *Nat. Rev. Neurosci.* 6, 810–818. doi: 10.1038/nrn1769

Milosevic, A., Noctor, S. C., Martínez-Cerdeño, V., Kriegstein, A. R., and Goldman, J. E. (2008). Progenitors from the postnatal forebrain subventricular zone differentiate into cerebellar-like interneurons and cerebellar-specific astrocytes upon transplantation. *Mol. Cell. Neurosci.* 39, 324–334. doi: 10.1016/j.mcn.2008.07.015

Molyneaux, B. J., Arlotta, P., Fame, R. M., MacDonald, J. L., MacQuarrie, K. L., and Macklis, J. D. (2009). Novel subtype-specific genes identify distinct subpopulations of callosal projection neurons. *J. Neurosci.* 29, 12343–12354. doi: 10.1523/JNEUROSCI.6108-08.2009

Molyneaux, B. J., Arlotta, P., Menezes, J. R. L., and Macklis, J. D. (2007). Neuronal subtype specification in the cerebral cortex. *Nat. Rev. Neurosci.* 8, 427–437. doi: 10.1038/nrn2151

Murtie, J. C., Macklin, W. B., and Corfas, G. (2007). Morphometric analysis of oligodendrocytes in the adult mouse frontal cortex. *J. Neurosci. Res.* 85, 2080–2086. doi: 10.1002/jnr.21339

Najm, F. J., Zaremba, A., Caprariello, A. V., Nayak, S., Freundt, E. C., Scacheri, P. C., et al. (2011). Rapid and robust generation of functional oligodendrocyte progenitor cells from epiblast stem cells. *Nat. Methods* 8, 957–962. doi: 10.1038/nmeth.1712

Nave, K. A., and Salzer, J. L. (2006). Axonal regulation of myelination by neuregulin 1. *Curr. Opin. Neurobiol.* 16, 492–500. doi: 10.1016/j.conb.2006.08.008

Nistor, G. I., Totoiu, M. O., Haque, N., Carpenter, M. K., and Keirstead, H. S. (2005). Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia* 49, 385–396. doi: 10.1002/glia.20127

Pakkenberg, B., and Gundersen, H. J. (1988). Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator. *J. Microsc.* 150, 1–20. doi: 10.1111/j.1365-2818.1988.tb04582.x

Parras, C. M., Galli, R., Britz, O., Soares, S., Galichet, C., Battiste, J., et al. (2004). Mash1 specifies neurons and oligodendrocytes in the postnatal brain. *EMBO J.* 23, 4495–4505. doi: 10.1038/sj.emboj.7600447

Patt, S., Labrakakis, C., Bernstein, M., Weydt, P., Cervós-Navarro, J., Nisch, G., et al. (1996). Neuron-like physiological properties of cells from human oligodendroglial tumors. *Neuroscience* 71, 601–611. doi: 10.1016/0306-4522(95)00468-8

Pouya, A., Satarian, L., Kiani, S., Javan, M., and Baharvand, H. (2011). Human induced pluripotent stem cells differentiation into oligodendrocyte progenitors and transplantation in a rat model of optic chiasm demyelination. *PLoS One* 6:e27925. doi: 10.1371/journal.pone.0027925

Psachoulia, K., Jamen, F., Young, K. M., and Richardson, W. D. (2009). Cell cycle dynamics of NG2 cells in the postnatal and ageing brain. *Neuron Glia Biol.* 5, 57–67. doi: 10.1017/s1740925x09990354

Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., et al. (2001). Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. *Development* 128, 2723–2733.

Richardson, W. D., Kessaris, N., and Pringle, N. (2006). Oligodendrocyte wars. *Nat. Rev. Neurosci.* 7, 11–18. doi: 10.1038/nrn1826

Riddle, A., Luo, N. L., Manese, M., Beardsley, D. J., Green, L., Rorvik, D. A., et al. (2006). Spatial heterogeneity in oligodendrocyte lineage maturation and not cerebral blood flow predicts fetal ovine periventricular white matter injury. *J. Neurosci.* 26, 3045–3055. doi: 10.1523/jneurosci.5200-05.2006

Rivers, L. E., Young, K. M., Rizzi, M., Jamen, F., Psachoulia, K., Wade, A., et al. (2008). PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. *Nat. Neurosci.* 11, 1392–1401. doi: 10.1038/nn.2220

Rodríguez-Peña, A. (1999). Oligodendrocyte development and thyroid hormone. *J. Neurobiol.* 40, 497–512. doi: 10.1002/(sici)1097-4695(19990915)40:4<497::aid-neu7>3.0.co;2-#

Rowitch, D. H., Lu, Q. R., Kessaris, N., and Richardson, W. D. (2002). An "oligarchy" rules neural development. *Trends Neurosci.* 25, 417–422. doi: 10.1016/s0166-2236(02)02201-4

Runyan, C. A., Schummers, J., Van Wart, A., Kuhlman, S. J., Wilson, N. R., Huang, Z. J., et al. (2010). Response features of parvalbumin-expressing interneurons suggest precise roles for subtypes of inhibition in visual cortex. *Neuron* 67, 847–857. doi: 10.1016/j.neuron.2010.08.006

Russell, D. S., and Rubinstein, L. J. (1959). *Pathology of Tumors of the Nervous System.* 1st Edn. London: Edward Arnold.

Shapiro, E., Biezuner, T., and Linnarsson, S. (2013). Single-cell sequencing-based technologies will revolutionize whole-organism science. *Nat. Rev. Genet.* 14, 618–630. doi: 10.1038/nrg3542

Spassky, N., Heydon, K., Mangatal, A., Jankovski, A., Olivier, C., Queraud-Lesaux, F., et al. (2001). Sonic hedgehog-dependent emergence of oligodendrocytes in the telencephalon: evidence for a source of oligodendrocytes in the olfactory bulb that is independent of PDGFRalpha signaling. *Development* 128, 4993–5004.

Stacpoole, S. R. L., Spitzer, S., Bilican, B., Compston, A., Káradóttir, R., Chandran, S., et al. (2013). High yields of oligodendrocyte lineage cells from human embryonic stem cells at physiological oxygen tensions for evaluation of translational biology. *Stem Cell Reports* 1, 437–450. doi: 10.1016/j.stemcr.2013.09.006

Stern, P. (2010). Glia. Glee for glia. Introduction. *Science* 330:773. doi: 10.1126/science.330.6005.773

Stevens, B., Porta, S., Haak, L. L., Gallo, V., and Fields, R. D. (2002). Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. *Neuron* 36, 855–868. doi: 10.1016/S0896-6273(02)01067-X

Sudarov, A., Turnbull, R. K., Kim, E. J., Lebel-Potter, M., Guillemot, F., and Joyner, A. L. (2011). Ascl1 genetics reveals insights into cerebellum local circuit assembly. *J. Neurosci.* 31, 11055–11069. doi: 10.1523/JNEUROSCI.0479-11.2011

Sun, W., and Dietrich, D. (2013). Synaptic integration by NG2 cells. *Front. Cell. Neurosci.* 7:255. doi: 10.3389/fncel.2013.00255

Sweet, H. O., Bronson, R. T., Johnson, K. R., Cook, S. A., and Davisson, M. T. (1996). Scrambler, a new neurological mutation of the mouse with abnormalities of neuronal migration. *Mamm. Genome* 7, 798–802. doi: 10.1007/s003359900240

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872. doi: 10.1016/j.cell.2007.11.019

Taveggia, C., Feltri, M. L., and Wrabetz, L. (2010). Signals to promote myelin formation and repair. *Nat. Rev. Neurol.* 6, 276–287. doi: 10.1038/nrneurol.2010.37

Tekki-Kessaris, N., Woodruff, R., Hall, A. C., Gaffield, W., Kimura, S., Stiles, C. D., et al. (2001). Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon. *Development* 128, 2545–2554.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147. doi: 10.1126/science.282.5391.1145

Tomassy, G. S., Berger, D. R., Chen, H.-H., Kasthuri, N., Hayworth, K. J., Vercelli, A., et al. (2014). Distinct profiles of myelin distribution along single axons of pyramidal neurons in the neocortex. *Science* 344, 319–324. doi: 10.1126/science.1249766

Tomassy, G. S., Lodato, S., Trayes-Gibson, Z., and Arlotta, P. (2010). Development and regeneration of projection neuron subtypes of the cerebral cortex. *Sci. Prog.* 93, 151–169. doi: 10.3184/003685010x12705764469952

Trapp, B. D., and Nave, K. A. (2008). Multiple sclerosis: an immune or neurodegenerative disorder? *Annu. Rev. Neurosci.* 31, 247–269. doi: 10.1146/annurev.neuro.30.051606.094313

Tripathi, R. B., Clarke, L. E., Burzomato, V., Kessaris, N., Anderson, P. N., Attwell, D., et al. (2011). Dorsally and ventrally derived oligodendrocytes have similar electrical properties but myelinate preferred tracts. *J. Neurosci.* 31, 6809–6819. doi: 10.1523/JNEUROSCI.6474-10.2011

Tudor, M., Akbarian, S., Chen, R. Z., and Jaenisch, R. (2002). Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain. *Proc. Natl. Acad. Sci. U S A* 99, 15536–15541. doi: 10.1073/pnas.242566899

Vallstedt, A., Klos, J. M., and Ericson, J. (2005). Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. *Neuron* 45, 55–67. doi: 10.1016/j.neuron.2004.12.026

Verkhratsky, A., and Butt, A. M. (2007). *Glial Neurobiology.* Malden, MA: John Wiley and Sons.

Viganò, F., Möbius, W., Götz, M., and Dimou, L. (2013). Transplantation reveals regional differences in oligodendrocyte differentiation in the adult brain. *Nat. Neurosci.* 16, 1370–1372. doi: 10.1038/nn.3503

Virchow, R. L. K. (1859). *Die Cellularpathologie in ihrer Begründung auf Physiologische und Pathologsgische Gewebelehre . . . 2, neu durchgesehene Aufl.* Berlin: August Hirschwald.

Wake, H., Lee, P. R., and Fields, R. D. (2011). Control of local protein synthesis and initial events in myelination by action potentials. *Science* 333, 1647–1651. doi: 10.1126/science.1206998

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., et al. (2013). Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. *Cell Stem Cell* 12, 252–264. doi: 10.1016/j.stem.2012.12.002

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63. doi: 10.1038/nrg2484

Ware, M. L., Fox, J. W., González, J. L., Davis, N. M., Lambert de Rouvroit, C., Russo, C. J., et al. (1997). Aberrant splicing of a mouse disabled homolog, mdab1, in the scrambler mouse. *Neuron* 19, 239–249. doi: 10.1016/s0896-6273(00)80936-8

Wichterle, H., Lieberam, I., Porter, J. A., and Jessell, T. M. (2002). Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110, 385–397. doi: 10.1016/s0092-8674(02)00835-8

Xu, Q., Cobos, I., De La Cruz, E., Rubenstein, J. L., and Anderson, S. A. (2004). Origins of cortical interneuron subtypes. *J. Neurosci.* 24, 2612–2622. doi: 10.1523/jneurosci.5667-03.2004

Xu, X., Cai, J., Fu, H., Wu, R., Qi, Y., Modderman, G., et al. (2000). Selective expression of Nkx-2.2 transcription factor in chicken oligodendrocyte progenitors and implications for the embryonic origin of oligodendrocytes. *Mol. Cell. Neurosci.* 16, 740–753. doi: 10.1006/mcne.2000.0916

Zhang, Y., and Barres, B. A. (2010). Astrocyte heterogeneity: an underappreciated topic in neurobiology. *Curr. Opin. Neurobiol.* 20, 588–594. doi: 10.1016/j.conb.2010.06.005

Zhang, L., and Goldman, J. E. (1996). Developmental fates and migratory pathways of dividing progenitors in the postnatal rat cerebellum. *J. Comp. Neurol.* 370, 536–550. doi: 10.1002/(sici)1096-9861(19960708)370:4<536::aid-cne9>3.0.co;2-5

Zhang, X., Santuccione, A., Leung, C., and Marino, S. (2011). Differentiation of postnatal cerebellar glial progenitors is controlled by Bmi1 through BMP pathway inhibition. *Glia* 59, 1118–1131. doi: 10.1002/glia.21184

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 May 2014; paper pending published: 30 May 2014; accepted: 03 July 2014; published online: 28 July 2014.

*Citation: Tomassy GS and Fossati V (2014) How big is the myelinating orchestra? Cellular diversity within the oligodendrocyte lineage: facts and hypotheses. Front. Cell. Neurosci. **8**:201. doi: 10.3389/fncel.2014.00201*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in
CELLULAR NEUROSCIENCE

MINI REVIEW ARTICLE
published: 22 December 2014
doi: 10.3389/fncel.2014.00434

Subventricular zone progenitors in time and space: generating neuronal diversity

Eduardo B. Sequerra*

Instituto do Cérebro, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil

Edited by:
Caroline Rouaux, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Liliana Bernardino, University of Beira Interior, Portugal
Carlos Vicario-Abejón, Instituto Cajal, Consejo Superior de Investigaciones Científicas (CSIC), Spain
Erica Butti, Ospedale San Raffaele, Italy

***Correspondence:**
Eduardo B. Sequerra, Instituto do Cérebro, Universidade Federal do Rio Grande do Norte, Av. Nascimento de Castro, 2155, Natal, 59056-450, RN, Brazil
e-mail: ebsequerra@gmail.com

The adult mammalian brain harbors a population of cells around their lateral ventricles capable of giving rise to new neurons throughout life. The so-called subventricular zone (SVZ) is a heterogeneous germinative niche in regard to the neuronal types it generates. SVZ progenitors give rise to different olfactory bulb (OB) interneuron types in accordance to their position along the ventricles. Here, I review data showing the difference between progenitors located along different parts of the SVZ axes and ages. I also discuss possible mechanisms for the origin of this diversity.

Keywords: heterogeneity, interneuron sub-type, morphogens, transcription factors, cellular differentiation

SVZ PROGENY IN SPACE AND TIME

The SVZ is one of the main neural stem cell niches in the adult mammalian brain. SVZ progenitors continuously give rise to new neurons that migrate to and differentiate in the ipsilateral olfactory bulb (OB; Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994). Once there, newly generated neurons differentiate into multiple neuronal types that participate in the OB local circuitry (Luskin, 1993; Brill et al., 2009; Merkle et al., 2014). Progenitors located at different positions of the antero-posterior and dorso-ventral axes of the lateral ventricles originate these neuronal types in a spatial-segregated manner (**Figure 1**; Merkle et al., 2007). However, it is still debated to which extent this co-relation between progenitor position and neuronal type generated is due to an internal program or distinct environmental factors impinging onto progenitors (for discussion see, Sequerra et al., 2013).

The first evidence supporting that the SVZ is indeed a heterogeneous territory came from a series of studies performed at Marla Luskin's lab. This group described that neuroblasts migrating to the OB proliferate (Menezes et al., 1995). However, many of them leave the cell cycle along the pathway forming a posterior to anterior gradient of proliferation along the RMS (Coskun and Luskin, 2001). The progenitors located along different points of dorso-ventral and antero-posterior axes of the ventricular wall display different cell cycle kinetics and cell type density (Falcão et al., 2012). Retroviral labeling of the proliferative progenitors in the anterior SVZ (Luskin, 1993) or at the horizontal limb of the rostral migratory stream (Smith and Luskin, 1998), demonstrated that progenitors of granular neurons leave the cell cycle earlier (more posteriorly) than the ones that will generate the periglomerular (PG) neurons. These data, later reproduced by other group (Hack et al., 2005), show that different populations of newly generated OB neurons segregate very early, while still migrating. But how early would such segregation take place?

During the embryonic development of the nervous system, different neuronal types are generated in morphogenetic territories that are formed through the establishment of gradients of diffusive molecules and the subsequent expression of type-specific transcription factors (TFs; Jessell, 2000). In the embryonic telencephalon, different types of neurons are generated according to their position in the dorso-ventral axis: cholinergic in the ventral ventricular zone (VZ), GABAergic in the ventro-lateral and glutamatergic in the dorsal (Marín and Rubenstein, 2001). Therefore, a question to be addressed was whether the spatial segregation of the SVZ germinative niche could represent a continuum of that in the embryonic telencephalon. Since the adult neural stem cells of the SVZ are directly derived from radial glia cells (Alves et al., 2002; Tramontin et al., 2003; Merkle et al., 2004), it is possible to label their radial process at different pial locations during early postnatal stages and then track the progeny of the generated SVZ progenitors (Merkle et al., 2007; Ventura and Goldman, 2007). Surprisingly for that time, the dorsal radial glia, that during embryogenesis gives rise to glutamatergic cortical neurons (Schuurmans et al., 2004), was shown to contribute inhibitory OB interneurons after embryogenesis (Merkle et al., 2007; Ventura and Goldman, 2007), and the progeny of radial glia located at different positions of the lateral ventricle dorso-ventral and antero-posterior axes give rise to different OB interneuron subtypes (**Figure 1**; Kelsch et al., 2007;

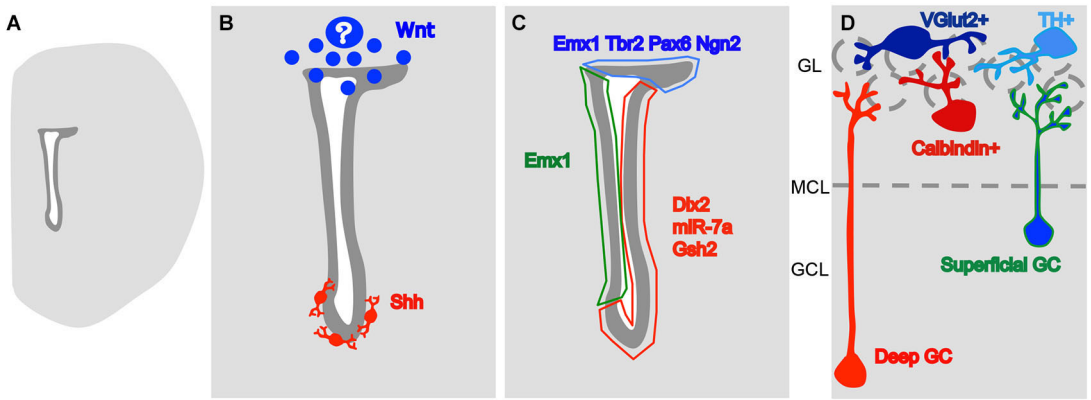


FIGURE 1 | A model for the origin of heterogeneity in the SVZ. **(A)** Drawing of a coronal slice of one hemisphere, representing the lateral ventricle (in white) and the SVZ (in dark gray). **(B)** The morphogens so far described to influence the generation of different OB interneuron subtypes. Wnt is produced dorsally, by an unknown source, and Shh is produced by neurons at the vicinity of the ventral SVZ. **(C)** These signaling molecules probably lead to differential expression of type-specific gene regulators dorsally (in blue) on the lateral wall, close to the striatum (in red) and on the medial wall, close to the septum (in green). **(D)** The expression of these regulators leads to the generation of OB interneuron type in a space-segregated manner. Neurons in blue are generated dorsally while the neurons in red come from ventral positions and the type in green and blue come both from the medial and dorsal SVZs. GCL- granular cell layer, MCL- mitral cell layer (represented by a dashed line), GL- glomerular layer (the glomeruli are represented by dashed circles).

Merkle et al., 2007; Paez-Gonzalez et al., 2014). Collectively, these data indicate that the adult ventricular wall is also divided in morphogenetic territories specialized in forming specific neuronal types.

Interestingly, different OB interneuron types are not only generated in a spatially segregated manner but also in a different time dynamics. De Marchis et al. (2007) injected Fluorogold in the anterior SVZ of neonatal and adult mice and described their progeny according to the neuronal types generated in the glomerular layer (GL). Batista-Brito et al. (2008) analyzed mice that had an inducible Cre-recombinase regulated by the Dlx1/2 enhancer, which is expressed in SVZ type C cells, in different time points, from embryogenesis to postnatal and adults. Li et al. (2011) analyzed the identity of new neurons labeled with BrdU at different ages. All three groups found that different OB interneuron types are generated in very specific time patterns, some exclusively during embryogenesis and first postnatal week, like the Parvalbumin$^+$ cells of the external plexiform layer (EPL; Batista-Brito et al., 2008; Li et al., 2011), but others keep their production consistently during life, like the granular and PG Calretinin$^+$ cells (De Marchis et al., 2007; Batista-Brito et al., 2008; Li et al., 2011). Although most of the Tyrosine hydroylase (TH)$^+$ PG neurons are generated embryonically and during the first postnatal week (Li et al., 2011), there are contradictory data in respect to the tendency of adult-generated neurons to give rise to this OB subtype. While Fluorogold (De Marchis et al., 2007) and BrdU injections (Whitman and Greer, 2007) suggest that a higher percentage of neurons born in the adult differentiate into TH$^+$ PG neurons, lineage analysis of the inducible Dlx1/2 enhancer progeny indicates that the percentage of cells that choose this type declines from embryogenesis to P30 (Batista-Brito et al., 2008). Independent of this contradiction, these data collectively show that the multiple types of OB subtypes are generated in a time-dependent fashion, suggesting dynamics in the activity of the SVZ during life. Since the different types are generated in different spatial domains it will be interesting to see if these domains are differentially regulated during time or if they can even fluctuate in size.

Different granular neuron subtypes are also mostly generated in segregated periods. The administration of BrdU or retroviral injections in the SVZ during the first postnatal week reveals that during this period superficial granular neurons are generated, whereas neurons generated in adults assume preferentially a deeper position (in respect to their position in the granular layer; Lemasson et al., 2005; Kelsch et al., 2007).

Although the main populations produced by the adult SVZ are granular or PG neurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994), this region also produces other interneuron populations in a smaller number. The EPL has a much lower cell density than the granule cell layer (GCL) and GL. However, the EPL interneurons are generated at the SVZ with a peek during the first postnatal days but continuously along adulthood (Winner et al., 2002; Batista-Brito et al., 2008; Yang, 2008; Li et al., 2011). Merkle et al. (2014) recently described populations of EPL and mitral cell layer interneurons that are generated in the ventral SVZ during adulthood.

Through the analysis of the progeny of Neurog2$^+$ progenitors, Brill et al. (2009) described the addition of a glutamatergic population to the PG population. PG neurons derived from these progenitors do not express Calretinin, TH, Sp8 or Calbindin and express the Vesicular glutamate transporter 2 (VGlut2; Brill et al., 2009). The morphologies of PG neurons from Neurog2$^+$ lineage also vary over time. While neurons generated at embryonic stages project into the glomeruli, the posnatally-generated ones keep their dendrites around these structures (Winpenny et al., 2011). The adult-generated cells project to two or three glomeruli

(Brill et al., 2009), a morphology typical of short-axon cells (Pinching and Powell, 1971). The neurochemical identity of short-axon neurons though, is currently under check. These cells were firstly described as glutamatergic since they do not have an active GAD_{65} promoter and the interglomerular transmission, attributed to these cells based on their morphology, is inhibited by ionotrophic glutamate receptors antagonists (Aungst et al., 2003). However, further histological (Kosaka and Kosaka, 2008; Kiyokage et al., 2010) and more careful functional analysis of their projection to the external tufted cells (Liu et al., 2013), show that interglomerular projecting neurons are actually GABAergic and dopaminergic instead of glutamatergic. The large soma TH^+ cells in the GL, presumably short axon cells, are generated during embryogenesis and first postnatal week (Kosaka and Kosaka, 2009) and some of these neurons, co-expressing Parvalbumin at the border between the EPL and the GL, are generated in the postnatal but not the adult SVZ (Yang, 2008). Therefore, it remains unclear the role of the $VGlut2^+$, with short axon cell-like morphology, interneurons in the adult OB circuitry.

The segregation of the SVZ into multiple progenitor domains and windows leads us to ask what are the mechanisms involved in the generation of this diversity. For having some insights about this mostly unanswered question, I will review the existence of signaling pathways in the SVZ that can regulate the formation of domains for subtype-specific neurogenesis. I will also review the gene transcription regulators that have a regionally restricted pattern of expression and, therefore, can be downstream of these signaling pathways.

ESTABLISHMENT OF MORPHOGENETIC TERRITORIES IN THE SVZ

Being the SVZ a heterogeneous territory in respect to the neuronal populations it produces, how are these spatial differences formed? As said above, the embryonic ventricular cells are divided in morphogenetic territories and as the adult SVZ derives from it, the information can be passed on as an internal program. Alternatively, new information can be added in the postnatal/adult milieu either maintaining the original program or overwriting it.

The ventral adult SVZ display an active Sonic Hedgehog (Shh) signaling (**Figure 1B**; Ihrie et al., 2011). The disruption of this signaling in the ventral SVZ leads to a shift of production of OB interneurons to dorsal phenotypes, superficial granular cells and TH^+ PG neurons. The dorsal SVZ however, is irresponsive to Shh administration, unless a constitutively active Smo (a Shh receptor) is induced (Ihrie et al., 2011). Therefore, the adult morphogenetic environment constantly maintains the ventral SVZ territory. Although SVZ progenitors can respond to their environment, their receptor composition differs, and their plasticity is limited.

In opposition to ventral Shh, the dorsal SVZ displays Wnt signaling (**Figure 1B**; Azim et al., 2014). The activation of this signaling contributes to the activation of the dorsal TF Tbr2 (Azim et al., 2014). The knock down of the downstream players of Wnt/Planar cell polarity signaling, Dvl2 or Vangl2, leads to a specific decrease in the generation of superficial granular neurons (Hirota et al., 2012), that are typically generated in more dorsal parts of the SVZ (**Figure 1D**; Merkle et al., 2007). It is still not clear at what point interfering with Wnt signaling leads to the generation of ventrally-generated interneuron subtypes, however, it seems clear that dorsal TFs in the SVZ are positively affected by this molecule.

Therefore, the SVZ regionalization seems to be actively maintained during the adult life. Since the production of different interneuron subtypes is age-dependent it will be interesting to see how these signaling pathways fluctuate in time.

THE SVZ MOLECULAR LANDSCAPE

The differential expression of morphogens along the SVZ axes leads to the next step in cell line segregation that is an internal cascade of molecular events that lead to their specification into an OB neuronal subtype. TFs and microRNAs are expressed in discrete regions of the SVZ niche (**Figure 1C**).

Neurog2, Tbr2 and Tbr1, which are involved in specification of $VGlut2^+$ interneurons of the GL, are restricted to the dorsal part of the SVZ around the lateral ventricles (Brill et al., 2009). Neurog2 and Tbr2 are expressed by fast proliferating, type C cells while Tbr1 is expressed later in the lineage (Brill et al., 2009). Pax6, a TF that takes part both in TH^+ PG neuron (Hack et al., 2005; Kohwi et al., 2005; Brill et al., 2008) and in superficial granular neuron specification (Kohwi et al., 2005), is mostly expressed by the dorsal SVZ and by few cells in the lateral SVZ (Brill et al., 2008; de Chevigny et al., 2012a). The Emx1 lineage, that gives rise to superficial granule neurons and preferentially gives rise to $Calretinin^+$ interneurons, is restricted to the dorsal and septal SVZs (Kohwi et al., 2007; Young et al., 2007). Dlx2, which works in conjunction with Pax6 to specify dopaminergic PG neurons (Brill et al., 2008), is expressed mainly by cells of the lateral wall of the ventricle (where it has no clear role interneuron type specification) but the dorsally generated population turns it on after migrating as neuroblasts in the RMS (de Chevigny et al., 2012a). Pax6 restriction to the dorsal aspect of the SVZ is regulated by an opposing gradient of the micro-RNA miR-7a, inhibiting its translation. The inhibition of miR-7a expression leads to an increase in dopaminergic PG neuron production (de Chevigny et al., 2012b). The Gsh2 lineage is located at the lateral wall of the ventricle and seems to be the lineage that produces $Calbindin^+$ PG neurons (Young et al., 2007). Merkle et al. (2014) identified a subpopulation of SVZ cells located at the anterior region of the ventral lateral ventricles, that expresses Nkx6.2 and gives rise to EPL and mitral cell layer interneurons. Other TFs were shown to be restricted to a region of the SVZ, like Nkx2.1 and Dbx1 (Young et al., 2007), but their contribution to the generation of the different subtypes is not available yet.

Therefore, the postnatal/adult SVZ seems to be compartmentalized. During development, the neuroepithelium gets divided in physical structures, called neuromeres, which differentially express adhesion molecules (Redies and Takeichi, 1996). Actually, EphB1, EphB2, ephrin-B and VCAM1 are expressed exclusively at the lateral wall of the SVZ (Conover et al., 2000; Kokovay et al., 2012) while EphB3 is expressed on the septal side (del Valle et al., 2011). It will be important in the future to test how the differential expression of these molecules restricts cell-cell communication and migration and, consequently, the access of different populations to region-specific signaling molecules.

Mellitzer et al. (1999) have shown that ephrin signaling restricts communication through GAP junctions to cells in the same neuromere. The postnatal dorsal SVZ (Freitas et al., 2012) and the adult lateral wall (Lacar et al., 2011) have local nets of astrocytes connected by GAP junctions but it is not clear at what extent these nets connect between compartments at the borders. A definite proof of the existence of physical compartments is still missing.

RELEVANCE TO HUMAN ADULT SVZ PROGENITOR HETEROGENEITY

Although the dynamics of OB interneuron subtype is very well described in mice, the relevance of this phenomenon for other species is starting to be understood. Of particular interest for medical sciences is the observation that in adult human OB there is little or no addition of new neurons (Sanai et al., 2011; Bergmann et al., 2012). However, for our discussion about the origin of the different OB interneuron subtypes, what is important is the source of these neurons. Is there a diversity of progenitors in the adult human brain capable of generating the OB interneuron pool? There is a massive reduction of neuroblast generation in the human lateral ventricles after the first year of life (Sanai et al., 2011) although the complete disappearance of neurogenesis in this region is controversial. There are groups that successfully detected neuronal markers, like PSA-NCAM, Dcx and and classIII-ßtubulin around the lateral ventricles (Curtis et al., 2007; Wang et al., 2011), on the olfactory tubercule (Wang et al., 2011) and around the olfactory ventricle (Curtis et al., 2007). Although these neuroblasts do not seem to be resulting in the addition of new neurons to the OB, they are possibly a source for the recently documented addition of interneurons to the human adult striatum (Ernst et al., 2014). There is not a current molecular analysis of the heterogeneity of neuronal progenitors in the human SVZ although we can expect it to be a common feature in primates. This idea is supported by the demonstration that both macaques (Tonchev et al., 2006) and marmosets (Azim et al., 2013) express type-specific TFs in the SVZ. Marmosets have a specific reduction of $Tbr2^{+}$ progenitors from first days of life to adulthood (Azim et al., 2013). Therefore, although the species differences have to be investigated, the theoretical piece of information collected in experimentation with rodents can still be relevant for the better understanding of the human SVZ heterogeneity of neuronal precursors.

CONCLUSION

The data published so far shows that the different neuronal lineages generated after birth to the OB are spatially and temporally segregated. Both morphogen signaling and molecular internal programs affect the specification of these cells.

Although we have some information on the internal programs of different interneuron subtypes and about their site and time of origin, little is known about how segregation between clones is kept. The criticisms lie around our technical limitations on testing the SVZ neural stem cells on their level of commitment. The argument supporting that clones in different locations are following an internal program is in dissonance with the observation that all the TFs cited above start to be expressed on type C cells or neuroblasts (for review, see Sequerra et al., 2013), not affecting the slow dividing stem cell on the top of the lineage. And the culture methods used so far for isolating stem cells, preferentially select fast dividing progenitors instead of the slow ones (for review, see Pastrana et al., 2011). Even the lack of response to Shh by dorsal progenitors detected by Ihrie et al. (2011) can be due to a lack of an inductive signal that makes these progenitors competent of responding to it (Waddington, 1940).

Although there are many similarities between the segregation of the embryonic and adult periventricular stem cell niches, there are important differences too. The adult brain is much bigger and less suitable for the establishment of molecular gradients. Instead, the adult SVZ has an intricate net of blood vessels (Mercier et al., 2002; Shen et al., 2008; Tavazoie et al., 2008; Snapyan et al., 2009) and axons (Höglinger et al., 2004, 2014; Paez-Gonzalez et al., 2014; Tong et al., 2014) bringing new players to the niche. Actually, the source of Shh to the ventral SVZ is composed of neurons located at the septum, preoptic nuclei and the stria terminalis, neurons that project into the SVZ (**Figure 1B**; Ihrie et al., 2011). The elimination of dopaminergic projections from the substantia nigra decreases proliferation in the SVZ (Baker et al., 2004) and it was recently shown that the substantia nigra and the ventral tegmented area project to distinct regions of the lateral ventricle wall (Höglinger et al., 2014). It is possible then, that activity in distinct brain regions differentially modulate SVZ progenitors depending on their location. Therefore, although some signaling pathways and molecular tools are re-used in the adult SVZ, the way they are played can be completely new, not seen in embryos.

There is clearly a lot to be done from now on. Many studies in the past considered the SVZ a homogeneous population. Molecules that were tested for general neurogenesis (for review, Lim and Alvarez-Buylla, 2014), for example, can be affecting a specific population and not others. New studies have to take into account the influence of signaling molecules to different SVZ populations, the differential expression of molecular determinants along the ventricle axes, and the consequences of changes in these dynamics to the generation of the different OB interneuron populations.

REFERENCES

Altman, J. (1969). Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J. Comp. Neurol.* 137, 433–457. doi: 10.1002/cne.901370404

Alves, J. A., Barone, P., Engelender, S., Fróes, M. M., and Menezes, J. R. (2002). Initial stages of radial glia astrocytic transformation in the early postnatal anterior subventricular zone. *J. Neurobiol.* 52, 251–265. doi: 10.1002/neu.10087

Aungst, J. L., Heyward, P. M., Puche, A. C., Karnup, S. V., Hayar, A., Szabo, G., et al. (2003). Centre-surround inhibition among bulb glomeruli. *Nature* 426, 623–629. doi: 10.1038/nature02185

Azim, K., Fischer, B., Hurtado-Chong, A., Draganova, K., Cantù, C., Zemke, M., et al. (2014). Persistent Wnt/ß-Catenin signaling determines dorsalization of the postnatal subventricular zone and neural stem cell specification into oligodendrocytes and glutamatergic neurons. *Stem Cells* 32, 1301–1312. doi: 10.1002/stem.1639

Azim, K., Zweifel, S., Klaus, F., Yoshikawa, K., Amrein, I., and Raineteau, O. (2013). Early decline in progenitor diversity in the marmoset lateral ventricle. *Cereb. Cortex* 23, 922–931. doi: 10.1093/cercor/bhs085

Baker, S. A., Baker, K. A., and Hagg, T. (2004). Dopaminergic nigrostriatal projections regulate neural precursor proliferation in the adult mouse subventricular zone. *Eur. J. Neurosci.* 20, 575–579. doi: 10.1111/j.1460-9568.2004.03486.x

Batista-Brito, R., Close, J., Machold, R., and Fishell, G. (2008). The distinct temporal origins of olfactory bulb interneuron subtypes. *J. Neurosci.* 28, 3966–3975. doi: 10.1523/jneurosci.5625-07.2008

Bergmann, O., Liebl, J., Bernard, S., Alkass, K., Yeung, M. S. Y., Steier, P., et al. (2012). The age of olfactory bulb interneurons in humans. *Neuron* 74, 634–639. doi: 10.1016/j.neuron.2012.03.030

Brill, M. S., Ninkovic, J., Winpenny, E., Hodge, R. D., Ozen, I., Yang, R., et al. (2009). Adult generation of glutamatergic olfactory bulb interneurons. *Nat. Neurosci.* 12, 1524–1533. doi: 10.1038/nn.2416

Brill, M. S., Snapyan, M., Wohlfrom, H., Ninkovic, J., Jawerka, M., Mastick, G. S., et al. (2008). A Dlx2- and Pax6-dependent transcriptional code for periglomerular neuron specification in the adult olfactory bulb. *J. Neurosci.* 28, 6439–6452. doi: 10.1523/jneurosci.0700-08.2008

Conover, J. C., Doetsch, F., Garcia-Verdugo, J., Gale, N. W., Yancopoulos, G. D., and Alvarez-Buylla, A. (2000). Disrutpion of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. *Nat. Neurosci.* 3, 1091–1097. doi: 10.1038/80606

Coskun, V., and Luskin, M. B. (2001). The expression pattern of the cell cycle inhibitor p19(INK4d) by progenitor cells of the rat embryonic telencephalon and neonatal anterior subventricular zone. *J. Neurosci.* 21, 3092–3103.

Curtis, M. A., Kam, M., Nannmark, U., Andreson, M. F., Axell, M. Z., Wikkelso, C., et al. (2007). Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science* 315, 1243–1249. doi: 10.1126/science.1136281

de Chevigny, A., Coré, N., Follert, P., Gaudin, M., Barbry, P., Béclin, C., et al. (2012b). miR-7a regulation of Pax6 controls spatial origin of forebrain dopaminergic neurons. *Nat. Neurosci.* 15, 1120–1126. doi: 10.1038/nn.3142

de Chevigny, A., Core, N., Follert, P., Wild, S., Bosio, A., Yoshikawa, K., et al. (2012a). Dynamic expression of the pro-dopaminergic transcription factors Pax6 and Dlx2 during postnatal olfactory bulb neurogenesis. *Front. Cell. Neurosci.* 6:6. doi: 10.3389/fncel.2012.00006

De Marchis, S., Boveti, S., Carletti, B., Hsieh, Y., Garzotto, D., Peretto, P., et al. (2007). Generation of distinct types of periglomerular olfactory bulb interneurons during development and in adult mice: implication for intrinsic properties of the subventricular zone progenitor population. *J. Neurosci.* 27, 657–664. doi: 10.1523/jneurosci.2870-06.2007

del Valle, K., Theus, M. H., Bethea, J. R., Liebl, D. J., and Ricard, J. (2011). Neural progenitors proliferation is inhibited by EphB3 in the developing subventricular zone. *Int. J. Dev. Neurosci.* 29, 9–14. doi: 10.1016/j.ijdevneu.2010.10.005

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., et al. (2014). Neurogenesis in the striatum of the adult human brain. *Cell* 156, 1072–1083. doi: 10.1016/j.cell.2014.01.044

Falcão, A. M., Palha, J. A., Ferreira, A. C., Marques, F., Sousa, N., and Sousa, J. C. (2012). Topographical analysis of the subependymal zone neurogenic niche. *PLoS One* 7:e38647. doi: 10.1371/journal.pone.0038647

Freitas, A. S., Xavier, A. L. R., Furtado, C. M., Hedin-Pereira, C., Fróes, M. M., and Menezes, J. R. L. (2012). Dye coupling and connexin expression by cortical radial glia in the early postnatal subventricular zone. *Dev. Neurobiol.* 72, 1482–1497. doi: 10.1002/dneu.22005

Hack, M. A., Saghatelyan, A., de Chevigny, A., Pfeifer, A., Ashery-Padan, R., Lledo, P., et al. (2005). Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nat. Neurosci.* 8, 865–872. doi: 10.1038/nn1479

Hirota, Y., Sawada, M., Kida, Y. S., Huang, S., Yamada, O., Sakagushi, M., et al. (2012). Roles of planar cell polarity signaling in maturation of neuronal precursor cells in the postnatal mouse olfactory bulb. *Stem Cells* 30, 1726–1733. doi: 10.1002/stem.1137

Höglinger, G. U., Arias-Carrión, O., Ipach, B., and Oertel, W. H. (2014). Origin of the dopaminergic innervation of adult neurogenic areas. *J. Comp. Neurol.* 522, 2336–2348. doi: 10.1002/cne.23537

Höglinger, G. U., Rizk, P., Muriel, M. P., Duyckaerts, C., Oertel, W. H., Cailie, I., et al. (2004). Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nat. Neurosci.* 7, 726–735. doi: 10.1038/nn1265

Ihrie, R. A., Shah, J. K., Harwell, C. C., Levine, J. H., Guinto, C. D., Lezameta, M., et al. (2011). Persistent sonic hedgehog signaling in adult brain determines neural stem cell positional identity. *Neuron* 71, 250–262. doi: 10.1016/j.neuron.2011.05.018

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* 1, 20–29. doi: 10.1038/35049541

Kelsch, W., Mosley, C. P., Lin, C. W., and Lois, C. (2007). Distinct mammalian precursors are commited to generate neurons with defined dendritic projection partterns. *PLoS Biol.* 5:e300. doi: 10.1371/journal.pbio.0050300

Kiyokage, E., Pan, Y., Shao, Z., Kobayashi, K., Szabo, G., Yanagawa, Y., et al. (2010). Molecular identity of periglomerular and short axon cells. *J. Neurosci.* 30, 1185–1196. doi: 10.1523/jneurosci.3497-09.2010

Kohwi, M., Osumi, N., Rubenstein, J. R., and Alvarez-Buylla, A. (2005). Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. *J. Neurosci.* 25, 6997–7003. doi: 10.1523/jneurosci.1435-05.2005

Kohwi, M., Petryniak, M. A., Long, J. E., Ekker, M., Obata, K., Yanagawa, Y., et al. (2007). A subpopulation of olfactory bulb GABAergic interneurons is derived from Emx1- and Dlx5/6-expressing progenitors. *J. Neurosci.* 27, 6878–6891. doi: 10.1523/jneurosci.0254-07.2007

Kokovay, E., Wang, Y., Kusek, G., Wurster, R., Lederman, P., Lowry, N., et al. (2012). VCAM1 is essential to maintain the structure of the SVZ niche and acts as an environmental sensor to regulate SVZ lineage progression. *Cell Stem Cell* 11, 220–230. doi: 10.1016/j.stem.2012.06.016

Kosaka, T., and Kosaka, K. (2008). Tyrosine hydroxylase-positive GABAergic juxtaglomerular neurons are the main source of the interglomerular connections in the mouse main olfactory bulb. *Neurosci. Res.* 60, 349–354. doi: 10.1016/j.neures.2007.11.012

Lacar, B., Young, S. Z., Platel, J., and Bordey, A. (2011). Gap junction-mediated calcium waves define communication networks among murine postnatal neural progenitor cells. *Eur. J. Neurosci.* 34, 1895–1905. doi: 10.1111/j.1460-9568.2011.07901.x

Lemasson, M., Sahatelyan, A., Olivo-Marin, J., and Lledo, P. (2005). Neonatal and adult neurogenesis provide distinct populations of newborn neurons to the mouse olfactory bulb. *J. Neurosci.* 25, 6816–6825. doi: 10.1523/jneurosci.1114-05.2005

Li, X., Sun, C., Lin, C., Ma, T., Madhavan, M. C., Campbell, K., et al. (2011). The transcription factor Sp8 is required for the production of parvalbumin-expressing interneurons in the olfactory bulb. *J. Neurosci.* 31, 8450–8455. doi: 10.1523/jneurosci.0939-11.2011

Lim, D., and Alvarez-Buylla, A. (2014). Adult neural stem cells stake their ground. *Trends Neurosci.* 37, 563–571. doi: 10.1016/j.tins.2014.08.006

Liu, S., Planchez, C., Shao, Z., Puche, A., and Shipley, M. T. (2013). Olfactory bulb short axon cell release of GABA and dopamine produces a temporally biphasic inhibition-excitation response in external tufted cells. *J. Neurosci.* 33, 2916–2926. doi: 10.1523/jneurosci.3607-12.2013

Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. *Science* 264, 1145–1148. doi: 10.1126/science.8178174

Luskin, M. B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11, 173–189. doi: 10.1016/0896-6273(93)90281-u

Marín, O., and Rubenstein, J. L. (2001). A long, remarkable journey: tangential migration in the telencephalon. *Nat. Rev. Neurosci.* 2, 780–790. doi: 10.1038/35097509

Mellitzer, G., Xu, Q., and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* 400, 77–81. doi: 10.1038/21907

Menezes, J. R. L., Smith, C. M., Nelson, K. C., and Luskin, M. B. (1995). The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. *Mol. Cell. Neurosci.* 6, 496–508. doi: 10.1006/mcne.1995.0002

Mercier, F., Kitasako, J. T., and Hatton, G. I. (2002). Anatomy of the brain neurogenic zones revisited: fractones and the fibroblast/macrophage network. *J. Comp. Neurol.* 451, 170–188. doi: 10.1002/cne.10342

Merkle, F. T., Fuentealba, L. C., Sanders, T. A., Magno, L., Kessaris, N., and Alvarez-Buylla, A. (2014). Adult neural stem cells in distinct microdomains generate previously unknown interneuron types. *Nat. Neurosci.* 17, 207–214. doi: 10.1038/nn.3610

Merkle, F. T., Mirzadeh, Z., and Alvarez-Buylla, A. (2007). Mosaic organization of neural stem cells in the adult brain. *Science* 317, 381–384. doi: 10.1126/science.1144914

Merkle, F. T., Tramontin, A. D., García-Verdugo, J. M., and Alvarez-Buylla, A. (2004). Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc. Natl. Acad. Sci. U S A* 101, 17528–17532. doi: 10.1073/pnas.0407893101

Paez-Gonzalez, P., Asrican, B., Rodriguez, E., and Kuo, C. T. (2014). Identification of distinct $ChAT^{+}$ neurons and activity-dependent control of postnatal SVZ neurogenesis. *Nat. Neurosci.* 17, 934–942. doi: 10.1038/nn.3734

Pastrana, E., Silva-Vargas, V., and Doetsch, F. (2011). Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell* 8, 486–498. doi: 10.1016/j.stem.2011.04.007

Pinching, A. J., and Powell, T. P. S. (1971). The neuron types of the glomerular layer of the olfactory bulb. *J. Cell Sci.* 9, 305–345.

Redies, C., and Takeichi, M. (1996). Cadherins in the developing nervous system: an adhesive code for segmental and functional subdivisions. *Dev. Biol.* 180, 413–423. doi: 10.1006/dbio.1996.0315

Sanai, N., Nguyen, T., Ihrie, R. A., Mirzadeh, Z., Tsai, H., Wong, M., et al. (2011). Corridors of migrating neurons in the human brain and their decline during infancy. *Nature* 478, 382–386. doi: 10.1038/nature10487

Schuurmans, C., Armant, O., Nieto, M., Stenman, J. M., Britz, O., Klenin, N., et al. (2004). Sequential phases of cortical specification involve Neurogenein-dependent and –independent pathways. *EMBO J.* 23, 2892–2902. doi: 10.1038/sj.emboj.7600278

Sequerra, E. B., Costa, M. R., Menezes, J. R. L., and Hedin-Pereira, C. (2013). Adult neural stem cells: plastic or restricted neuronal fates? *Development* 140, 3303–3309. doi: 10.1242/dev.093096

Shen, Q., Wang, Y., Kokovay, E., Lin, G., Chuang, S., Goderie, S. K., et al. (2008). Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. *Cell Stem Cell* 3, 289–300. doi: 10.1016/j.stem.2008.07.026

Smith, C. M., and Luskin, M. B. (1998). Cell cycle length of olfactory bulb neuronal progenitors in the rostral migratory stream. *Dev. Dyn.* 213, 220–227. doi: 10.1002/(sici)1097-0177(199810)213:2<220::aid-aja7>3.0.co;2-i

Snapyan, M., Lemasson, M., Brill, M. S., Blais, M., Massouh, M., Ninkovic, J., et al. (2009). Vasculature guides migrating neuronal precursors in the adult mammalian forbrain via brain-derived neurotrophic factor signaling. *J. Neurosci.* 29, 4172–4188. doi: 10.1523/jneurosci.4956-08.2009

Tavazoie, M., Van der Veken, L., Silva-Vargas, V., Louissaint, M., Colonna, L., Zaidi, B., et al. (2008). A specialized vascular niche for adult neural stem cells. *Cell Stem Cell* 3, 279–288. doi: 10.1016/j.stem.2008.07.025

Tonchev, A. B., Yamashima, T., Sawamoto, K., and Okano, H. (2006). Transcription factor protein expression patterns by neural or neuronal progenitor cells of adult monkey subventricular zone. *Neurosci.* 139, 1355–1367. doi: 10.1016/j.neuroscience.2006.01.053

Tong, C. K., Chen, J., Cebrián-Silla, A., Mizradeh, Z., Obernier, K., Guinto, C. D., et al. (2014). Axonal control of the adult neural stem cell niche. *Cell Stem Cell* 14, 500–511. doi: 10.1016/j.stem.2014.01.014

Tramontin, A. D., García-Verdugo, J. M., Lim, D. A., and Alvarez-Buylla, A. (2003). Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb. Cortex* 13, 580–587. doi: 10.1093/cercor/13.6.580

Ventura, R. E., and Goldman, J. E. (2007). Dorsal radial glia generate olfactory bulb interneurons in the postnatal murine brain. *J. Neurosci.* 27, 4297–4302. doi: 10.1523/jneurosci.0399-07.2007

Waddington, C. H. (1940). *Organisers and Genes.* Cambridge: Cambridge University Press.

Wang, C., Liu, F., Liu, Y., Zhao, C., You, Y., Wang, L., et al. (2011). Identification and characterization of neuroblasts in the subventricular zone and rostral migratory stream of the adult human brain. *Cell Res.* 21, 1534–1550. doi: 10.1038/cr.2011.83

Whitman, M. C., and Greer, C. A. (2007). Adult-generated neurons exhibit diverse developmental fates. *Dev. Neurobiol.* 67, 1079–1093. doi: 10.1002/dneu.20389

Winner, B., Cooper-Khun, C. M., Aigner, R., Winkler, J., and Kuhn, H. G. (2002). Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. *Eur. J. Neurosci.* 16, 1681–1689. doi: 10.1046/j.1460-9568.2002.02238.x

Winpenny, E., Lebel-Potter, M., Fernandez, M. E., Brill, M. S., Götz, M., Guillemot, F., et al. (2011). Sequential generation of olfactory bulb glutamatergic neurons by Neurog2-expressing precursor cells. *Neural Dev.* 6:12. doi: 10.1186/1749-8104-6-12

Yang, Z. (2008). Postnatal subventricular zone progenitors give rise mot only to granular and periglomerular interneurons but also to interneurons in the external plexiform layer of the rat olfactory bulb. *J. Comp. Neurol.* 506, 347–358. doi: 10.1002/cne.21557

Young, K. M., Fogarty, M., Kessaris, N., and Richardson, W. D. (2007). Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J. Neurosci.* 27, 8286–8296. doi: 10.1523/jneurosci.0476-07.2007

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 September 2014; accepted: 01 December 2014; published online: 22 December 2014.

*Citation: Sequerra EB (2014) Subventricular zone progenitors in time and space: generating neuronal diversity. Front. Cell. Neurosci. **8**:434. doi: 10.3389/fncel.2014.00434*

This article was submitted to the journal Frontiers in Cellular Neuroscience.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 18 March 2015
doi: 10.3389/fncel.2015.00078

PPARβ/δ and PPARγ maintain undifferentiated phenotypes of mouse adult neural precursor cells from the subventricular zone

Carolina Bernal[1*†], *Claudia Araya*[1], *Verónica Palma*[2*] and *Miguel Bronfman*[1‡]

[1] Department of Cell and Molecular Biology, Faculty of Biological Sciences, Center for Aging and Regeneration, Pontifical Catholic University of Chile, Santiago, Chile, [2] Laboratory of Stem Cells and Development, Faculty of Science, FONDAP Center for Genome Regulation, University of Chile, Santiago, Chile

The subventricular zone (SVZ) is one of the main niches of neural stem cells in the adult mammalian brain. Stem and precursor cells in this region are the source for neurogenesis and oligodendrogesis, mainly in the olfactory bulb and corpus callosum, respectively. The identification of the molecular components regulating the decision of these cells to differentiate or maintain an undifferentiated state is important in order to understand the modulation of neurogenic processes in physiological and pathological conditions. PPARs are a group of transcription factors, activated by lipid ligands, with important functions in cellular differentiation and proliferation in several tissues. In this work, we demonstrate that mouse adult neural precursor cells (NPCs), *in situ* and *in vitro*, express PPARβ/δ and PPARγ. Pharmacological activation of both PPARs isoforms induces proliferation and maintenance of the undifferentiated phenotype. Congruently, inhibition of PPARβ/δ and PPARγ results in a decrease of proliferation and loss of the undifferentiated phenotype. Interestingly, PPARγ regulates the level of EGFR in adult NPCs, concurrent with it is function described in embryonic NPCs. Furthermore, we describe for the first time that PPARβ/δ regulates SOX2 level in adult NPCs, probably through a direct transcriptional regulation, as we identified two putative PPAR response elements in the promoter region of Sox2. EGFR and SOX2 are key players in neural stem/precursor cells self-renewal. Finally, rosiglitazone, a PPARγ ligand, increases PPARβ/δ level, suggesting a possible cooperation between these two PPARs in the control of cell fate behavior. Our work contributes to the understanding of the molecular mechanisms associated to neural cell fate decision and places PPARβ/δ and PPARγ as interesting new targets of modulation of mammalian brain homeostasis.

Keywords: neural stem/precursor cells, PPARβ/δ, PPARγ, subventricular zone, SOX2, EGFR

OPEN ACCESS

Edited by:
Cecilia Hedin-Pereira, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Marcos R. Costa, Federal University of Rio Grande do Norte, Brazil
Eduardo Bouth Sequerra, Federal University of Rio Grande do Norte, Brazil

***Correspondence:**
Carolina Bernal, Department of Cell and Molecular Biology, Faculty of Biological Sciences, Center for Aging and Regeneration, Pontifical Catholic University of Chile, Santiago, Chile
carolina.bernal@unige.ch;
Verónica Palma, Laboratory of Stem Cells and Development, Faculty of Science, FONDAP Center for Genome Regulation, University of Chile, Las Palmeras 3425, 7800024 Ñuñoa, Santiago, Chile
vpalma@uchile.cl

†Present Address:
Carolina Bernal, Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva-4, Switzerland

‡*Passed away in January of 2014.*

Received: *08 November 2014*
Accepted: *21 February 2015*
Published: *18 March 2015*

Citation:
Bernal C, Araya C, Palma V and Bronfman M (2015) PPARβ/δ and PPARγ maintain undifferentiated phenotypes of mouse adult neural precursor cells from the subventricular zone. Front. Cell. Neurosci. 9:78. doi: 10.3389/fncel.2015.00078

Introduction

The subventricular zone of the lateral ventricles (SVZ) in the adult mammalian brain is one of two major CNS neural stem cell niches. There, neural stem cells self-renew and differentiate into neurons, oligodendrocytes and astrocytes (Gage et al., 1998; Doetsch et al., 1999; Rietze et al., 2001; Rietze and Reynolds, 2006). Within the SVZ, Epidermal Growth Factor Receptor (EGFR)

expressing stem cells (type B cells) give rise to rapidly dividing transit-amplifying progeny (type C cells) which also express EGFR, which in turn generate immature neuroblasts (type A cells) (Doetsch et al., 2002). B and C cell populations are responsible of making neurospheres in cultures (Doetsch et al., 2002; Pastrana et al., 2009); and will be referred here as neural precursor cells (NPCs). Regulation of NPCs cell fate involves a complex and coordinated network of extrinsic and intrinsic signaling pathways. Among the molecules that regulate NPCs pool proliferation and maintenance, Sonic Hedgehog (Shh) and Epidermal Growth Factor (EGF) have been addressed as important mitogenic signals (Reynolds et al., 1992; Doetsch et al., 2002; Palma et al., 2005; Reinchisi et al., 2013; Alvarez-Buylla and Ihrie, 2014). In NPCs, EGFR signaling functions as a control system of cell proliferation, not only by responding to its own ligands but also by serving as a nodal element for a variety of other stimuli acting through downstream effectors of EGFR signaling pathways (Sibilia et al., 2007; Hu et al., 2010; Reinchisi et al., 2013). Cell-intrinsic components committed in cell fate decisions involve transcription factors, some of which play a role in self-renewal, such as SOX2, BMI1 and TLX (Graham et al., 2003; Ferri et al., 2004; Shi et al., 2004; Molofsky et al., 2005; Qu et al., 2010), while others, like MASH1 (Casarosa et al., 1999; Cau et al., 2002), act in neuronal differentiation. Although much progress has been made in understanding NPCs function in the adult brain, the detailed understanding of events regulating the delicate balance between self-renewal capacity and stem cell fate is still far from being clarified.

Peroxisome proliferator-activated receptors (PPARs), a subgroup of the nuclear receptor superfamily, are ligand-activated transcription factors (Issemann and Green, 1990, 1991). Three isotypes of PPARs have been described in vertebrates, PPARα, PPARβ/δ and PPARγ, which are highly conserved between species (Issemann and Green, 1990, 1991; Dreyer et al., 1992; Schmidt et al., 1992; Kliewer et al., 1994). PPARα is barely expressed in the central nervous system of adult rodents (Braissant et al., 1996). In contrast, a high expression level of PPARβ/δ is observed in the developing neural tube and epidermis Braissant and Wahli, 1998; Keller et al., 2000). In adult rodents, PPARβ/δ is abundantly and ubiquitously expressed, although some tissues such as brain, adipose tissue, and skin have higher mRNA level (Kliewer et al., 1994; Braissant et al., 1996). PPARβ/δ has important functions in proliferation, differentiation and cellular survival in several cell types. In skin, induction of keratinocyte proliferation by several stimuli, such as tetradecanoylphorbol acetate, is associated with up-regulation of PPARβ/δ level in the epidermis (Michalik et al., 2005). Moreover, PPARβ/δ-mutants decrease the number of proliferative keratinocytes and display increased apoptosis in early hair follicles (Di-Poi et al., 2005). PPARβ/δ-null mice are also smaller than wild type littermates and their brains present alterations in the myelinization of the corpus callosum (Peters et al., 2000). Although PPARβ/δ is abundantly expressed in the brain, a possible role of this factor modulating NPCs behavior has not yet been studied.

Regarding PPARγ, expression pattern analysis of this factor shows a transient peak of expression in the central nervous system between E13.5 and E15.5 (Braissant and Wahli, 1998; Keller et al., 2000). Interestingly, NPCs cultures (neurosphere assay) obtained at E13.5 from PPARγ+/− mice shows diminished cellular viability and EGFR level (Wada et al., 2006). On the other hand, NPCs obtained from wild type embryos and treated with rosiglitazone (PPARγ agonist) increase cellular viability and EGFR level, suggesting a role of this isotype in NPCs self-renewal (Wada et al., 2006). *In vivo* treatments with PPARγ agonists, namely pioglitazone and rosiglitazone, also increase both cellular proliferation and differentiation in the SVZ (Morales-Garcia et al., 2011). Recently, Ghoochani et al. evaluated PPARγ level during induced neuronal differentiation of mouse embryonic stem cells (mESC) *in vitro*. They observed an increase in PPARγ level in NPCs, which dropped in mature neurons. PPARγ antagonist decreased the expression of terminal differentiation markers suggesting a role of this transcription factor in the maintenance of the neural stem/precursor phenotype (Ghoochani et al., 2012).

The aim of the present study was to evaluate the potential role of PPARβ/δ and PPARγ in mouse adult NPCs. Our experiments establish the presence of both receptors in precursor cells *in situ* and *in vitro*. We also show that PPARγ regulates proliferation and maintenance of the precursor phenotype and modulates EGFR level in adult NPCs, a result that complements the function of this factor in embryonic mice NPCs (Wada et al., 2006). Finally, we describe for the first time that PPARβ/δ maintains the undifferentiated phenotype of adult NPCs and regulates SOX2 level, a key component of self-renewal. Our results identify PPARγ and PPARβ/δ as regulators of adult neural precursor cell behavior.

Materials and Methods

Reagents and Antibodies

GW0742 and GSK0660 were purchased from Sigma-Aldrich (St. Lois, MO, USA). Rosiglitazone, GW9662 and Bisphenol A diglycidyl ether (BADGE) are from Cayman Chemical Company (Ann Arbor, MI, USA). 5-bromo-2′deoxyuridine is from Sigma-Aldrich (St. Lois, MO, USA). Anti-PPARβ/δ and anti-Myc were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PPARγ, anti-GFAP (Glial fibrillary acidic protein), anti-DCX (Doublecortin) and anti-SOX2 are from Cell Signaling Technology (Beverly, MA, USA). Anti-Nestin and anti-EGFR are from Millipore (Billerica, MA, USA), anti-Galactocerebroside C (GalC) was purchased from Sigma-Aldrich (St. Lois, MO, USA), anti-5-bromo-2′deoxyuridine is from Abcam (Cambridge, MA, USA) and anti-βIII-Tubulin is from Promega (Madison, WI, USA). Restriction enzymes are all from New England Biolabs (Ipswich, MA, USA). GoTaq Flexi DNA Polymerase and RT-PCR reagents were purchased from Promega (Madison, WI, USA) and Invitrogen (Grand Island, NY, USA). siRNA-PPARβ/δ was purchased from Santa Cruz Biotechonology, siRNA-control and siGlo-Green Transfection Indicator were obtained from Thermo Fisher Scientific, Dharmacon Inc (Lafayette, CO, USA).

Generation of Reporter and Expression Vectors

Three direct tandems of the peroxisome proliferator response element (PPRE) sequence from the acyl-CoA Oxidase gene were obtained from an original vector, donated by Dr. R. M. Evans (Howard Hughes Medical Institute, The Salk Institute

for Biological Studies, La Jolla, CA) (Forman et al., 1995). This sequence was digested with BamHI and HindIII restriction enzymes and was inserted into the commercial vector tkLuc (ATCC, Manassas, VA, USA), specifically, in the 5′ region of the thymidine kinase promoter. This new vector was denominated tkPPRELuc. A luciferase gene was deleted from both vectors (tkLuc and tkPPRELuc) by enzymatic digestion with XhoI and SmaI, and replaced with full-length E. coli βGalactosidase gene, which was obtained from commercial vector pCMVβ (Clontech, Mountain View, CA, USA), by digestion with XhoI and SalI. These vectors were named tkβGal and tkPPREβGal, respectively.

For Myc-PPARβ/δ vector construction, mouse cDNA of PPARβ/δ was obtained from vector pCMX-PPARβ/δ (donated by Dr. R. M. Evans), by digestion with BamHI and HindIII restriction enzymes, and inserted in pCDNA3-NLS-Myc vector (donated by Dr. Hugo Olguín, Cellular and Molecular Biology Department, P. Catholic University of Chile; Olguin et al., 2007). These restriction sites delete the NLS sequence. For expression and functional analysis of the Myc-PPARβ/δ vector, HEK293 cells were transiently transfected with Lipofectamine-2000 Reagent (Invitrogen, Grand Island, NY, USA). Expression was analyzed by western blot and cellular localization by immunofluorescence. Activity was evaluated for luciferase reporter assay. We did not observe statistical differences in the activity and localization between both vectors (**Supplementary Figure 1**). All constructs were verified by DNA sequencing.

Isolation and Culture of Adult Mice NPCs

This study was carried out in strict accordance with the recommendations of the Biosecurity Guide of CONICYT (Comisión Nacional de Investigación Científica y Tecnológica). The Bioethical Committees of Pontifical Catholic University of Chile and University of Chile approved all protocols. We used three-month-old C57bl/6 mice. NPCs culture was prepared according to published protocols (Rietze and Reynolds, 2006; Brewer and Torricelli, 2007). Briefly, the lateral walls of the ventricles, i.e., striatal SVZ, of adult mice brains were dissected, incubated with papain and dissociated with a fire-polished glass pipette in the presence of DNaseI and Ovomucoid (Worthington Biochemical Corporation, Lakewood, NJ). NPCs were cultured as neurospheres in neurobasal medium supplemented with B27 minus vitamin A, 200 mM L-Glutamine (Invitrogen), penicillin/streptomycin (Invitrogen) and 10 ng/ml EGF (Invitrogen). After 7 days in culture, primary neurospheres were dissociated, centrifuged at 110× g, re-suspended and seeded at a density of 10.000 cells/cm^2 and cultured in the same conditions, in order to generate secondary neurospheres. For all assays we used NPCs seeded as adherent cells. Secondary neurospheres were dissociated into single cells using trypsin-EDTA (Invitrogen), plated onto poly-l-ornithine/laminin (Sigma-Aldrich and Invitrogen, respectively), and cultured as monolayers in a complete medium (neurobasal medium supplemented with B27 minus vitamin A, penicillin/streptomycin, 200 mM L-Glutamine and 10 ng/ml EGF). For differentiation assays, cells were cultivated in neurobasal medium supplemented with B27 minus vitamin A, 200 mM L-Glutamine, penicillin/streptomycin and 0.1% fetal bovine serum, without EGF, in absence or presence of drugs.

Western Blot

Cells were washed with phosphate buffered saline (PBS), lysed and centrifuged at 14.000 xg for 10 min at 4°C. For EGFR detection, lysis buffer was 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2 and 0,5% Nonidet P-40 (Garcion et al., 2004). For detection of all other proteins, we used RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40 and 1% sodium deoxycholate). Both buffers were supplemented with protease and phosphatase inhibitors mixture (Roche Applied Science, Mannheim, Germany). Proteins were resolved by SDS-PAGE and transferred into PVDF membranes. Blots were subsequently incubated with antibodies as follows: Anti-PPARβ/δ (1:1000), anti-PPARγ (1:1000), anti-EGFR (1:1000), anti-Nestin (1:5000), anti SOX2 (1:2000). For detection, horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used followed by enhanced chemiluminescence detection (Perkin Elmer Inc., Waltham, MA). Optical density was quantified with ImageJ software.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from NPCs using Trizol Reagent (Invitrogen. Grand Island, NY, USA) and treated with DNase I (Promega. Madison, WI, USA). 2 μg of RNA was reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) and Oligo(dT), according to the manufacturer's instructions. The following primers were used for PCR reactions: forward-PPARβ/δ 5′-GCA GCC TCT TCC TCA ATG AC-3′ and reverse-PPARβ/δ 5′-CCG TCT TCT TTA GCC ACT GC-3′, forward-PPARγ 5′-CTG GCC TCC CTG ATG AAT AA-3′and reverse-PPARγ 5′-ACG TGC TCT GTG ACG ATC TG-3′, Forward-GADPH 5′-TGA CCA CAG TCC ATG CCA TC-3 and reverse-GADPH 5′-GAC GGA CAC ATT GGG GGT AG-3′. The following PCR conditions were used: 94°C for 5 min, followed by 35 cycles of 94°C (30 s), 55°C or 60°C (30 s) and 72°C (30 s). GoTaq Flexi DNA Polymerase and 2 mM MgCl2 were used in all reactions (Promega. Madison, WI, USA). Fragments were analyzed with agarose gel electrophoresis (2%) and SYBR-Safe staining (Invitrogen).

BrdU Incorporation Assays

For *in vitro* BrdU-incorporation assays, NPCs were incubated with 10 μM BrdU for 6 h previous to fixation in 4% paraformaldehyde. Samples were incubated 10 min in HCl 2 M, thrice in Sodium Borate Buffer 0,1 M pH 8,5 (10 min each time) and permeabilized/blocked in PBS 0.1% Triton-×100 and 5% normal donkey serum for 30 min. Anti-BrdU antibody (1:500, Abcam. Cambridge, MA, USA) was incubated for 2 h at 37°C. Cy2-conjugated anti-rat IgG was used as a secondary antibody (1:500, Abcam) and incubated for 1 h at room temperature. BrdU-positive cells were evaluated using an Epifluorescent Axioplan Microscope and AxioCam MRm (Zeiss). BrdU positive cells were counted in 15 randomly selected fields from three different coverslips, for each experiment. We used DAPI for total cells count. At least three independent experiments were carried out for each assay.

For *in vivo* BrdU-incorporation assays, mice were intraperitoneally injected with 100 mg BrdU/Kg of animal body weight for 5 days. At day 5, mice were anesthetized and perfused intracardially with PBS, followed by cold 4% paraformaldehyde solution. Brains were collected and post-fixed overnight in 4% paraformaldehyde, followed by 24 h immersion in a 20% sucrose solution. Brains were included in OCT. Coronal sections (30 μm) from SVZ were processed for immunofluorescence. Briefly, slices were incubated 20 min in 0.13 M NaBH4 and washed with PBS, then incubated 10 min in HCl 2 M, 10 min in Sodium Borate Buffer 0,1 M pH 8,5, thrice in TBS and permeabilized/blocked in TBS 0.1% Triton-×100 and 5% normal donkey serum for 30 min. Primary antibodies, anti-BrdU (1:1000) and anti-PPARβ/δ (1:100), were incubated for 48 h at 4°C. Alexa Fluor secondary antibodies (Invitrogen) or Cy2 secondary antibody (Abcam) were incubated for 1 h at room temperature. This protocol was modified from Valero et al. (2005) and Wojtowicz and Kee (2006).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, permeabilized/blocked in PBS-0.1%Triton-X100/5% normal donkey serum for 1 h and incubated in primary antibodies at 4°C overnight. The following primary antibodies were used: anti-PPARβ/δ (1:100), anti-β-Galactosidase (1:1000), anti-Nestin (1:1000), anti-DCX (1/500), anti-SOX2 (1:200) and anti-Myc (1:500). Alexa-Fluor secondary antibodies (1:1000) were incubated 1 h at room temperature. DAPI (Invitrogen) was used for nuclei detection. Samples were examined in an Epifluorescent Axioplan Microscope and AxioCam MRm (Zeiss), or in a Fluoview 1000 Confocal Microscope (Olympus). ImageJ Program was used to analyze and quantify the images.

Nucleofection of Mouse Adult NPCs

Nucleofection of adult NPCs was performed, using the mouse NSC NucleofectorTM Kit and optimized protocols provided by the manufacturer (Amaxa Biosystem, Cologne, Germany). Live and dead cells were counted by trypan blue staining in Neubauer hemocytometer after nucleofection and cells were plated onto poly-l-ornithine/laminin coated coverslips in a medium supplemented with growth factors. 24 h after nucleofection, cells were treated with PPAR ligands, for time and concentrations as indicated in the results section. For PPAR reporter assay, images were acquired with an Epifluorescent Axioplan Microscope and AxioCam MRm (Zeiss). Cells were delimited and βGalactosidase fluorescence was quantified using ImageJ.

Transfection of siRNA

Cells were seeded onto poly-l-ornithine/laminin coated coverslip in a complete medium supplemented with EGF. Cells were co-transfected with siGlo-Green/siRNA-Control or siGlo-Green/siRNA-PPARβ/δ using DharmaFECT 3 transfection reagent (Dharmacon), according to the manufacturer's instructions. Transfected cells were maintained in complete medium with EGF for 48 h, the medium was replaced every day. Silencing of PPARβ/δ was evaluated by western blot and followed by anti-SOX2 immunofluorescence. Images were taken with an Epifluorescent Axioplan Microscope and AxioCam MRm (Zeiss). SOX2 fluorescence was quantified in siGloGreen positive cells. As SOX2 is a nuclear factor, nucleus was delimited in DAPI positive area and fluorescence of SOX2 was quantified in this region using ImageJ program.

Statistical Analysis

Mann Whitney Test and One-Way ANOVA-Bonferroni were used to analyze the statistical differences of means. $p < 0.05$ (95% confidence intervals) was considered significant. Prism Program was used for all analysis. Values are expressed as mean ± standard error of the mean (SEM).

Results

PPARβ/δ and PPARγ are Present in Mouse Adult SVZ NPCs and Activities of these Receptors are Inducible by Exogenous Ligands

In order to evaluate if PPARβ/δ and PPARγ are expressed in proliferating NPCs in the SVZ, adult mice were injected for 5 days intraperitoneally with BrdU followed by immunostaining on coronal brain sections. We observed that both PPARβ/δ and PPARγ are expressed in BrdU positive cells in the SVZ of adult mice, as detected by co-labeling with BrdU immunostaining (**Figure 1A**). Both PPARs have a nuclear expression. As previously described (Braissant et al., 1996), PPARβ/δ expression is also observed in striatum and cortex. In order to establish a possible role of PPARs in these progenitors, we prepared primary cultures of NPCs from the SVZ of adult mice. PPARβ/δ and PPARγ are expressed in NPCs *in vitro* (**Figures 1B,C**) displaying mainly a nuclear localization, as revealed by immunofluorescence (**Figure 1D**).

Next, we sought to find out if PPARs are transcriptionally active *in vitro* and if their activity is inducible by exogenous ligands in these cells. In order to detect PPAR transcriptional activity, we generated βGalactosidase reporter vectors, called tkPPREβGal and tkβGal (see Material and Methods Section), which allowed us to detect PPAR activity *in situ* by immunofluorescence. This method was applied due to low plasmid transfection efficiency of NPCs (15.53 ± 5.43% for Nucleofection and 4.40 ± 1.71 for Lipofectamine-2000 Reagent; Mean ± SD). To be able to identify cells carrying βGalactosidase vectors and subsequently quantify fluorescence intensity of βGalactosidase on these cells, we performed co-transfection with an EGFP vector. Co-transfection efficiency in NPCs in our system was 56.85 ± 14.49% (mean ± SD), as evaluated by co-nucleofection of EGFP and dsRed-N1 vectors. Transfected cells were treated for 24 h with a PPARβ/δ agonist (GW0742) or vehicle (DMSO). In control condition, we observed that NPCs transfected with tkβGal presented a 3.8-fold lower intensity of fluorescence vs. cells transfected with tkPPREβGal. When NPCs transfected with the tkPPREβGal vector were treated with GW0742, we detected a 5-fold increase in the fluorescence intensity with respect to cells treated with DMSO (**Figures 1E,F**). In line with this observation, Rosiglitazone (PPARγ agonist) increased the fluorescence intensity 7 times with respect to the control.

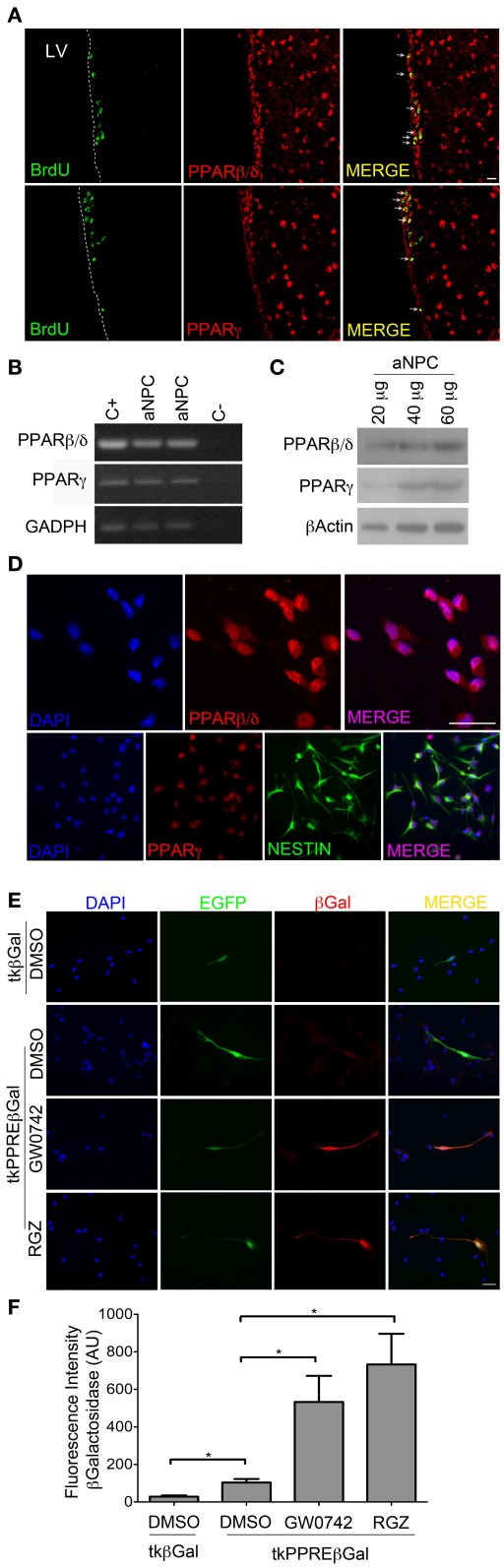


FIGURE 1 | PPARβ/δ and PPARγ are expressed in adult NPCs and their transcriptional activity is inducible by exogenous ligands. (A) Adult mice were injected with 100 mg BrdU/Kg animal body weight for labeling of *(Continued)*

FIGURE 1 | Continued
proliferative cells. Brain coronal sections of 30 µm were immunostained with anti-PPARβ/δ or anti- PPARγ (red) and anti-BrdU (green) antibodies. Samples were analyzed by confocal microscopy. **(B)** RT-PCR for PPARβ/δ and PPARγ: RNA was extracted from SVZ NPCs. Neocortical and adipose tissues from adult mice were used as positive controls (C+), respectively. **(C)** Western blots anti-PPARβ/δ and PPARγ. **(D)** Immunofluorescence anti-PPARβ/δ and PPARγ. **(E,F)** *In situ* transcriptional activity assay: Adult NPCs were transiently co-transfected with pEGFP-C1 reporter vector and tkPPREβGal (PPAR activity transcriptional reporter vector) or tkβGal (empty vector). Cells were treated with GW0742 1 µM, Rosiglitazone 1 µM (RGZ) or DMSO (vehicle) for 24 h. Immunofluorescence anti-βGalactosidase (βGal) was performed. Fluorescence intensity was quantified by ImageJ program. Graphic in **(E)** represent the mean fluorescence intensity ± s.e.m (*$p < 0.05$). AU, Arbitrary Units.

These results reveal endogenous transcriptional activity of PPARs in adult SVZ-NPCs, and also that their activities can be induced by exogenous ligands.

PPARβ/δ and PPARγ Agonists Induce Proliferation of NPCs

Having established that PPARβ/δ is expressed in NPCs and that this activity is inducible by exogenous ligand *in vitro*, we next evaluated if PPARβ/δ plays a role in NPCs proliferation by performing a BrdU incorporation assay. NPCs were seeded as a monolayer in presence of 10 ng/ml of EGF and treated with PPARβ/δ ligands. A BrdU pulse was performed 6 h previous to the experiment end-point. Cell proliferation showed an increase of almost 30% in cultures treated with PPARβ/δ agonist (GW0742) for 24 h and this effect was reverted by incubation with a PPARβ/δ antagonist (GSK0660), suggesting that this increase is indeed PPARβ/δ-dependent (**Figures 2A,B**). We should note that we did not observe any effect of antagonist treatment in basal conditions in a 24 h treatment. Nevertheless, when cells were treated with the same antagonist for a longer time period (48 h), we did observe a small but statistically significant decrease in the percentage of BrdU positive cells (32.12 ± 0.66 v/s 27.28 ± 1.04; $p = 0.003$) (**Figures 2C,E**). We did not see differences in the percentage of activated caspase-3, as evaluated by immunofluorescence (**Figures 2D,F**), indicating that apoptosis does not account for this decrease. Rosiglitazone, a PPARγ agonist, also induced an increase of proliferation in NPCs treated for 24 h, an effect that is reverted by a PPARγ antagonist (BADGE). Unlike the PPARβ/δ antagonist, BADGE is able to decrease basal level of BrdU incorporation at 24 h (**Figures 2G,H**).

PPARβ/δ Antagonist Drives Differentiation of NPCs Even in Presence of EGF

In addition to regulating proliferation, PPARβ/δ could also act by maintaining the NPCs pool. In order to evaluate this hypothesis, NPCs, seeded as single cells over poly-L-ornithine/laminin, were treated with the PPARβ/δ antagonist for 48 h, in presence of 10 ng/ml EGF. A precursor phenotype was evaluated for Nestin, GFAP and SOX2 expression. In control samples, over 95% of the cells were positive for SOX2 and Nestin expression. Nestin positive cells showed a relative homogenous morphology, with two or three thin processes of different lengths (**Figure 3A**). In contrast,

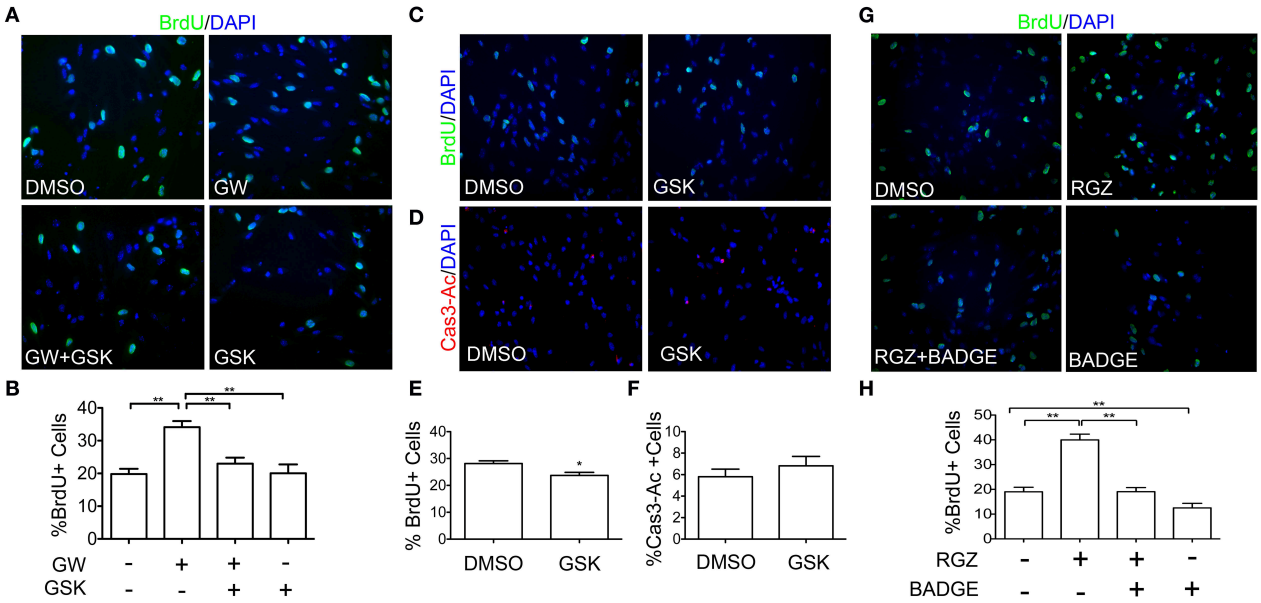


FIGURE 2 | PPARβ/δ and PPARγ ligands regulate cell proliferation. (A) NPCs cultures were treated with a vehicle (DMSO), 1 μM GW0742 (GW; PPARβ/δ agonist), 5 μM GSK0660 (GSK; PPARβ/δ antagonist) or both, for 24 h, in the presence of 10 ng/ml EGF. BrdU was added to the cultures followed by anti-BrdU immunodetection. Graphic in **(B)** represents the mean percentage of BrdU positive cells ± s.e.m. of three independent experiments $N = 3$; $^{**}p < 0.01$. **(C)** Cells were treated with 5 μM GSK0660 or a vehicle (DMSO) for 48 h in the presence of 10 ng/ml EGF. BrdU was added to the cultures 6 h prior to fixation. Immunostaining with anti-BrdU was performed. The Graph in **(E)** represents the mean percentage of BrdU positive cells ± s.e.m. ($^{*}p = 0.012$, Mann Whitney Test). **(D)** Cells were treated with 5 μM GSK0660 or a vehicle (DMSO) for 48 h in the presence of 10 ng/ml EGF. Immunofluorescence anti-Activated Caspase3 (Cas3-Ac) was performed to identify apoptotic cells. The Graph in **(F)** represents the mean percentage of Caspase3-Ac positive cells ± s.e.m. of three independent experiments. No statistical differences were observed ($N = 3$; $p = 0.4338$). **(G)** Adult NPCs were treated with a vehicle (DMSO), 1 μM Rosiglitazone (RGZ; PPARγ agonist), 50 μM of BADGE (PPARγ antagonist) or both, for 24 h, in the presence of 10 ng/ml EGF. Immunodetection with anti-BrdU is shown. The Graph in **(H)** represents the mean percentage of BrdU positive cells ± s.e.m. of three independent experiments $N = 3$; $^{**}p < 0.01$.

a significant decrease in Nestin positive cells in cultures treated with the drug was observed (95.10 ± 0.55% vs. 69.06 ± 2.74%; $p < 0.0001$) (**Figures 3A,B**). In addition, a decrease in fluorescence intensity was also observed as shown in **Figure 3A**. GFAP during late cortical embryogenesis and in adult SVZ is considered a marker of many terminally differentiated astrocytes, but has also been reported as a NSC/Radial glia marker. Therefore, in order to check if GFAP labeling is related to NPCs or to mature astrocyte, we co-labeled the cells with the stem cell marker SOX2. In control conditions, 89.41 ± 5.66% of the GFAP positive cells are SOX2+/GFAP+ vs. 10.60 ± 5.66% SOX−/GFAP+; cultures treated with the antagonist (5 μM GSK0660) changed these percentages to 46.90 ± 7.34% vs. 53.10 ± 7.34%, for SOX2+/GFAP+ and SOX2−/GFAP+, respectively (**Figures 3C,D**). In agreement with these results, we also observed a clear change in the morphology of these cells. In the presence of the PPARβ/δ antagonist, GFAP positive cells presented a greater number of processes and the projections were thicker, a suggestive morphology of mature astrocytes (**Figure 3C**). In addition, treatment with this inhibitor resulted in an increase in the percentage of neurons in the culture. It should be noted that a spontaneous but minimal neuronal differentiation is observed in presence of growth factors when monolayer cultures are maintained for 3 days or more in normal conditions (medium supplemented with 10 ng/ml EGF), as evidenced by a 0.50 ± 0.14% of DCX positive cells. But in cultures treated with the PPARβ/δ antagonist, the percentage increased to 1.12 ± 0.14% ($p < 0.0001$) and some cells even displayed morphologies characteristic of mature neurons in contrast with DCX positive cell morphologies observed in the controls (**Figures 3E,F**). Finally, regarding oligodendrocytes, neither in the controls nor after treatments, we were able to observe changes, as evaluated by an immunofluorescence anti-GalC (data not shown). From these results, it clearly emerges that PPARβ/δ participates in the maintenance of the precursor phenotype.

PPARβ/δ Regulates SOX2 Level in NPCs *In Vitro*

SOX2 is an important regulator of NPCs self-renewal and since we observed a decrease in its expression level after treatment with the PPARβ/δ antagonist by immunostaining (**Figures 3C,D**), we were interested in evaluating if this receptor regulates SOX2 directly. Indeed, PPARβ/δ antagonist decreased SOX2 expression below the basal level, acting in a dose dependent manner, whereas the agonist increased SOX2 level (**Figure 4A**). In order to characterize the PPARβ/δ overexpression effect, we took two issues into consideration: 1) adult NPCs present low transfection efficiency, complicating the analysis by western blot and 2) PPARβ/δ is already expressed in basal conditions in adult NPCs.

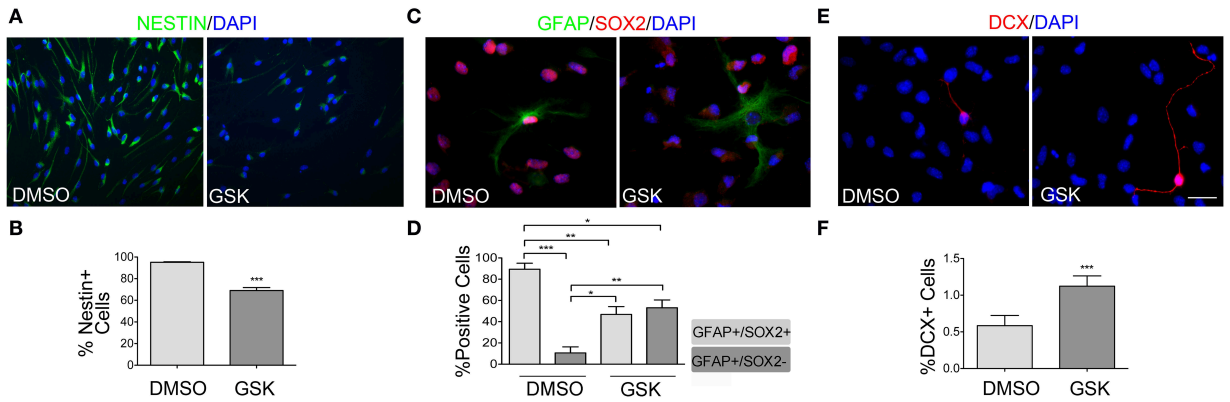


FIGURE 3 | PPARβ/δ antagonist diminished precursor phenotype. Adult NPCs were treated with 5 μM GSK0660 or the vehicle (DMSO) for 48 h in the presence of 10 ng/ml EGF. Different markers were evaluated by immunofluorescence. Quantification of percentage in different conditions was performed and shown in the graphs. **(A)** Immunofluorescence anti-Nestin. **(C)** Immunofluorescence anti-GFAP/SOX2. In GSK0660 treated cells we detected a change in the percentage of GFAP+/SOX+ and GFAP+/SOX2− cells, as observed in the graph. Control shows a representative image of GFAP+/SOX2+ cells, the predominant population. GSK0660 shows a representative image of GFAP+/SOX2− cells, whose population increased in this condition. In both conditions, light gray bars represent GFAP+/SOX2+ cells and dark gray bars represent GFAP+/SOX2− cells. Percentages expressed are in respect to the total GFAP+ cells. **(E)** Immunofluorescence anti-DCX. Graph in **(B,D,F)**, represent the mean percentage of positive cells ± s.e.m. of three independent experiments. Bars correspond to 20 μm. Mann Whitney Test was applied in **(B,F)** and One-way ANOVA-Bonferroni Test was applied in **(D)**. (*$p < 0.05$, **$p = 0.01$ to 0.001, ***$p < 0.001$).

To overcome these difficulties, we generated a PPARβ/δ expression vector with an additional tag (Myc-PPARβ/δ), allowing us thereby to identify individual transfected cells that were overexpressing PPARβ/δ. The expression and functionality of the Myc-PPARβ/δ vector were evaluated first in a Hek293 cell line. We did not observe any change in cell behavior and activity of Myc-PPARβ/δ in comparison to PPARβ/δ without the tag (**Supplementary Figure 1**).

NPCs were transfected with Myc-PPARβ/δ and ectopic expression of PPARβ/δ in the cells was evaluated by anti-Myc immunofluorescence. Transfected cells were treated with PPARβ/δ agonist (GW0742) or vehicle (DMSO) for 24 h, followed by an anti-SOX2/anti-Myc co-immunofluorescence. The fluorescence intensity of SOX2 was evaluated in both Myc-PPARβ/δ positive and negative cells, in presence or absence of the agonist. In control conditions (DMSO) we were able to observe a 1.78-fold increase in the SOX2 fluorescence intensity in Myc-PPARβ/δ positive cells with respect to Myc-PPARβ/δ negative cells. When cells were cultured in the presence of PPARβ/δ agonist (GW0742), the fluorescence intensity increased in both conditions, Myc-PPARβ/δ negative and positive cells, but the increment in Myc-PPARβ/δ positive cells were much greater (1.76-fold vs. 2.44-fold, in Myc-PPARβ/δ negative and positive cells, respectively, both treated with GW0742 compared to Myc-PPARβ/δ negative cells treated with the vehicle). We did not observe statistical differences between Myc-PPARβ/δ positive cells treated with GW0742 or vehicle (**Figure 4B**).

PPARβ/δ is Necessary for SOX2 Expression in Mouse Adult NPCs from the SVZ

In order to establish if PPARβ/δ is necessary for SOX2 maintenance, we subsequently did a knock down of this factor in NPCs by using siRNA, co-transfecting either siGlo-Green with siRNA-PPARβ/δ or the siRNA-control. Transfection efficiency was higher than 80% (Percentage of siGLO-Green positive cells). Interestingly, we observed an average decrease of 0.6-fold in the fluorescence intensity of SOX2 in cells transfected with siRNA-PPARβ/δ with respect to the control ($p < 0.0001$; **Figures 4C,D**). Next, NPCs were transfected with siRNA-PPARβ/δ or siRNA-control and treated with 1 μM GW0742 for 24 h in the presence of 10 ng/ml of EGF, followed by anti-PPARβ/δ and anti-SOX2 Western blots. An expected increase of SOX2 level was observed in control cells treated with the agonist, as already observed (**Figures 4A,E**). Moreover, PPARβ/δ knockdown decreased SOX2 level while PPARβ/δ agonist in this condition was not able to increase the level of SOX2 (**Figure 4E**).

Thus, our results show that PPARβ/δ contributes toward the maintenance of the precursor phenotype and regulates SOX2 level in adult NPCs. Since PPARβ/δ is a transcription factor that regulates the expression of its target genes by recognizing specific sequence denominated PPRE (PPAR response element) in the regulatory region, we analyzed the mouse *Sox2* gene (Gene ID 20674) for the presence of possible response elements in the promoter region. Note that the Mat Inspector Program revealed two putative PPREs in the positions −203 to −225 (GTCTTGGTGCTGTTTACCCACTT) and −244 to −266 (CCGTTTTCAGCAACAGGTCACGG), in respect of the transcription site, suggesting a direct transcriptional regulation of *Sox2* by PPARβ/δ. Further *in vitro* studies will be required to confirm this *in silico* result.

PPARγ and PPARβ/δ Regulate EGFR level in NPCs

As already shown, PPARβ/δ regulates SOX2 level in NPCs *in vitro*. SOX2 is an important transcription factor necessary in

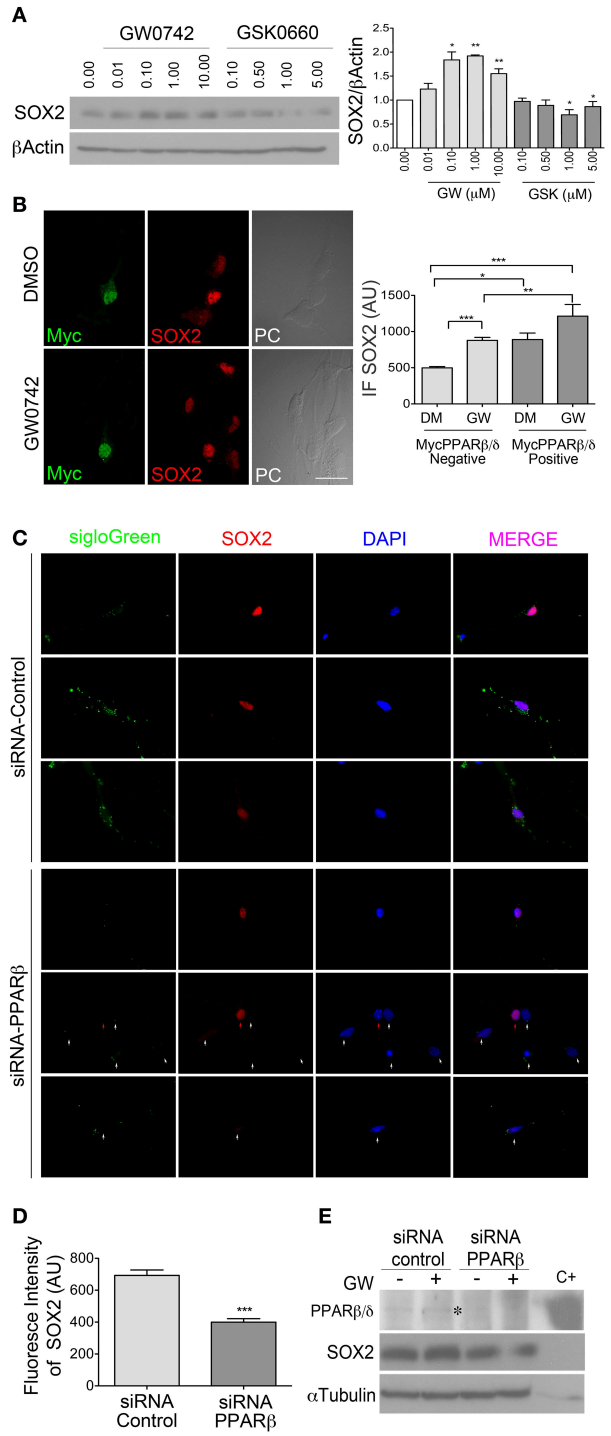


FIGURE 4 | PPARβ/δ regulate SOX2 level in adult NPCs. (A) NPCs were treated for 24 h at different concentrations of GW0742 or GSK0660 as indicated, in the presence of 10 ng/ml EGF. Total proteins were analyzed by western blot for anti-SOX2. Graph represents the mean ± s.e.m. of three independent experiments. One-Way ANOVA-Bonferroni Test was applied. **(B)** Myc-PPARβ/δ expression vector was transiently transfected. Cells were treated with 1 μM GW0742 (agonist) or a vehicle (DMSO) for 24 h.Immunofluorescence anti-SOX2/anti-Myc was realized.

(Continued)

FIGURE 4 | Continued

SOX2 fluorescence intensity was quantified in both Myc positive and negative cells. Graph represents the mean ± s.e.m. of three independent experiments.One-Way ANOVA-Bonferroni Test was applied. **(C)** Adult NPCs were transiently co-transfected with siGLO-Green and siRNA-PPARβ/δ or siRNA-control, in the presence of 10 ng/ml of EGF. SOX2 fluorescence intensity was quantified in siGLO-Green positive cells. Representative images of cells observed and quantified are shown. White arrows shows siGLO-Green positive cell. Red arrow shows a siGLO-Green negative cell. Graph in **(D)** represents the mean ± s.e.m. **(E)** NPCs were transfected with siRNA-PPARβ/δ or siRNA-control, under the same condition as in **(C)**. Cells transfected were treated with 1 μM GW0742 (GW) for 24 h in the presence of 10 ng/ml of EGF. Western blots anti-PPARβ/δ and anti-SOX2, Total protein extract from HEK293 cells transiently transfected with pCMX- PPARβ/δ was used as a positive control (C+). (*$p < 0.05$, **$p = 0.01$–0.001, ***$p < 0.001$, Mann Whitney Test). DM, DMSO; GW, GW0742; AU, Arbitrary Units.

the maintenance of undifferentiated phenotypes of these cells. Interestingly, it generates a positive feedback loop with EGFR, also involved in self-renewal of NPCs (Hu et al., 2010). Additionally, Wada et al. reported recently that PPARγ regulates EGFR level in embryonic mouse NPCs (E13.5–E14.5), increasing thereby their cellular viability. We therefore decided to evaluate if PPARγ has the same effect on adult NPCs as on embryonic ones and, in addition, if PPARβ/δ is also able to regulate EGFR level.

We observed that the PPARγ agonist (Rosiglitazone) increased EGFR level at 12 h and this effect lasted up to 24 h (**Figures 5A,B**). This effect is reverted by the antagonist (BADGE), suggesting PPARγ dependence. Moreover, an increase in the EGFR level at short times (15–30 min) in respect to time zero was also observed in both control and treated cells, consistent with data reported by Hu et al. and probably due to the addition of EGF to the culture (Hu et al., 2010). Nevertheless, we did not observe statistically significant differences between treatments at these short times (data not shown). Finally, when PPARγ was overexpressed in adult NPCs, concomitant to the higher proliferation rate of NPCs cultures, we found increased EGFR level, even in basal conditions (**Figure 5C**). Remarkably, 24 h treatment with the agonist also increased PPARβ/δ protein level in a concentration-dependent manner indicating a possible collaborative function between PPARs (**Figure 5D**).

With regard to the PPARβ/δ function over EGFR level, NPCs were treated for 24 h with PPARβ/δ agonist (GW0742) and EGFR protein was evaluated by western blot. We did not observe any difference between treatment and control (**Figure 5E**). However, when cells were treated with the antagonist (GSK0660) for 48 h at a concentration of 10 μM, protein level of EGFR was clearly diminished (**Figure 5F**).

We conclude that PPARγ regulates EGFR level in adult NPCs. Additionally, that the inhibition of PPARβ/δ alone is able to modulate EGFR level, with a different kinetic action regarding PPARγ, suggesting an indirect effect of PPARβ/δ over EGFR. Finally, PPARγ regulates PPARβ/δ level suggesting a cooperative effect in the maintenance of NPCs undifferentiated phenotype.

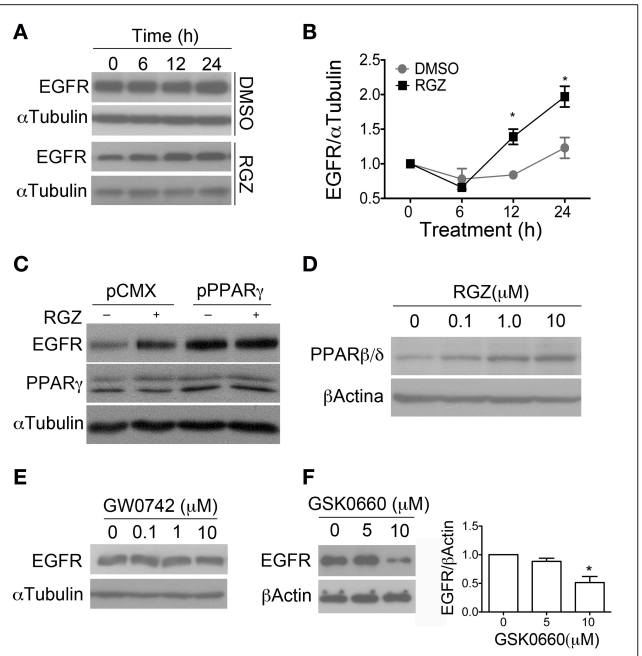


FIGURE 5 | PPARγ regulate EGFR level in adult NPCs. (A) NPCs were treated with 1 μM rosiglitazone (RGZ) or a vehicle (DMSO), for the times indicated. Proteins were analyzed by western blot anti-EGFR. αTubulin was used as a loading control. In **(B)** graph represents the mean ± s.e.m. of three independent experiments (One-way ANOVA-Bonferroni test; * $p < 0.05$). **(C)** NPCs were transfected with PPARγ1 expression vector (pPPARγ) or empty vector (pCMX) and treated with 1 μM Rosiglitazone (RGZ) or a vehicle (DMSO) for 24 h. Total proteins were analyzed by western blot for anti-EGFR. Western blot with anti-PPARγ is also shown. Only PPARγ overexpression increases EGFR level up to 3-fold. **(D)** Adult NPCs were treated for 24 h with RGZ at the concentrations indicated in the figure. PPARβ/δ level were analyzed by western blot. Adult NPCs were treated with GW0742 **(E)** and GSK0660 **(F)** at different concentrations. Level of EGFR was evaluated by western blot.

Discussion

In this study we reveal a new role of PPARs acting in maintenance of adult NPCs undifferentiated phenotype *in vitro*. We demonstrate the presence of PPARβ/δ and PPARγ in proliferative cells in the SVZ *in situ* and describe their mainly nuclear expression pattern in NPCs cultures. PPARs appear to have endogenous activity in these cells and their activity can be induced by exogenous ligands, as shown by pharmacological activation, thereby allowing a detailed functional analysis of PPARs in NPCs. Our results, therefore, imply an endogenous activity of PPARs regulating NPCs behavior.

PPARβ/δ is a transcription factor expressed, with high level in selected tissues such as skin, fat and brain. Over 90% of PPARβ/δ-null mice are not viable and die at early embryonic stages, mainly due to placental disorders (Barak et al., 2002). The surviving mutant mice have disorders in myelination of the central nervous system and decreased adipose mass. In adipose tissue and skin, PPARβ/δ has a role in apoptosis, proliferation and differentiation (Peters et al., 2000; Di-Poi et al., 2005). Here we show that PPARβ/δ contributes to maintain undifferentiated phenotype and regulates proliferation of adult NPCs *in vitro*. These two important functions are involved in self-renewal of NPCs, an essential property *in vivo* to maintain the NPCs pool in specific regions of the adult brain.

One of the most important factors involved in NPCs self-renewal is SOX2. Adult Sox2-KO mice present reduced proliferation and neurogenesis in SVZ and Hippocampus (Ferri et al., 2004). Interestingly, we show that PPARβ/δ is a regulator of SOX2 levels. Moreover, PPARβ/δ is necessary for SOX2 expression. Consistent with this notion, we found two putative PPRE elements in the promoter region of this factor, suggesting a direct role in its transcriptional control. SOX2 is not only necessary for maintenance of the embryonic and adult neural stem/precursor phenotype, it also has been demonstrated that this factor is essential for inner cell mass embryonic stem cells, as its ablation causes early embryonic lethality (Avilion et al., 2003). Moreover, SOX2 is one of four-transcription factors necessary for induction of fibroblast to pluripotent cells (Takahashi et al., 2006; Takahashi and Yamanaka, 2006). Thus, understanding the mechanism and identifying factors involved in the regulation of SOX2 expression is a relevant topic.

We described that inhibition of PPARβ/δ decreases the level of SOX2, but also changes the phenotype of these cells, inducing differentiation. Adult NPCs culture display high percentage of SOX2 and Nestin positive cells (∼95%). The evaluation of the PPARβ/δ activation effect on population phenotypes is technically difficult, but it would be interesting to address if ectopic expression of SOX2 and even PPARβ/δ activation on specific-phenotype committed precursor (to neuron, astrocyte or oligodendrocyte; SOX2−/Nestin−) can return them into an earlier precursor stage (SOX+/Nestin+).

Interestingly, SOX2 presents a positive feedback with EGFR in embryonic NPCs involved in self-renewal (Hu et al., 2010). EGFR is a membrane receptor of several extracellular ligands, including EGF, but also can be transactivated by external inputs via interaction with other pathways, such as SHH, acting as a nodal mediator in the control of cellular behavior (Reinchisi et al., 2013). *In vivo*, EGFR is expressed in the SVZ, mainly in activated neural stem cells (B-cells) and in the transit-amplifying C-cell population (Doetsch et al., 2002; Pastrana et al., 2009). This population, in response to EGF, increases its proliferation and maintains the multipotent characteristic (Doetsch et al., 2002). *In vitro*, maintenance of adult mice NPCs cultures requires EGF; deprivation of this ligand in the medium induces spontaneously differentiation to neurons, astrocyte and oligodendrocytes. Interestingly, PPARβ/δ is a mediator of the EGF-EGFR pathway in skin, one of the organs where this transcription factors is highly expressed. In HaCat keratinocytes, EGF induces up-regulation of PPARβ/δ expression, increased DNA binding and promotes its transcriptional activity. Conversely, PPARβ/δ knockdown leads to decreased EGF-mediated cell proliferation (Liang et al., 2008b). Cells pretreated with a PPARβ/δ agonist, show a smaller percentage of apoptosis induced by TNF-α and an increased protective effect of EGF (Liang et al., 2008a), suggesting a PPARβ/δ-dependent mechanism in EGF-stimulated cell proliferation and survival. Furthermore, PPARβ/δ is involved in cell proliferation of other cells types such as endothelial cells (Piqueras et al., 2007), pre-adipocytes (Hansen et al., 2001),

human breast, prostate, gastric and hepatocellular carcinoma cells (Glinghammar et al., 2003; Stephen et al., 2004; Nagy et al., 2011). Our results show that a PPARβ/δ antagonist is able to decrease level of EGFR and proliferation of cells after 48 h of treatment, but no increase was observed when cells were treated with the agonist. Although at this moment we have no explanation for this last result, we can not rule out that the effect in this context requires a longer period of analysis. Additional approaches addressing the relationship of PPARβ/δ and EGFR signaling pathways will have to be undertaken in order to obtain a better understanding of how and when these pathways interact to control NPCs behavior.

Our data suggest that regulation of EGFR level and proliferation of cells by PPARβ/δ antagonists can be consequences associated with changes in the phenotype of the NPCs, as we show that antagonists induce differentiation, even in presence of EGF. In addition, the absence of changes in EGFR level after activation of PPARβ/δ seem to be contradictory with the evidence of the positive feedback between EGFR and SOX2 in neural precursor cells, but this could just be due to the timing evaluated in this study. Also, it might be possible that PPARβ/δ acts as a mediator of the EGF-EGFR pathway modulating self-renewal function through the regulation of SOX2, however this hypothesis requires more experimental evidence to be confirmed.

On the other hand, we also show that PPARγ regulates EGFR level as well as proliferation of adult NPCs. Rosiglitazone, a PPARγ agonist, increases the level of EGFR at 12 h post-treatment, which is maintained at least until 24 h. Overexpression of this transcription factor is sufficient to induce an increase of EGFR level. Concomitant with this effect, ligands of PPARγ regulate proliferation of adult NPCs, indicating that this PPAR isoform is involved in the control of proliferation and maintenance of NPCs, probably acting through the transcriptional control of EGFR. We observed that cultures deprived of EGF in order to induce differentiation of NPCs and treated with Rosiglitazone, present a higher number of Nestin positive cells at 48 h of treatment. Even if the effect of Rosiglitazone is not sufficient to maintain the cells in undifferentiated phenotype after 72 h of EGF deprivation (differentiation assays; data not shown), we observe a slower rate of differentiation. Wada et al. reported an increase of cell viability in embryonic NPCs treated with rosiglitazone (PPARγ agonist) and interestingly, stated that NPCs obtained from embryonic PPARγ-KO mice (E13.5) have decreased size and numbers. Moreover, this group reported regulation of EGFR by PPARγ, and suggested that regulation of proliferation by PPARγ could be mediated by regulation of EGFR (Wada et al., 2006). Consistent with these data, Burrows et al. showed that level and density of EGFR are important for cell fate and proliferation of NPCs, and that cell response depends, at least partially, on ligand concentration (Burrows et al., 1997). However, our observations are in disagreement with results from *in vitro* experiments published by Morales-Garcia et al. (Morales-Garcia et al., 2011). They proposed that pioglitazone and rosiglitazone, PPARγ agonists, induce differentiation of NPCs *in vitro*. One explanation for these opposite findings could be the different model systems used (rat vs. mouse), but also, and more importantly, the concentration of the drugs used in the study. Morales-Garcia used 30 μM of rosiglitazone in a pre-treatment of 7 days, previous to the differentiation assay (Morales-Garcia et al., 2011). Rosiglitazone binds to the PPARγ ligand-binding domain with a Kd of 43 nM and activated a luciferase reporter vector with an approximately EC50 of 0.1 uM (Lehmann et al., 1995) and pioglitazone and rosiglitazone at a concentration 10 μM, induce activation of PPARα, in COS-1 cells (Sakamoto et al., 2000). Moreover, Wada et al. showed that in embryonic neurospheres, rosiglitazone has a biphasic effect. At low concentrations, this agonist induces proliferation and maintenance of a stem/precursor state, but at a concentration 30 μM or more, rosiglitazone induces apoptosis (Wada et al., 2006). However, Morales et al., showed an interesting effect *in vivo*, with an increase of proliferation in the SVZ that consequently leads to increase in neurogenesis, revealing that the size of the NSC population in the SVZ is important in the regulation of neurogenesis. Thus, the *in vivo* pharmacological activation of PPARγ, by thiazolidinedione, could be an early event in the increase of the NPCs population with the consequent increase in neurogenesis (and as such not necessary a direct induction of differentiation). Accordingly, when Ghoochani et al. evaluated the expression of PPARγ in the process of differentiation from embryonic stem cells to neurons, they observed that the level of PPARγ were increased in the formation of NPCs but then decreased in terminal neuronal differentiation (Ghoochani et al., 2012). Added to that, pharmacological treatment with ligands at different times of the differentiation process revealed that inactivation of PPARγ at early stage in mESC decreased the formation of NPCs, and later of neurons and astrocytes (Ghoochani et al., 2012).

Our results demonstrate that PPARs seem to be active endogenously, at least *in vitro*. Thus, an interesting question is to answer which endogenous ligands can be mediating the effect of PPARs in neural precursor cells. PPARs are described to be nuclear transcription factors and sensors of the lipid metabolism. Known endogenous PPAR ligands in the brain include omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA). These ligands have been described to have neuroprotective effects (reviewed in Michael-Titus and Priestley, 2014) and to be involved in proliferation and differentiation of NPCs (Dyall et al., 2010; Sakayori et al., 2011). Another interesting family of endogenous ligands of PPARs is the endocannabinoids (Reviewed in Pistis and Melis, 2010). Activation of the endocannabinoid system in NPCs by a synthetic ligand increases cellular proliferation as well as both number and size of neurospheres (Aguado et al., 2005). CB1 receptor, a key component of this system, was described to be required for neurospheres formation (Aguado et al., 2005) and even more, for neurogenesis *in vivo* (Jin et al., 2004). The roles described for all these ligands in neural precursor cells, positions them as the main candidates to mediate PPAR functions in NPCs, but further investigation will be required to identify specific ligands associated to PPAR function in NPCs.

Finally, we observed that PPARγ agonist is also able to increase PPARβ/δ level, suggesting a possible cooperative effect of these two isoforms of PPAR, which suggest a possible mechanism in NPCs where PPARγ and PPARβ/δ could be mediators between EGFR and SOX2 positive feedbacks, contributing thereby to maintain an undifferentiated phenotype in adult SVZ-NPCs.

Author Contribution

CB, MB designed the research. CB, MB and VP analyzed data. CB and CA performed the research. CB and VP wrote the paper.

Acknowledgments

We thank Dr. Francisca Bronfman for her support, comments and permanent encouragement. We thank Dr. María Estela Andrés for plasmid donation and for her constructive suggestions during the development of this work and also Dr. Hugo Olguín for plasmid donation. We are grateful to Dr. Ariel Ruiz i Altaba, Sonakshi Mishra, Débora Vega and Dr. Monika Kuciak for every suggestion on the manuscript and permanent encouragement. Miguel Bronfman's work was supported by FONDECYT-1095177; Veronica Palma's work is supported by FONDAP-15090007. Carolina Bernal was beneficiary of a CONICYT Fellowship for Ph. D. studies.

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2015.00078/abstract

Supplementary Figure 1 | pMyc-PPARβ. (A) Double anti-Myc/anti-PPARβ immunofluorescence. Representative confocal microscopy images are shown. **(B)** Myc-PPARβ expression was evaluated by western blot with anti-PPARβ and anti-Myc antibodies. pPPARβ was used as a control of wild type protein overexpression. pCDNA3-Myc and pCMX are empty vectors for pMyc-PPARβ and pPPARβ, respectively. **(C–E)** Functional analysis of pMyc-PPARβ by luciferase reporter assay. Cells were transfected with the vectors as indicated plus PPRE luciferase reporter vector and pCMXβ as a control. Luciferase activity was measured and shown in respect to control vector (pCMVβ). **(C)** Cells were treated with PPARβ/d agonist (GW, 1 μM) or vehicle (DMSO) for 24 h. **(D)** Different concentrations of GW0742 were used to evaluate activity and to compare both vectors. **(E)** Cells were pre-treated with antagonist (GSK) for 1 h and then co-treated with PPARβ/d agonist (GW) and antagonist (GSK) for additional 24 h (*$p < 0.05$, **$p = 0.01$–0.001, ***$p < 0.001$; One-Way ANOVA-Bonferroni test). GW, GW0742; GSK, GSK0660; DM, DMSO.

References

Aguado, T., Monory, K., Palazuelos, J., Stella, N., Cravatt, B., Lutz, B., et al. (2005). The endocannabinoid system drives neural progenitor proliferation. *FASEB J.* 19, 1704–1706. doi: 10.1096/fj.05-3995fje

Alvarez-Buylla, A., and Ihrie, R. A. (2014). Sonic hedgehog signaling in the postnatal brain. *Semin. Cell Dev. Biol.* 33, 105–111. doi: 10.1016/j.semcdb.2014.05.008

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 17, 126–140. doi: 10.1101/gad.224503

Barak, Y., Liao, D., He, W., Ong, E. S., Nelson, M. C., Olefsky, J. M., et al. (2002). Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. *Proc. Natl. Acad. Sci. U.S.A.* 99, 303–308. doi: 10.1073/pnas.012610299

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* 137, 354–366.

Braissant, O., and Wahli, W. (1998). Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. *Endocrinology* 139, 2748–2754.

Brewer, G. J., and Torricelli, J. R. (2007). Isolation and culture of adult neurons and neurospheres. *Nat. Protoc.* 2, 1490–1498. doi: 10.1038/nprot.2007.207

Burrows, R. C., Wancio, D., Levitt, P., and Lillien, L. (1997). Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron* 19, 251–267. doi: 10.1016/S0896-6273(00)80937-X

Casarosa, S., Fode, C., Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* 126, 525–534.

Cau, E., Casarosa, S., and Guillemot, F. (2002). Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development* 129, 1871–1880.

Di-Poi, N., Ng, C. Y., Tan, N. S., Yang, Z., Hemmings, B. A., Desvergne, B., et al. (2005). Epithelium-mesenchyme interactions control the activity of peroxisome proliferator-activated receptor beta/delta during hair follicle development. *Mol. Cell. Biol.* 25, 1696–1712. doi: 10.1128/MCB.25.5.1696-1712.2005

Doetsch, F., Caille, I., Lim, D. A., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97, 703–716. doi: 10.1016/S0092-8674(00)80783-7

Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J. M., and Alvarez-Buylla, A. (2002). EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron* 36, 1021–1034. doi: 10.1016/S0896-6273(02)01133-9

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., and Wahli, W. (1992). Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* 68, 879–887. doi: 10.1016/0092-8674(92)90031-7

Dyall, S. C., Michael, G. J., and Michael-Titus, A. T. (2010). Omega-3 fatty acids reverse age-related decreases in nuclear receptors and increase neurogenesis in old rats. *J. Neurosci. Res.* 88, 2091–2102. doi: 10.1002/jnr.22390

Ferri, A. L., Cavallaro, M., Braida, D., Di Cristofano, A., Canta, A., Vezzani, A., et al. (2004). Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development* 131, 3805–3819. doi: 10.1242/dev.01204

Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995). 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 83, 803–812. doi: 10.1016/0092-8674(95)90193-0

Gage, F. H., Kempermann, G., Palmer, T. D., Peterson, D. A., and Ray, J. (1998). Multipotent progenitor cells in the adult dentate gyrus. *J. Neurobiol.* 36, 249–266.

Garcion, E., Halilagic, A., Faissner, A., and ffrench-Constant, C. (2004). Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. *Development* 131, 3423–3432. doi: 10.1242/dev.01202

Ghoochani, A., Shabani, K., Peymani, M., Ghaedi, K., Karamali, F., Karbalaei, K., et al. (2012). The influence of peroxisome proliferator-activated receptor gamma(1) during differentiation of mouse embryonic stem cells to neural cells. *Differentiation* 83, 60–67. doi: 10.1016/j.diff.2011.08.009

Glinghammar, B., Skogsberg, J., Hamsten, A., and Ehrenborg, E. (2003). PPARdelta activation induces COX-2 gene expression and cell proliferation in human hepatocellular carcinoma cells. *Biochem. Biophys. Res. Commun.* 308, 361–368. doi: 10.1016/S0006-291X(03)01384-6

Graham, V., Khudyakov, J., Ellis, P., and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* 39, 749–765. doi: 10.1016/S0896-6273(03)00497-5

Hansen, J. B., Zhang, H., Rasmussen, T. H., Petersen, R. K., Flindt, E. N., and Kristiansen, K. (2001). Peroxisome proliferator-activated receptor delta (PPARdelta)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling. *J. Biol. Chem.* 276, 3175–3182. doi: 10.1074/jbc.M005567200

Hu, Q., Zhang, L., Wen, J., Wang, S., Li, M., Feng, R., et al. (2010). The EGF receptor-sox2-EGF receptor feedback loop positively regulates the self-renewal of neural precursor cells. *Stem Cells* 28, 279–286. doi: 10.1002/stem.246

Issemann, I., and Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347, 645–650. doi: 10.1038/347645a0

Issemann, I., and Green, S. (1991). Cloning of novel members of the steroid hormone receptor superfamily. *J. Steroid Biochem. Mol. Biol.* 40, 263–269. doi: 10.1016/0960-0760(91)90191-7

Jin, K., Xie, L., Kim, S. H., Parmentier-Batteur, S., Sun, Y., Mao, X. O., et al. (2004). Defective adult neurogenesis in CB1 cannabinoid receptor knockout mice. *Mol. Pharmacol.* 66, 204–208. doi: 10.1124/mol.66.2.204

Keller, J. M., Collet, P., Bianchi, A., Huin, C., Bouillaud-Kremarik, P., Becuwe, P., et al. (2000). Implications of peroxisome proliferator-activated receptors (PPARS) in development, cell life status and disease. *Int. J. Dev. Biol.* 44, 429–442. Available online at: http://www.ijdb.ehu.es/web/paper.php?doi=11032176

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., et al. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. USA.* 91, 7355–7359. doi: 10.1073/pnas.91.15.7355

Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.* 270, 12953–12956. doi: 10.1074/jbc.270.22.12953

Liang, P., Jiang, B., Huang, X., Xiao, W., Zhang, P., Yang, X., et al. (2008a). Anti-apoptotic role of EGF in HaCaT keratinocytes via a PPARbeta-dependent mechanism. *Wound Repair Regen.* 16, 691–698. doi: 10.1111/j.1524-475X.2008.00419.x

Liang, P., Jiang, B., Yang, X., Xiao, X., Huang, X., Long, J., et al. (2008b). The role of peroxisome proliferator-activated receptor-beta/delta in epidermal growth factor-induced HaCaT cell proliferation. *Exp. Cell Res.* 314, 3142–3151. doi: 10.1016/j.yexcr.2008.06.013

Michael-Titus, A. T., and Priestley, J. V. (2014). Omega-3 fatty acids and traumatic neurological injury: from neuroprotection to neuroplasticity? *Trends Neurosci.* 37, 30–38. doi: 10.1016/j.tins.2013.10.005

Michalik, L., Feige, J. N., Gelman, L., Pedrazzini, T., Keller, H., Desvergne, B., et al. (2005). Selective expression of a dominant-negative form of peroxisome proliferator-activated receptor in keratinocytes leads to impaired epidermal healing. *Mol. Endocrinol.* 19, 2335–2348. doi: 10.1210/me.2005-0068

Molofsky, A. V., He, S., Bydon, M., Morrison, S. J., and Pardal, R. (2005). Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes Dev.* 19, 1432–1437. doi: 10.1101/gad.1299505

Morales-Garcia, J. A., Luna-Medina, R., Alfaro-Cervello, C., Cortes-Canteli, M., Santos, A., Garcia-Verdugo, J. M., et al. (2011). Peroxisome proliferator-activated receptor gamma ligands regulate neural stem cell proliferation and differentiation *in vitro* and *in vivo*. *Glia* 59, 293–307. doi: 10.1002/glia.21101

Nagy, T. A., Wroblewski, L. E., Wang, D., Piazuelo, M. B., Delgado, A., Romero-Gallo, J., et al. (2011). beta-Catenin and p120 mediate PPARdelta-dependent proliferation induced by *Helicobacter pylori* in human and rodent epithelia. *Gastroenterology* 141, 553–564. doi: 10.1053/j.gastro.2011.05.004

Olguin, H. C., Yang, Z., Tapscott, S. J., and Olwin, B. B. (2007). Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. *J. Cell Biol.* 177, 769–779. doi: 10.1083/jcb.200608122

Palma, V., Lim, D. A., Dahmane, N., Sánchez, P., Brionne, T. C., Herzberg, C. D., et al. (2005). Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. *Development* 132, 335–344. doi: 10.1242/dev.01567

Pastrana, E., Cheng, L. C., and Doetsch, F. (2009). Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. *Proc. Natl. Acad. Sci. U.S.A.* 106, 6387–6392. doi: 10.1073/pnas.0810407106

Peters, J. M., Lee, S. S., Li, W., Ward, J. M., Gavrilova, O., Everett, C., et al. (2000). Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). *Mol. Cell. Biol.* 20, 5119–5128. doi: 10.1128/MCB.20.14.5119-5128.2000

Piqueras, L., Reynolds, A. R., Hodivala-Dilke, K. M., Alfranca, A., Redondo, J. M., Hatae, T., et al. (2007). Activation of PPARbeta/delta induces endothelial cell proliferation and angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* 27, 63–69. doi: 10.1161/01.ATV.0000250972.83623.61

Pistis, M., and Melis, M. (2010). From surface to nuclear receptors: the endocannabinoid family extends its assets. *Curr. Med. Chem.* 17, 1450–1467. doi: 10.2174/092986710790980014

Qu, Q., Sun, G., Li, W., Yang, S., Ye, P., Zhao, C., et al. (2010). Orphan nuclear receptor TLX activates Wnt/beta-catenin signalling to stimulate neural stem cell proliferation and self-renewal. *Nat. Cell Biol.* 12, 31–39. doi: 10.1038/ncb2001

Reinchisi, G., Parada, M., Lois, P., Oyanedel, C., Shaughnessy, R., Gónzalez, A., et al. (2013). Sonic Hedgehog modulates EGFR dependent proliferation of neural stem cells during late mouse embryogenesis through EGFR transactivation. *Front. Cell. Neurosci.* 7:166. doi: 10.3389/fncel.2013.00166

Reynolds, B. A., Tetzlaff, W., and Weiss, S. (1992). A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* 12, 4565–4574.

Rietze, R. L., and Reynolds, B. A. (2006). Neural stem cell isolation and characterization. *Meth. Enzymol.* 419, 3–23. doi: 10.1016/S0076-6879(06)19001-1

Rietze, R. L., Valcanis, H., Brooker, G. F., Thomas, T., Voss, A. K., and Bartlett, P. F. (2001). Purification of a pluripotent neural stem cell from the adult mouse brain. *Nature* 412, 736–739. doi: 10.1038/35089085

Sakamoto, J., Kimura, H., Moriyama, S., Odaka, H., Momose, Y., Sugiyama, Y., et al. (2000). Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. *Biochem. Biophys. Res. Commun.* 278, 704–711. doi: 10.1006/bbrc.2000.3868

Sakayori, N., Maekawa, M., Numayama-Tsuruta, K., Katura, T., Moriya, T., and Osumi, N. (2011). Distinctive effects of arachidonic acid and docosahexaenoic acid on neural stem?/progenitor cells. *Genes Cells.* 16, 778–790. doi: 10.1111/j.1365-2443.2011.01527.x

Schmidt, A., Endo, N., Rutledge, S. J., Vogel, R., Shinar, D., and Rodan, G. A. (1992). Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol. Endocrinol.* 6, 1634–1641.

Shi, Y., Chichung Lie, D., Taupin, P., Nakashima, K., Ray, J., Yu, R. T., et al. (2004). Expression and function of orphan nuclear receptor TLX in adult neural stem cells. *Nature* 427, 78–83. doi: 10.1038/nature02211

Sibilia, M., Kroismayr, R., Lichtenberger, B. M., Natarajan, A., Hecking, M., and Holcmann, M. (2007). The epidermal growth factor receptor: from development to tumorigenesis. *Differentiation* 75, 770–787. doi: 10.1111/j.1432-0436.2007.00238.x

Stephen, R. L., Gustafsson, M. C., Jarvis, M., Tatoud, R., Marshall, B. R., Knight, D., et al. (2004). Activation of peroxisome proliferator-activated receptor delta stimulates the proliferation of human breast and prostate cancer cell lines. *Cancer Res.* 64, 3162–3170. doi: 10.1158/0008-5472.CAN-03-2760

Takahashi, K., Ichisaka, T., and Yamanaka, S. (2006). Identification of genes involved in tumor-like properties of embryonic stem cells. *Methods Mol. Biol.* 329, 449–458. doi: 10.1385/1-59745-037-5:449

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Valero, J., Weruaga, E., Murias, A. R., Recio, J. S., and Alonso, J. R. (2005). Proliferation markers in the adult rodent brain: bromodeoxyuridine and proliferating cell nuclear antigen. *Brain Res. Brain Res. Protoc.* 15, 127–134. doi: 10.1016/j.brainresprot.2005.06.001

Wada, K., Nakajima, A., Katayama, K., Kudo, C., Shibuya, A., Kubota, N., et al. (2006). Peroxisome proliferator-activated receptor gamma-mediated regulation of neural stem cell proliferation and differentiation. *J. Biol. Chem.* 281, 12673–12681. doi: 10.1074/jbc.M513786200

Wojtowicz, J. M., and Kee, N. (2006). BrdU assay for neurogenesis in rodents. *Nat. Protoc.* 1, 1399–1405. doi: 10.1038/nprot.2006.224

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
CELLULAR NEUROSCIENCE

ORIGINAL RESEARCH ARTICLE
published: 17 February 2015
doi: 10.3389/fncel.2015.00037

Ontogeny of CX3CR1-EGFP expressing cells unveil microglia as an integral component of the postnatal subventricular zone

Anna L. Xavier[1,2†], Flavia R. S. Lima[3], Maiken Nedergaard[2] and João R. L. Menezes[1]*

[1] *Programa em Ciências Morfológicas, Programa de Diferenciação Celular, Laboratório de Neuroanatomia Celular, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[2] *Center for Translational Neuromedicine, University of Rochester Medical School, Rochester, NY, USA*
[3] *Laboratório de Morfogênese Celular, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Edited by:
Marcos R. Costa, Federal University of Rio Grande do Norte, Brazil

Reviewed by:
Athena Soulika, UC Davis, USA
Felipe Ortega, Universidad Complutense de Madrid, Spain

***Correspondence:**
João R. L. Menezes, Programa em Ciências Morfológicas, Programa de Diferenciação Celular, Laboratório de Neuroanatomia Celular, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, s/n, Rio de Janeiro 21941-902, Brazil
e-mail: jmenezes@icb.ufrj.br

†Present address:
Anna L. Xavier, Neurosciences Paris-Saclay Institute, UMR 9197, CNRS - Université Paris-Sud, Gif-sur-Yvette 91190, France

The full spectrum of cellular interactions within CNS neurogenic niches is still poorly understood. Only recently has the monocyte counterpart of the nervous system, the microglial cells, been described as an integral cellular component of neurogenic niches. The present study sought to characterize the microglia population in the early postnatal subventricular zone (SVZ), the major site of postnatal neurogenesis, as well as in its anterior extension, the rostral migratory stream (RMS), a pathway for neuroblasts during their transit toward the olfactory bulb (OB) layers. Here we show that microglia within the SVZ/RMS pathway are not revealed by phenotypic markers that characterize microglia in other regions. Analysis of the transgenic mice strain that has one locus of the constitutively expressed fractalkine CX3CR1 receptor replaced by the gene encoding the enhanced green fluorescent protein (EGFP) circumvented the antigenic plasticity of the microglia, thus allowing us to depict microglia within the SVZ/RMS pathway during early development. Notably, microglia within the early SVZ/RMS are not proliferative and display a protracted development, retaining a more immature morphology than their counterparts outside germinal layers. Furthermore, microglia contact and phagocyte radial glia cells (RG) processes, thereby playing a role on the astroglial transformation that putative stem cells within the SVZ niche undergo during the first postnatal days.

Keywords: microglia, CX3CR1, subventricular zone, rostral migratory stream, neurogenesis

INTRODUCTION

Most often neurogenesis occurs in discrete regions known as germinal or germinative zones (Götz and Huttner, 2005; Franco and Müller, 2013). Interactions of specific cellular and molecular components of the neurogenic niche determine the progeny output (Jones and Wagers, 2008; Pathania et al., 2010; Lim and Alvarez-Buylla, 2014). In postnatal germinal zones, such as the adult telencephalic subventricular zone (SVZ) (Lim and Alvarez-Buylla, 2014) and subgranular layer of the hippocampus dentate gyrus (Seri et al., 2004), a common set of cells with distinct features are observed (Miller and Gauthier-Fisher, 2009), including quiescent multipotent neural stem cell with astrocytic characteristics, support cells, intermediary progenitors, immediate progeny, blood vessels and a specialized extracellular matrix (Tavazoie et al., 2008; Miller and Gauthier-Fisher, 2009). In the last few years it has been demonstrated that the monocyte counterpart of the nervous system, the microglial cell, is a full component of neurogenic niches (Mercier et al., 2002; Sierra et al., 2010; Olah et al., 2011; Cunningham et al., 2013). However, its importance, function, and interactions are yet to be fully uncovered.

Microglial cells constitute the main mesoderm-derived macrophage population of the central nervous system (CNS) (Prinz and Mildner, 2011) and are distinguished from other CNS cell types by their small cell soma, as well as by the expression of specific macrophage markers (Vilhardt, 2005). Monocytes precursors generated in the yolk sac invade the early embryonic nervous parenchyma as ameboid microglial cells (Chan et al., 2007; Ginhoux et al., 2010, 2013). As development progresses, microglia within the CNS parenchyma undergo differentiation, changing from ameboid morphology into ramified cells, rather deceitfully known as resting state (Nimmerjahn et al., 2005). Ramified microglia are typically distributed throughout the adult, healthy CNS (Imamoto and Leblond, 1978; Cuadros and Navascués, 1998; Dalmau et al., 2003; Hanisch and Kettenmann, 2007). In the course of an insult microglia revert to an ameboid morphology, which usually indicates their active state (Perry et al., 1993; Hanisch and Kettenmann, 2007). Moreover, microglia are involved in several events of brain development, such as phagocytosis, neurito- and synaptogenesis, synaptic pruning, myelination, astrocyte proliferation and differentiation, and vasculogenesis (Giulian et al., 1988; Pow et al., 1989; Hamilton

and Rome, 1994; Presta et al., 1995; Honda et al., 1999; Navascués et al., 2000; Streit, 2001; Rochefort et al., 2002; Marín-Teva et al., 2004; Shin et al., 2004; Checchin et al., 2006; Bessis et al., 2007; Nakanishi et al., 2007; Paolicelli et al., 2011; Kettenmann et al., 2013). Recently, microglia have also been shown to play an important role regulating neural progenitor physiology (Monje et al., 2003; Ziv et al., 2006; Sierra et al., 2010; Arnò et al., 2014; Su et al., 2014).

Here we investigate the ontogenesis, distribution and cellular interactions of microglia residing in the early postnatal SVZ, and its anterior extension, the rostral migratory stream (RMS). This region represents the major neurogenic niche in the mammalian brain that generates mostly interneurons destined for the olfactory bulb (OB) from birth to senescence (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994). During the first two postnatal weeks a peak on proliferation is observed within the SVZ and its main progenitor cell, the radial glia (RG), undergo a process known as astrocytic transformation (Voigt, 1989; Misson et al., 1991; Freitas et al., 2012). During astrocytic transformation a set of RG transforms into astrocytes destined to populate the overlying mantle layers and/or into resident astrocytes of the SVZ/RMS pathway. Our results reveal that at this critical period microglia is already present in this germinal layer and greatly outnumber the microglia cells observed in the overlying cerebral cortex (CTX). Besides, SVZ/RMS microglia exhibit a more protracted differentiation rate compared to the regions outside this germinal zone. Importantly, during the first postnatal week SVZ/RMS microglia interact with RGs, the putative stem cells of this niche, possibly using RG processes as scaffold for its migration. Furthermore, SVZ/RMS microglia seem to engulf RGs processes, thus playing a key role in RG astrocytic transformation and possibly acting on progenitor regulation.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

CX3CR1-encoding the green fluorescent protein (EGFP) mice on the C57BL/6J background were purchased from Jackson Labs (Strain Name B6.129P-CX3CR1tm1Litt/J, stock number 005582). Wild type Swiss mice, raised in our own colony, were also used. For both strains, mice at postnatal day (P) 0 up to P7 were used in our analysis. All experiments were performed in conformity with NIH (National Institute of Health, USA) guidelines for animal care and in accordance with protocols approved by both, the Animal Use Committees at the University of Rochester (UCAR-2011-021) and the Committee of Ethics on Animal Handling and Care at the Federal University of Rio de Janeiro (CEUA/DAHEICB 052; ICB/CCS—UFRJ).

Tissue harvesting

Heterozygous animals ($CX3CR1^{+}/EGFP^{+}$) and Swiss mice at P1 and P7 (n = 12 animals for each age, both strains) were deeply anesthetized by isoflurane inhalation (chamber atmosphere containing 4% isoflurane). Upon cessation of reflexes, mice were transcardially perfused with phosphate buffered saline 0.1 M (PBS, pH 7.4, Sigma Aldrich) and paraformaldehyde 4% (PFA, Sigma Aldrich, in PBS 0.1 M pH 7.4). Brains were dissected and post fixed in PFA 4% for 3–6 h at room temperature (RT). Histological sections (50–100 μm) were obtained in vibratome (Vibratome Series 3000, Vibratome Co.) and kept in PBS containing azide 0.1% (Fisher Scientific) at 4°C for immunohistochemistry analysis (see Section Immunohistochemistry).

BrdU administration

Short pulses of the thymidine analog BrdU (5-bromo-2′-deoxyuridine; Sigma Aldrich) were performed in order to evaluate microglia proliferation along the SVZ niche. CX3CR1-EGFP mice (P1 and P7, n = 6 animals for each age) received a single pulse of BrdU into the intraperitoneal cavity (i.p. injection; 150 mg Kg^{-1}) and were euthanized 1 h after BrdU administration. Proliferative cells were revealed by immunohistochemistry (described below), using a primary antibody that reacts with BrdU incorporated into single stranded DNA.

IMMUNOHISTOCHEMISTRY

Histological sections were blocked for 1 h at RT in a PBS containing 0.1% Triton-X (Sigma Aldrich) solution added with 5% normal donkey serum (NDS, Vector Labs) and incubation with specific antibodies against microglial/monocyte markers (Iba1; 1:500, Wako, CD68, F4/80 and CD11c; 1:100, AbD Serotec), neuroblasts (DCX; 1:1000, Millipore) and astroglial lineage cells (GFAP; 1:250, Sigma Aldrich) was performed overnight at 4°C. Proliferative cells were revealed by using an anti-BrdU antibody (1:100, AbD Serotec). To allow labeling of nuclear DNA, before blockage, sections were treated for 1 h with HCl 1M (RT) under agitation (Tang et al., 2007). Staining was revealed by 2-hour incubation period (RT) with appropriated secondary antibodies conjugated to Cy3 or Cy5 fluorophores (1:250, Jackson ImmunoResearch). DAPI (4′,6-Diamidino-2-phenylindole, 1:1000, Sigma Aldrich) was used for nuclear counterstaining and slides were mounted with ProLong Antifade (Life Technologies). Immunolabeled brain sections were analyzed and imaged using a confocal microscope (Olympus FluoView 500) with 40x (NA 1.30) and 60x oil-immersion (NA 1.25) objective lens (Olympus). Acquired images were adjusted for brightness and contrast using FIJI/ImageJ software.

FLUORO-GOLD TRACER INJECTIONS

Pups (P0 or P1) were anesthetized by isoflurane inhalation (chamber atmosphere containing 4% isoflurane), and under visual guidance, 100–200 nl of Fluoro-Gold (FG; hydroxystilbamidine methanesulfonate in 2% in deionized water; Fluorochrome, Englewood, CO) were injected unilaterally in the pial surface (1–0.5 mm from midline and 0.5 mm anterior to Bregma) using a glass micropipette (80–100 mm tip diameter) coupled to a pressure injector (Nanoliter 2000, WPI, Sarasota, USA). Animals were analyzed 2 or 7 days after pial injections (n = 6; injection site included cortical supragranular layers; animals with deep injections reaching the cortical subgranular layers or the white matter were discarded from analysis).

STATISTICAL ANALYSIS

Histograms are expressed as mean ± standard error (SEM). Raw data, obtained in distinct experimental approaches used in the

present work, were statistically analyzed using Prism (GraphPad Software, Inc.).

RESULTS

ANALYSIS OF CX3CR1-EGFP$^+$ CELLS DEPICTS MICROGLIA AS A CELLULAR COMPONENT OF THE EARLY POSTNATAL SVZ/RMS

Confocal microscopy analysis of brain sections obtained from newborn mice (P1) reveals that CX3CR1-EGFP$^+$ cells accumulate at the ventricular layers, VZ/SVZ (**Figure 1A**). CX3CR1-EGFP$^+$ cells are also distributed in the RMS core (**Figure 1B**), and within the OB layers (**Figure 1C**). In contrast, we observe very few CX3CR1-EGFP$^+$ cells in the cortical parenchyma (**Figure 1D**). In common, CX3CR1-EGFP$^+$ expressing cells in the SVZ, RMS, OB and CTX display immature/amoeboid morphology (**Figures 1A1–D1**), regardless of the significative difference on cell density between these regions [SVZ: $35 \times 10^3 \pm 4.3 \times 10^3$; RMS: $19.3 \times 10^3 \pm 1.3 \times 10^3$; OB: $20 \times 10^3 \pm 3 \times 10^3$; CTX: $4.4 \times 10^3 \pm 0.6 \times 10^3$; CX3CR1-EGFP$^+$ cells/mm^3; mean ± SEM; $p < 0.05$ for SVZ in comparison to RMS and OB, and for RMS and OB in comparison to CTX, and $p < 0.005$ for SVZ in comparison to CTX; 1way ANOVA Bonferroni's Multiple Comparison Test] (**Figure 1E**).

We next asked if the CX3CR1-EGFP$^+$ cells observed within the SVZ/RMS niche, OB and in the cortical parenchyma correspond solely to microglial cells, as the fractalkine receptor is also expressed by monocytes, subsets of natural killers and dendritic cells (Jung et al., 2000). Since the dendritic cell antigen CD11c was detected in a transgenic mice strain in postnatal SVZ cells that were also immunoreactive for microglial markers (Bulloch et al., 2008), we analyzed by immunohistochemistry if CX3CR1-EGFP$^+$ cells were co-labeled by CD11c. Notably, the majority of CX3CR1-EGFP expressing cells present in the SVZ/RMS, OB and cortical parenchyma correspond to microglia, as only a few cells restricted to the pial surface, are co-labeled by dendritic cell marker CD11c (**Figures 2A,B,B1,B2**).

Remarkably, analysis of brain sections obtained from CX3CR1-EGFP mice demonstrates that part of CX3CR1-EGFP$^+$ cells are not co-labeled by CD68 (**Figures 2C–E**). Of the total of microglial cells observed within the SVZ, CX3CR1-EGFP$^+$/CD68$^+$ cells corresponded to 30.3% and CX3CR1-EGFP$^+$/CD68$^-$ cells corresponded to 69.7% (**Figure 2I**). No CX3CR1-EGFP$^-$/CD68$^+$ cells were observed. Similarly, immunostaining for Iba1 revealed that 42.6% of the microglia in the SVZ are CX3CR1-EGFP$^+$/Iba1$^+$ and 57.4% CX3CR1-EGFP$^+$/Iba1$^-$ (**Figures 2F–I**), indicating that the SVZ microglia are a heterogeneous population (Olah et al., 2011).

At later stages (P7), CX3CR1-EGFP expressing microglia present in the SVZ retains their immature morphology (**Figures 3A,A1**). Despite the dense population of ramified microglia outside its borders, CX3CR1-EGFP$^+$ cells along

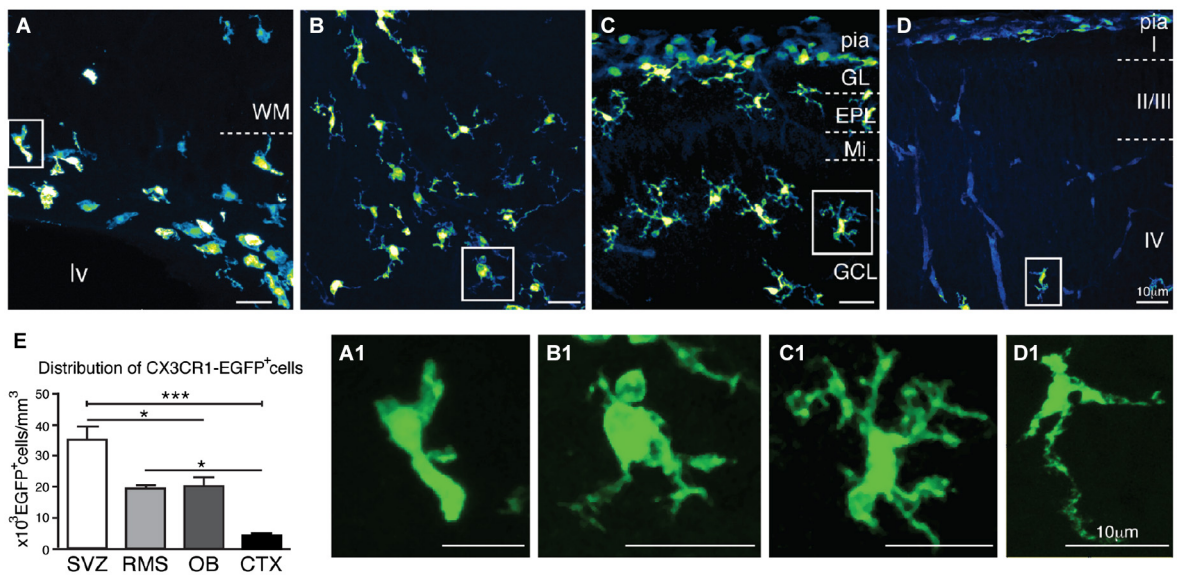


FIGURE 1 | Characterization of CX3CR1-EGFP$^+$ cells morphology and distribution in newborn mice (P1). CX3CR1-EGFP$^+$ microglia accumulate at the ventricular layers **(A)**. Interestingly, the core of RMS has a significative number of microglia cells **(B)**, as well as the distinct OB layers **(C)**. In contrast, we observe very few CX3CR1-EGFP$^+$ cells in the cortical parenchyma **(D)**. As expected, CX3CR1-EGFP expressing cells exhibit immature/amoeboid morphology in all analyzed regions **(A1,B1,C1,D1)**. **(E)** CX3CR1-EGFP$^+$ cell density in newborn mice: SVZ: $35 \times 10^3 \pm 4.3 \times 10^3$; RMS: $19.3 \times 10^3 \pm 1.3 \times 10^3$; OB: $20 \times 10^3 \pm 3 \times 10^3$; CTX: $4.4 \times 10^3 \pm 0.6 \times 10^3$; cells/mm^3; mean ± SEM; $p < 0.05$ (*) for SVZ in comparison to RMS and OB, and for RMS and OB in comparison to CTX, and $p < 0.005$ (***) for SVZ in comparison to CTX; 1way ANOVA Bonferroni's Multiple Comparison Test]. CTX, cerebral cortex; EPL: external plexiform layer; GCL: granular cell layer; GL: glomerular layer; lv: lateral ventricle; Mi: mitral layer; OB: olfactory bulb; RMS: rostral migratory stream; SVZ: subventricular zone; WM: white matter. Scale bars: 10 μm.

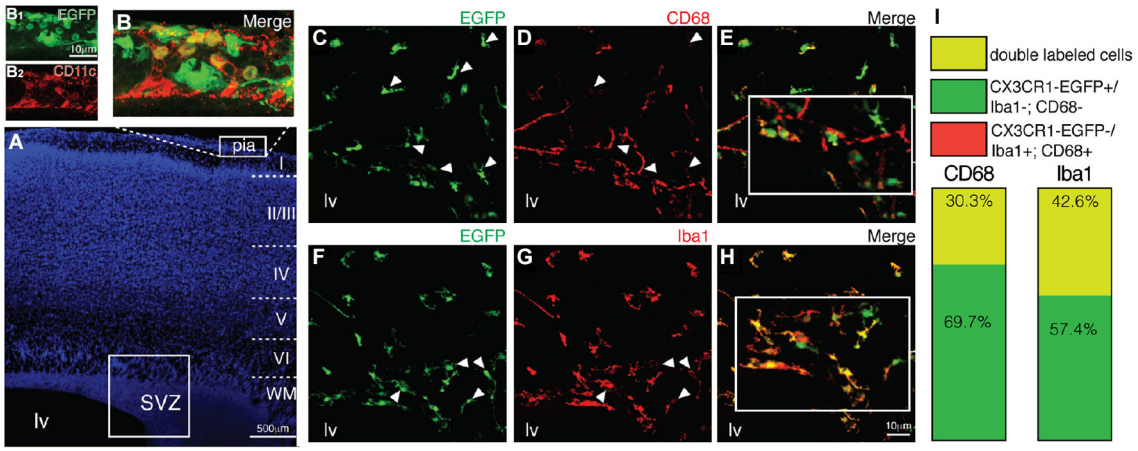


FIGURE 2 | Antigenic heterogeneity of CX3CR1-EGFP$^+$ cells morphology in early postnatal SVZ. **(A)** DAPI counterstaining unveils the cytoarchitecture of cortical parenchyma and ventricular region. Dendritic cells (CD11c$^+$, red) are restricted to the pial surface and some of them co-express CX3CR1-EGFP **(B,B1,B2)**. Immunolabeling of brain sections obtained from CX3CR1-EGFP mice with CD68 (**C–E**, red) and Iba1 (**F–H**, red) demonstrate that some of EGFP$^+$ cells (green) are not co-labeled by these common used microglia markers (white arrowheads). **(I)** The percentage of CX3CR1-EGFP$^+$/CD68$^+$ cells is 30.3% of the total of microglia observed and of CX3CR1-EGFP$^+$/Iba1$^+$ cells is 42.6%. SVZ: subventricular zone. Scale bars: 100 μm **(A)** and 10 μm (**B1,B2,C–H**).

the RMS also display immature/migratory morphology (**Figures 3B,B1**), similar to the microglial cells distributed within the OB layers (**Figures 3C,C1**). In contrast, we observe ramified CX3CR1-EGFP$^+$ microglia spanning all the cortical layers (**Figures 3D,D1**), and at this age, no significative differences on CX3CR1-EGFP$^+$ cell number are observed when comparing all analyzed regions [SVZ: $33 \times 10^3 \pm 3 \times 10^3$; RMS: $26.9 \times 10^3 \pm 3.7 \times 10^3$; OB: $30 \times 10^3 \pm 3.7 \times 10^3$; CTX: $36 \times 10^3 \pm 2.6 \times 10^3$; CX3CR1-EGFP$^+$ cells/mm^3; mean ± SEM; $p > 0.05$, 1way ANOVA Bonferroni's Multiple Comparison Test] (**Figure 3E**). Likewise observed in newborn mice, CX3CR1-EGFP$^+$ cells in the SVZ/RMS, regarding their immunoreactivity, remain a quite heterogeneous population at P7. Our results show that CX3CR1-EGFP$^+$/CD68$^+$ cells corresponded to 40.2% and CX3CR1-EGFP$^+$/CD68$^-$ cells represent 69.7% of microglia present in the SVZ (**Figures 4A–C,G**). Analysis of Iba1 immunoreactivity shows that CX3CR1-EGFP$^+$/Iba1$^+$ cells correspond to 20.8% of the microglia, whereas CX3CR1-EGFP$^-$/Iba1$^+$ cells correspond to 27.3%. However, the majority of microglia in the SVZ is CX3CR1-EGFP$^+$/Iba1$^-$ cells, corresponding to 51.9% (**Figures 4D–G**).

MICROGLIAL CELLS RESIDING IN THE SVZ NICHE ARE NOT PROLIFERATIVE DURING EARLY NEONATAL STAGES

Once inside the CNS, microglia precursors spread within the neural tissue, a process that includes cell proliferation and/or migration. In order to determine the spreading dynamics of microglial cells in the SVZ niche, we accessed the proliferation status of CX3CR1- EGFP$^+$ cells in the SVZ, RMS and OB during the first postnatal week. After a short pulse of BrdU (1 h before euthanasia), scarce CX3CR1-EGFP$^+$/BrdU$^+$ cells are observed in the lateral (lv) and olfactory ventricles (Olfv) of P1 mice (**Figures 5B,C**, respectively, and **Figure 5H**). Within the OB layers, only CX3CR1-EGFP$^+$/BrdU$^-$ are observed, indicating that the majority of microglial cells in the neonatal SVZ niche is quiescent (**Figures 5D,H**). At P7, we observe few CX3CR1-EGFP$^+$/BrdU$^+$ cells in the ventricular region (**Figures 5E,I**). Along the RMS, dividing microglia are detected in its borders, as well as some BrdU fragments are engulfed by microglia (**Figures 5F,I**). Remarkably, in the OB BrdU$^+$ cells distributed along the distinct layers are sparsely contacted by CX3CR1-EGFP$^+$ microglia and some CX3CR1-EGFP$^+$/BrdU$^+$ are observed (**Figures 5G,I**).

MICROGLIA CELLULAR INTERACTIONS WITHIN THE NEONATAL SVZ/RMS NICHE

We next sought to determine the microglial interactions with the typical cell types observed within the SVZ niche, namely the astroglial stem cell lineage (RGs and stem cell-like astrocytes/type B cells) and neuroblasts. Since previous studies show that tracer injections at the pial surface labels exclusively RG within the SZV (Freitas et al., 2012), we took advantage of the fact that at birth many RGs still maintain a long process touching the pial surface (Misson et al., 1991; Alves et al., 2002) to label this cell population. The neuroanatomical tracer Fluoro-Gold was injected in the pial surface of newborn mice (P0) and we followed labeled RGs up to the first postnatal week (P7). Immunohistochemistry analysis of brain sections obtained from injected animals reveals the transcellular labeling of microglia (F4/80$^+$ cells) neighboring labeled RG (**Figures 6B,B1,B2**). This is suggestive of a very intimate contact of microglia with RG, although we could not distinguish if this transcellular labeling was due to whole engulfment of RG by microglia, or partial phagocytosis of RG processes. Immunolabeling of RGs and astrocytes with GFAP antibody reveal a close apposition of

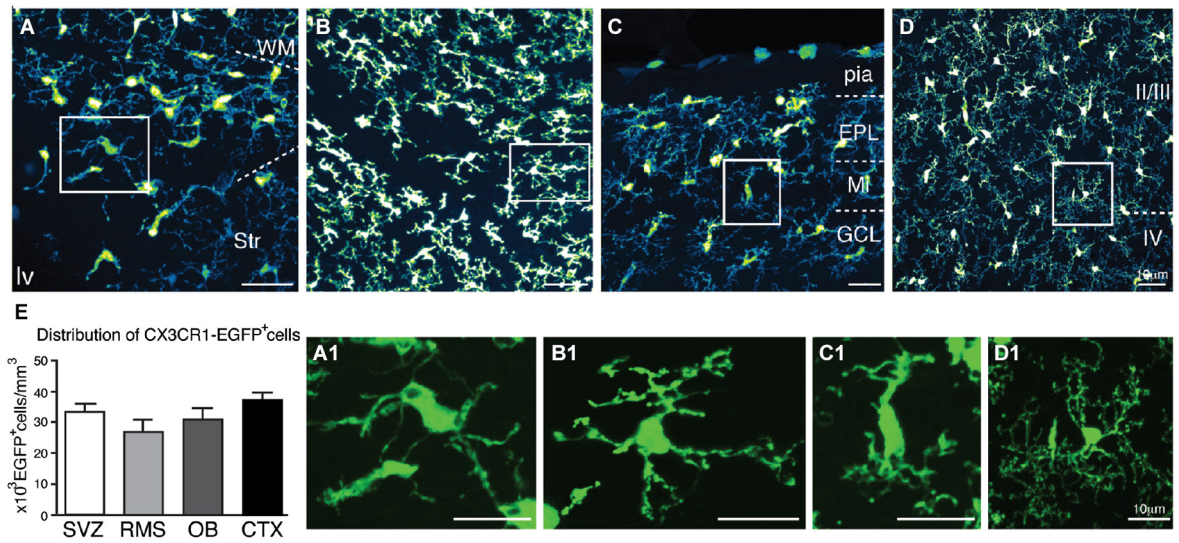


FIGURE 3 | Characterization of CX3CR1-EGFP$^+$ microglia morphology and density in neonatal mice. In P7 mice, microglia present in the SVZ retains immature morphology **(A,A1)**, differing from EGFP$^+$ cells present in the adjacent areas (WM and Str). In the RMS, CX3CR1-EGFP$^+$ cells also display immature/migratory morphology **(B,B1)**. Similar morphology is shown by CX3CR1-EGFP$^+$ cells in the OB **(C,C1)**. Within CTX layers, ramified CX3CR1-EGFP$^+$ microglia are homogenously distributed **(D,D1)**. **(E)** At this age, no significative differences on CX3CR1-EGFP$^+$ cell number are observed in the analyzed regions [SVZ: $33 \times 10^3 \pm 3 \times 10^3$; RMS: $26.9 \times 10^3 \pm 3.7 \times 10^3$; OB: $30 \times 10^3 \pm 3.7 \times 10^3$; CTX: $36 \times 10^3 \pm 2.6 \times 10^3$; cells/mm^3; mean ± SEM; $p > 0.05$, 1way ANOVA Bonferroni's Multiple Comparison Test]. EPL: external plexiform layer; GCL: granular cell layer; lv: lateral ventricle; Mi: mitral layer; WM: white matter. Scale bars: 10 μm.

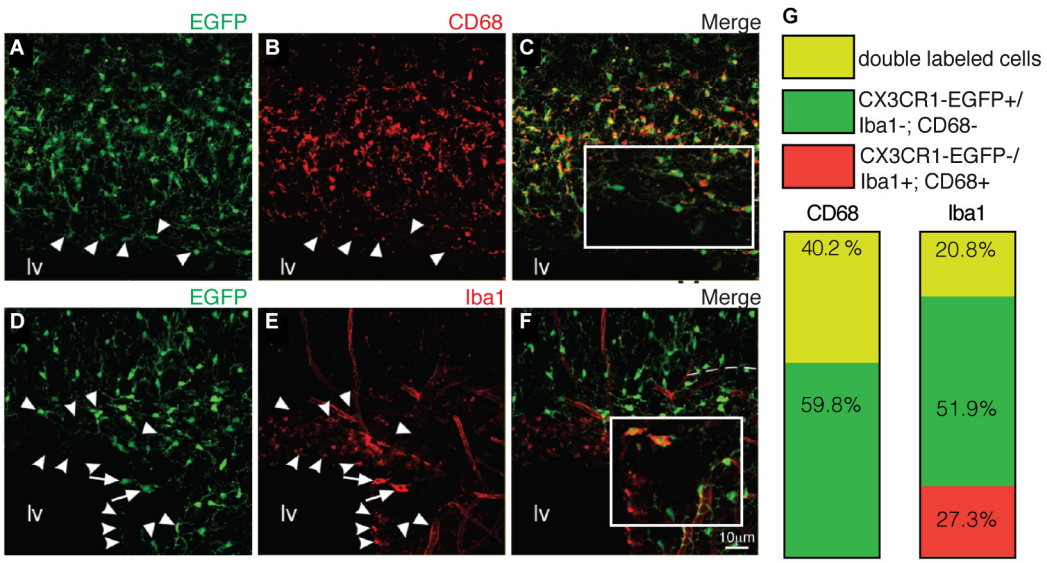


FIGURE 4 | Macrophage markers cannot depict the entire SVZ microglial population. (A–C) Similar to newborn mice, some of EGFP$^+$ cells (green) are not co-labeled by CD68 (red) (indicated by arrowheads). CX3CR1-EGFP$^+$/CD68$^+$ cells correspond to 40.2% of the microglia in the SVZ **(G)**. Immunostaining with Iba1 (**D–F**, red) reveals that CX3CR1-EGFP$^+$/Iba1$^+$ cells (arrows) correspond to 20.8%. and CX3CR1-EGFP$^+$/Iba1$^-$ cells (arrowheads) represent 51.9% of microglia **(G)**. Notably, some cells are solely labeled by Iba1, corresponding to 27.3% of the SVZ microglia (round arrowheads). lv: lateral ventricle. Scale bars: 10 μm.

microglia to astroglial processes (**Figure 6C**). Some microglia display a migratory morphology (**Figures 6C1,D,E**), indicating that microglia use radial processes to migrate within the cortical parenchyma. Remarkably, we also observe microglial cells enfolding GFAP$^+$ processes in the SVZ/WM border, where GFAP$^+$ cells accumulate during their putative astroglial transformation (**Figure 6C2**). Interestingly, along the SVZ/RMS of neonatal mice (P7) microglia are conspicuously distributed,

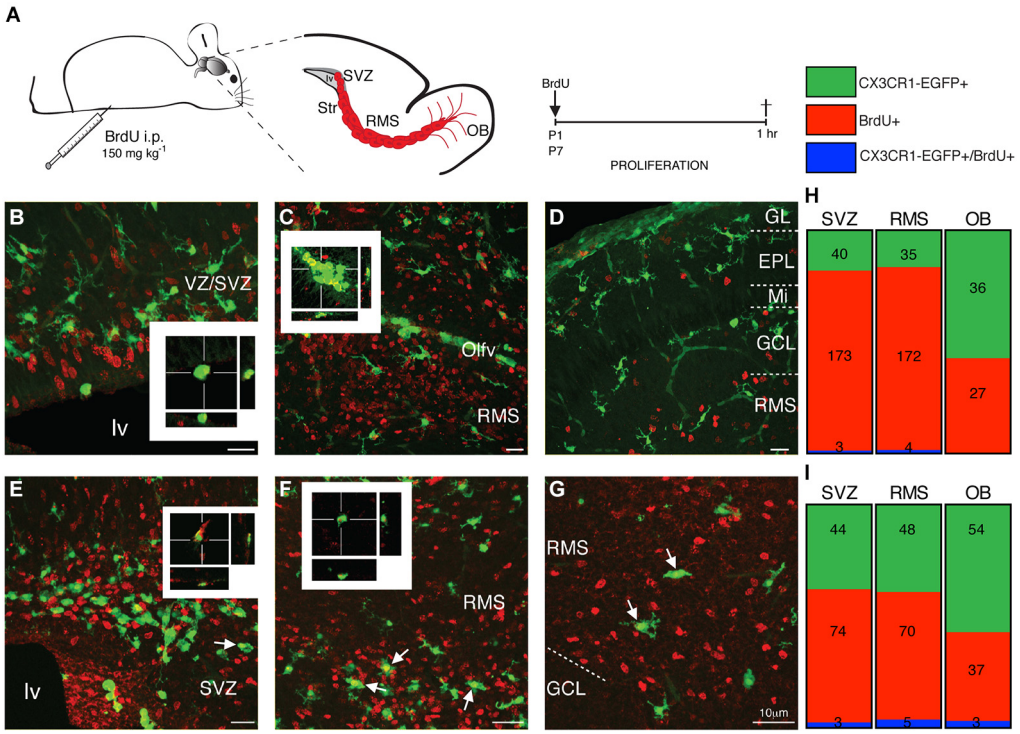


FIGURE 5 | Microglia residing in neonatal the SVZ, RMS and OB are quiescent cells. (A) Schematic representation of brain parasagittal section obtained from neonatal mice that received a single, short pulse of BrdU (i.p. injections of BrdU; 150 mg kg^{-1}). Sections containing the SVZ, RMS and OB were processed for BrdU immunostaining. **(B)** Dividing ($BrdU^+$ cells, red) are observed in the ventricular layers of P1 mice, but majority of SVZ microglia do not incorporate BrdU and only few cells are $EGFP^+$ (green)/$BrdU^+$, as depicted by optical sectioning. In the RMS of P1 mice, CX3CR1-$EGFP^+$/$BrdU^+$ cells are restricted into the olfactory ventricle **(C)**, while in the OB, we do not observe any CX3CR1-$EGFP^+$/$BrdU^+$ cell at this stage **(D)**. At P7, CX3CR1-$EGFP^+$ cells intermingle $BrdU^+$ cells, but few proliferative microglia are observed in the SVZ (**E**, arrows) and in the borders of RMS (**F**, arrows). Within OB layers, few CX3CR1-$EGFP^+$ cells do not incorporate BrdU (**G**, arrows). Quantification is shown, in absolute numbers, in **H** and **I**. EPL: external plexiform layer; GCL: granular cell layer; GL: glomerular layer; lv: lateral ventricle; Mi: mitral layer; OB: olfactory bulb; Olfv: olfactory ventricle; RMS: rostral migratory stream; SVZ: subventricular zone; WM: white matter; VZ: ventricular zone. Scale bars: 10 µm.

most often intermingling with the astrocyte compartment and outside of the chains of migratory neuroblasts (**Figures 6F, F1, F2**).

DISCUSSION

Here we demonstrate that microglia present within the early postnatal SVZ represents a copious population, which outnumber cortical microglia population during neonatal stages (**Figure 1E**). Our observations also show that SVZ microglia exhibit a remarkable antigenic plasticity (**Figures 2**, **4**) and quiescence (**Figure 5**), confirming and extending the concept of microglia regional heterogeneity (Carson et al., 2007; Olah et al., 2011). Furthermore, our analysis reveal that microglia is intimately associated to the astroglial compartment within the SVZ, since dye transfer between RG cells and microglial cells were observed, possibly a result of phagocytosis, and a spatial overlap with GFAP positive cells (**Figure 6**). This interaction could represent a direct microglia control over late cortical progenitors of the outer SVZ (Franco et al., 2012) or progenitors for interneurons of the OB layers (Merkle et al., 2007; Ventura and Goldman, 2007). Alternatively, suggest that microglia is involved in the astrocytic transformation of a subset of RG cells in the early postnatal SVZ/RMS as suggested in earlier publications (Schmechel and Rakic, 1979; Voigt, 1989; Misson et al., 1991; Alves et al., 2002; Freitas et al., 2012).

For several years, the presence of microglia within the early postnatal SVZ/RMS was either neglected (Dalmau et al., 2003), or undetected (Peretto et al., 2005). Only recently has microglia within the early SVZ been investigated (Shigemoto-Mogami et al., 2014). The previous underestimation of microglia within germinative layers, and specifically in the postnatal SVZ, may be due to the great phenotypic plasticity of microglia cells (Saijo and Glass, 2011), making detection by usual phenotypic markers unreliable. We have circumvented this limitation by using a transgenic animal in which the reporter gene encoding the green fluorescent protein (EGFP) was introduced in the locus of the constitutively expressed fractalkine CX3CR1 receptor (Jung et al., 2000), yielding a stable marker for this population. This results in a golgi-like cell labeling with EGFP, throughout the developing brain parenchyma, displaying several chracteristics of microglia. CX3CR1-$EGFP^+$ microglia in the SVZ were neither immunoreactive for neural nor blood vessel markers (data not shown), and also unlabeled by a dendritic cell marker CD11c. Interestingly and rather unexpected, common used

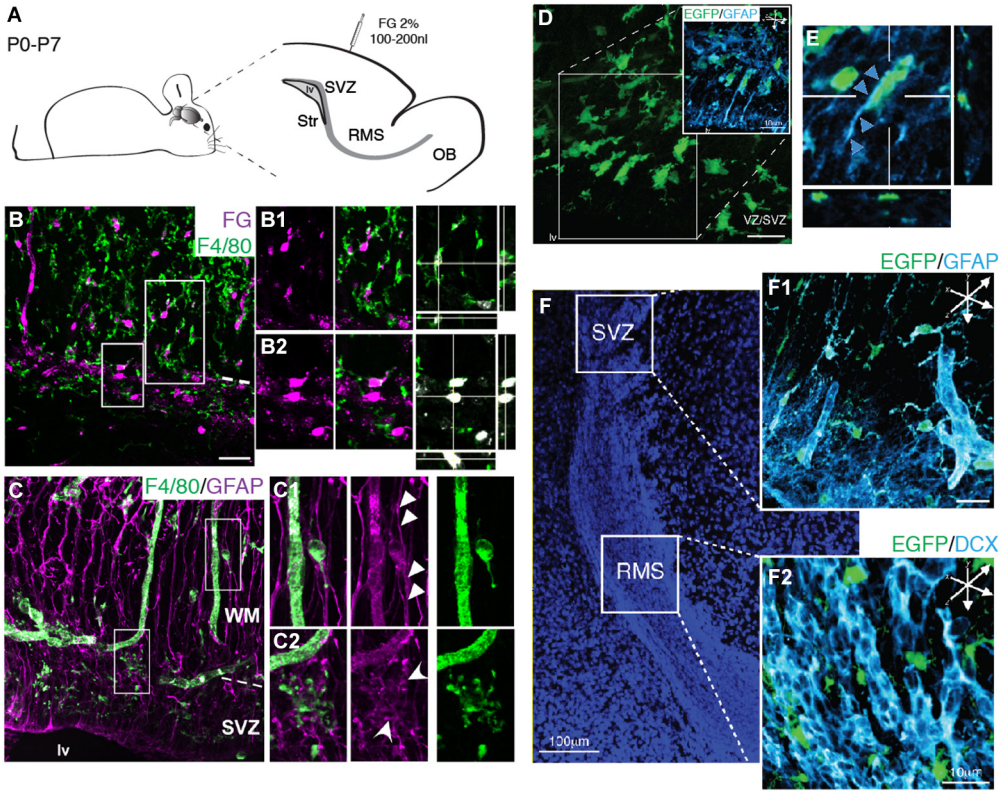


FIGURE 6 | Microglia cellular interactions within the SVZ niche. **(A)** Schematic representation of brain parasagittal section obtained from neonatal Swiss mice injected with FG (2%) in the pial surface. **(B)** Histological sections of injected mice and immunolabeled for F4/80 antigen reveal the overlapping of FG^+ cell soma (magenta) and $F4/80^+$ cells (green) in the SVZ and in the WM. RGs present in both the SVZ and overlaying WM are depicted in higher magnification in **(B1)** and **(B2)**, respectively. Optical sectioning shows that FG^+ cells are engulfed by $F4/80^+$ microglia. **(C)** Microglia ($F4/80^+$ cells, green) appose to RG processes (GFAP, magenta) that span the cortical parenchyma. **(C1)** Microglia displaying migratory morphology appose to RG processes spanning the WM (arrowheads). **(C2)** Within the SVZ, microglia displaying immature morphology are closely associated to $GFAP^+$ astroglial cells (round arrowheads). **(D)** In transgenic mice, CX3CR1-$EGFP^+$ cells (green) also associate to RG processes (GFAP, cyan), displaying migratory morphology, thus using astroglial processes as scaffold to invade the cortical parenchyma. **(E)** Orthogonal view of CX3CR1-$EGFP^+$ cells along $GFAP^+$ processes (arrowheads). **(F)**. SVZ and RMS delimit the cell dense region counterstained with DAPI. Within the SVZ, CX3CR1-$EGFP^+$ cells intermingle $GFAP^+$ cells **(F1)**, whereas in the RMS, microglia cells interleave neuroblasts chains (**F2**, DCX^+ cells, cyan).

markers for microglia only partially co-localize with CX3CR1-$EGFP^+$ cells. Since most of phenotypic markers used to reveal macrophages are membrane molecules related to cell-cell or cell-extracellular milieu interactions (Ling et al., 1991; Milligan et al., 1991; Chen et al., 2002; Gomez Perdiguero et al., 2013), this variability of antigen expression may reflect the influence of discrete signals present within this neurogenic niche, which may instruct and control identity and specialization of microglia.

The most striking morphological feature observed for microglia within the neonatal SVZ is their characteristic activated profile (**Figures 1**, **3A**), exhibiting ameboid morphology with few thick and short branches, also typical of immature microglia (Perry et al., 1993; Hanisch and Kettenmann, 2007). At P7, when cortical microglia already display a ramified "resting" morphology (Lima et al., 2001; Dalmau et al., 2003), SVZ microglia still retains the immature/activated profile. This may be a common feature for microglia resident of germinative layers, since the same reactive profile has been described for the embryonic cerebral cortical VZ/SVZ (Cunningham et al., 2013) and adult SVZ (Goings et al., 2006). Another outstanding difference of SVZ microglia is their relative quiescence (**Figure 5**), in contrast to actively proliferating microglia distributed throughout the cortical parenchyma during the first postnatal week (Mallat et al., 1997; Alliot et al., 1999; Dalmau et al., 2003). These regional differences could result from signals emanating from a progenitor enriched environment that has been shown to instruct resident microglia (Mosher et al., 2012; Linnartz and Neumann, 2013). It remains to be determined if this microglia behavior is dynamically controlled or represents an irreversible phenotype. It is interesting to note that microglia harvested from the adult SVZ, behave differently in culture, even after many *in vitro* passages (Walton et al., 2006), suggesting some stable and environment independent features for this microglial population.

A straightforward mechanism for any putative function for microglia over SVZ/RMS progenitors could lie on their intrinsic phagocytic activity. Phagocytosis of neural progenitors has been

shown to occur in the subgranular layer of the dentate gyrus (Sierra et al., 2010) and in the embryonic telencephalic ventricular zone (Cunningham et al., 2013). To test this hypothesis we have retrogradely labeled RG cells present in the SVZ by injecting the fluorescent tracer Fluoro-Gold at the pial surface. Previous results have shown that 2 days after pial injection of anatomical tracers only RG are labeled within the SVZ (Freitas et al., 2012). Interestingly, at 7 days post injection, we find the labeling of microglia. This can be explained as reminiscent of transcellular transfer of dyes to microglia by phagocytosis of retrogradely labeled cells, as observed in other systems (Thanos et al., 2000). Together this data suggests that microglia is actively phagocytizing RGs. However, alternatively this transcellular labeling may be due to gap junctional communication (Freitas et al., 2012) or partial phagocytosis of RG processes in a manner analogous to microglia stripping of neuronal synapses and processes described previously (Kettenmann et al., 2013). Although further investigation may be necessary to distinguish between these possibilities, the transcellular dye transfer and overlap of distribution with the astroglial compartment, as shown by double labeling with GFAP (**Figure 6C2**), indicates a consistent interaction between microglia and RGs. Nevertheless, we cannot rule out the hypothesis that microglia may also be phagocytizing neuroblasts en route to the OB layers, as shown to occur at the hippocampus dentate gyrus (Sierra et al., 2010).

Given their rapid response to diffusible signals and cell-cell interactions, microglia may represent a pivotal player to integrate short and long-range environmental cues within the germinal layers (Su et al., 2014). It has been documented that microglia can respond to neurotransmitters (Fontainhas et al., 2011); trophic factors (Ryu et al., 2012); peripheral cytokines and chemokines (Butovsky et al., 2006); humoral signaling from disease (Li and Graeber, 2012; Tsuda et al., 2013; Yu and Ye, 2014; Hu et al., 2015), membrane glycocalyx (Linnartz and Neumann, 2013) and progenitor secreted proteins (Mosher et al., 2012). On the executive side, microglia could exert their influence not only by its phagocytic activity, engulfing whole cells, processes, or stripping membranes (Kettenmann et al., 2013), but through the release of cytokines and trophic factors and (Nakajima et al., 2007; Cacci et al., 2008; Liao et al., 2008; Ueno et al., 2013). It is still unclear what specific roles microglia play over the generation (Shigemoto-Mogami et al., 2014), migration (Aarum et al., 2003) and addition of new neurons to the OB (Lazarini et al., 2012) and in response to insult (Goings et al., 2006). However, given the possible action of the selective phagocytosis of precursors and progeny (Sierra et al., 2010; Cunningham et al., 2013) microglia activity could contribute to the mismatch observed between the very restricted generative potential of SVZ neural progenitors *in situ* (Luskin, 1993; Lim and Alvarez-Buylla, 2014) and its wider capabilities when challenged *in vivo* or *in vitro* (Sequerra et al., 2010, 2013).

ACKNOWLEDGMENTS

We are thankful to Adiel Batista Nascimento for animal handling and care and to Carla M. Furtado and Celina Garcia for expert technical support. This work was supported by the National Institutes of Health (MN), the United States Department of Defense (MN), the Harold and Leila Y. Mathers Charitable Foundation (MN), Fundação de Amparo a Pesquisa do Rio de Janeiro (FAPERJ) (JRLM), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (JRLM). ALRX was recipient of graduate fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors declare no conflicts of interest.

REFERENCES

Aarum, J., Sandberg, K., Haeberlein, S. L., and Persson, M. A. (2003). Migration and differentiation of neural precursor cells can be directed by microglia. *Proc. Natl. Acad. Sci. U S A* 100, 15983–15988. doi: 10.1073/pnas.2237050100

Alliot, F., Godin, I., and Pessac, B. (1999). Microglia derive from progenitors, originating from the yolk sac and which proliferate in the brain. *Brain Res. Dev. Brain Res.* 117, 145–152. doi: 10.1016/s0165-3806(99)00113-3

Altman, J. (1969). Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J. Comp. Neurol.* 137, 433–457. doi: 10.1002/cne.901370404

Alves, J. A., Barone, P., Engelender, S., Fróes, M. M., and Menezes, J. R. L. (2002). Initial stages of radial glia astrocytic transformation in the early postnatal anterior subventricular zone. *J. Neurobiol.* 52, 251–265. doi: 10.1002/neu.10087

Arnò, B., Grassivaro, F., Rossi, C., Bergamaschi, A., Castiglioni, V., Furlan, R., et al. (2014). Neural progenitor cells orchestrate microglia migration and positioning into the developing cortex. *Nat. Commun.* 5:5611. doi: 10.1038/ncomms6611

Bessis, A., Béchade, C., Bernard, D., and Roumier, A. (2007). Microglial control of neuronal death and synaptic properties. *Glia* 55, 233–238. doi: 10.1002/glia.20459

Bulloch, K., Miller, M. M., Gal-Toth, J., Milner, T. A., Gottfried-Blackmore, A., Waters, E. M., et al. (2008). CD11c/EYFP transgene illuminates a discrete network of dendritic cells within the embryonic, neonatal, adult and injured mouse brain. *J. Comp. Neurol.* 508, 687–710. doi: 10.1002/cne.21668

Butovsky, O., Ziv, Y., Schwartz, A., Landa, G., Talpalar, A. E., Pluchino, S., et al. (2006). Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol. Cell. Neurosci.* 31, 149–160. doi: 10.1016/j.mcn.2005.10.006

Cacci, E., Ajmone-Cat, M. A., Anelli, T., Biagioni, S., and Minghetti, L. (2008). In vitro neuronal and glial differentiation from embryonic or adult neural precursor cells are differently affected by chronic or acute activation of microglia. *Glia* 56, 412–425. doi: 10.1002/glia.20616

Carson, M. J., Bilousova, T. V., Puntambekar, S. S., Melchior, B., Doose, J. M., and Ethell, I. M. (2007). A rose by any other name? The potential consequences of microglial heterogeneity during CNS health and disease. *Neurotherapeutics* 4, 571–579. doi: 10.1016/j.nurt.2007.07.002

Chan, W. Y., Kohsaka, S., and Razaie, P. (2007). The origin and cell lineage of microglia—new concepts. *Brain Res. Rev.* 53, 344–354. doi: 10.1016/j.brainresrev.2006.11.002

Checchin, D., Sennlaub, F., Levavasseur, E., Leduc, M., and Chemtob, S. (2006). Potential role of microglia in retinal blood vessel formation. *Invest. Ophthalmol. Vis. Sci.* 47, 3595–3602. doi: 10.1167/iovs.05-1522

Chen, L., Yang, P., and Kijlstra, A. (2002). Distribution, markers, and functions of retinal microglia. *Ocul. Immunol. Inflamm.* 10, 27–39. doi: 10.1076/ocii.10.1.27.10328

Cuadros, M. A., and Navascués, J. (1998). The origin and differentiation of microglial cells during development. *Prog. Neurobiol.* 56, 173–189. doi: 10.1016/s0301-0082(98)00035-5

Cunningham, C. L., Martínez-Cerdeño, V., and Noctor, S. C. (2013). Microglia regulate the number of neural precursor cells in the developing cerebral cortex. *J. Neurosci.* 33, 4216–4233. doi: 10.1523/jneurosci.3441-12.2013

Dalmau, I., Vela, J. M., González, B., Finsen, B., and Castellano, B. (2003). Dynamics of microglia in the developing rat brain. *J. Comp. Neurol.* 458, 144–157. doi: 10.1002/cne.10572

Fontainhas, A. M., Wang, M., Liang, K. J., Chen, S., Mettu, P., Damani, M., et al. (2011). Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission. *PLoS One* 6:e15973. doi: 10.1371/journal.pone.0015973

Franco, S. J., Gil-Sanz, C., Martinez-Garay, I., Espinosa, A., Harkins-Perry, S. R., Ramos, C., et al. (2012). Fate-restricted neural progenitors in the mammalian cerebral cortex. *Science* 337, 746–749. doi: 10.1126/science.1223616

Franco, S. J., and Müller, U. (2013). Shaping our minds: stem and progenitor cell diversity in the mammalian neocortex. *Neuron* 77, 19–34. doi: 10.1016/j.neuron.2012.12.022

Freitas, A. S., Xavier, A. L., Furtado, C. M., Hedin-Pereira, C., Fróes, M. M., and Menezes, J. R. L. (2012). Dye coupling and connexin expression by cortical radial glia in the early postnatal subventricular zone. *Dev. Neurobiol.* 72, 1482–1497. doi: 10.1002/dneu.22005

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., et al. (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330, 841–845. doi: 10.1126/science.1194637

Ginhoux, F., Lim, S., Hoeffel, G., Low, D., and Huber, T. (2013). Origin and differentiation of microglia. *Front. Cell. Neurosci.* 7:45. doi: 10.3389/fncel.2013.00045

Giulian, D., Young, D. G., Woodward, J., Brown, D. C., and Lachman, L. B. (1988). Interleukin-1 is an astroglial growth factor in the developing brain. *J. Neurosci.* 8, 709–714.

Goings, G. E., Kozlowski, D. A., and Szele, F. G. (2006). Differential activation of microglia in neurogenic versus non-neurogenic regions of the forebrain. *Glia* 54, 329–342. doi: 10.1002/glia.20381

Gomez Perdiguero, E., Schulz, C., and Geissmann, F. (2013). Development and homeostasis of "resident" myeloid cells: the case of the microglia. *Glia* 61, 112–120. doi: 10.1002/glia.22393

Götz, M., and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* 6, 777–788. doi: 10.1038/nrm1739

Hamilton, S. P., and Rome, L. H. (1994). Stimulation of in vitro myelin synthesis by microglia. *Glia* 11, 326–335. doi: 10.1002/glia.440110405

Hanisch, U. K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci.* 10, 1387–1394. doi: 10.1038/nn1997

Honda, S., Nakajima, K., Nakamura, Y., Imai, Y., and Kohsaka, S. (1999). Rat primary cultured microglia express glial cell line-derived neurotrophic factor receptors. *Neurosci. Lett.* 275, 203–206. doi: 10.1016/s0304-3940(99)00769-7

Hu, X., Leak, R. K., Shi, Y., Suenaga, J., Gao, Y., Zheng, P., et al. (2015). Microglial and macrophage polarization-new prospects for brain repair. *Nat. Rev. Neurol.* 11, 56–64. doi: 10.1038/nrneurol.2014.207

Imamoto, K., and Leblond, C. P. (1978). Radioautographic investigation of gliogenesis in the corpus callosum of young rats. II. Origin of microglial cells. *J. Comp. Neurol.* 180, 139–163. doi: 10.1002/cne.901800109

Jones, D. L., and Wagers, A. J. (2008). No place like home: anatomy and function of the stem cell niche. *Nat. Rev. Mol. Cell Biol.* 9, 11–21. doi: 10.1038/nrm2319

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M. J., Kreutzberg, G. W., Sher, A., et al. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol. Cell. Biol.* 20, 4106–4114. doi: 10.1128/mcb.20.11.4106-4114.2000

Kettenmann, H., Kirchhoff, F., and Verkhratsky, A. (2013). Microglia: new roles for the synaptic stripper. *Neuron* 77, 10–18. doi: 10.1016/j.neuron.2012.12.023

Lazarini, F., Gabellec, M. M., Torquet, N., and Lledo, P. M. (2012). Early activation of microglia triggers long-lasting impairment of adult neurogenesis in the olfactory bulb. *J. Neurosci.* 32, 3652–3664. doi: 10.1523/jneurosci.6394-11.2012

Li, W., and Graeber, M. B. (2012). The molecular profile of microglia under the influence of glioma. *Neuro Oncol.* 14, 958–978. doi: 10.1093/neuonc/nos116

Liao, H., Huang, W., Niu, R., Sun, L., and Zhang, L. (2008). Cross-talk between the epidermal growth factor-like repeats/fibronectin 6-8 repeats domains of Tenascin-R and microglia modulates neural stem/progenitor cell proliferation and differentiation. *J. Neurosci. Res.* 86, 27–34. doi: 10.1002/jnr.21454

Lim, D. A., and Alvarez-Buylla, A. (2014). Adult neural stem cells stake their ground. *Trends Neurosci.* 37, 563–571. doi: 10.1016/j.tins.2014.08.006

Lima, F. R., Gervais, A., Colin, C., Izembart, M., Neto, V. M., and Mallat, M. (2001). Regulation of microglial development: a novel role for thyroid hormone. *J. Neurosci.* 21, 2028–2038.

Ling, E. A., Kaur, C., and Wong, W. C. (1991). Expression of major histocompatibility complex and leukocyte common antigens in amoeboid microglia in postnatal rats. *J. Anat.* 177, 117–126.

Linnartz, B., and Neumann, H. (2013). Microglial activatory (immunoreceptor tyrosine-based activation motif)- and inhibitory (immunoreceptor tyrosine-based inhibition motif)-signaling receptors for recognition of the neuronal glycocalyx. *Glia* 61, 37–46. doi: 10.1002/glia.22359

Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. *Science* 264, 1145–1148. doi: 10.1126/science.8178174

Luskin, M. B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11, 173–189. doi: 10.1016/0896-6273(93)90281-u

Mallat, M., Calvo, C. F., and Dobbertin, A. (1997). Migration and proliferation of mononuclear phagocytes in the central nervous system. *Adv. Exp. Med. Biol.* 429, 99–108. doi: 10.1007/978-1-4757-9551-6_7

Marín-Teva, J. L., Dusart, I., Colin, C., Gervais, A., van Rooijen, N., and Mallat, M. (2004). Microglia promote the death of developing Purkinje cells. *Neuron* 41, 535–547. doi: 10.1016/s0896-6273(04)00069-8

Mercier, F., Kitasako, J. T., and Hatton, G. I. (2002). Anatomy of the brain neurogenic zones revisited: fractones and the fibroblast/macrophage network. *J. Comp. Neurol.* 451, 170–188. doi: 10.1002/cne.10342

Merkle, F. T., Mirzadeh, Z., and Alvarez-Buylla, A. (2007). Mosaic organization of neural stem cells in the adult brain. *Science* 317, 381–384. doi: 10.1126/science.1144914

Miller, F. D., and Gauthier-Fisher, A. (2009). Home at last: neural stem cell niches defined. *Cell Stem Cell* 4, 507–510. doi: 10.1016/j.stem.2009.05.008

Milligan, C. E., Cunningham, T. J., and Levitt, P. (1991). Differential immunochemical markers reveal the normal distribution of brain macrophages and microglia in the developing rat brain. *J. Comp. Neurol.* 314, 125–135. doi: 10.1002/cne.903140112

Misson, J. P., Austin, C. P., Takahashi, T., Cepko, C. L., and Caviness, V. S. Jr. (1991). The alignment of migrating neural cells in relation to the murine neopallial radial glial fiber system. *Cereb. Cortex* 1, 221–229. doi: 10.1093/cercor/1.3.221

Monje, M. L., Toda, H., and Palmer, T. D. (2003). Inflammatory blockade restores adult hippocampal neurogenesis. *Science* 302, 1760–1765. doi: 10.1126/science.1088417

Mosher, K. I., Andres, R. H., Fukuhara, T., Bieri, G., Hasegawa-Moriyama, M., He, Y., et al. (2012). Neural progenitor cells regulate microglia functions and activity. *Nat. Neurosci.* 15, 1485–1487. doi: 10.1038/nn.3233

Nakajima, K., Tohyama, Y., Maeda, S., Kohsaka, S., and Kurihara, T. (2007). Neuronal regulation by which microglia enhance the production of neurotrophic factors for GABAergic, catecholaminergic and cholinergic neurons. *Neurochem. Int.* 50, 807–820. doi: 10.1016/j.neuint.2007.02.006

Nakanishi, M., Niidome, T., Matsuda, S., Akaike, A., Kihara, T., and Sugimoto, H. (2007). Microglia-derived interleukin-6 and leukaemia inhibitory factor promote astrocytic differentiation of neural stem/progenitor cells. *Eur. J. Neurosci.* 25, 649–658. doi: 10.1111/j.1460-9568.2007.05309.x

Navascués, J., Calvente, R., Marín-Teva, J. L., and Cuadros, M. A. (2000). Entry, dispersion and differentiation of microglia in the developing central nervous system. *An. Acad. Bras. Cienc.* 72, 91–102. doi: 10.1590/s0001-37652000000100013

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308, 1314–1318. doi: 10.1126/science.1110647

Olah, M., Biber, K., Vinet, J., and Boddeke, H. W. (2011). Microglia phenotype diversity. *CNS Neurol. Disord. Drug Targets* 10, 108–118. doi: 10.2174/187152711794488575

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., et al. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science* 333, 1456–1458. doi: 10.1126/science.1202529

Pathania, M., Yan, L. D., and Bordey, A. (2010). A symphony of signals conducts early and late stages of adult neurogenesis. *Neuropharmacology* 58, 865–876. doi: 10.1016/j.neuropharm.2010.01.010

Peretto, P., Giachino, C., Aimar, P., Fasolo, A., and Bonfanti, L. (2005). Chain formation and glial tube assembly in the shift from neonatal to adult subventricular zone of the rodent forebrain. *J. Comp. Neurol.* 487, 407–427. doi: 10.1002/cne.20576

Perry, V. H., Anderson, P. B., and Gordon, S. (1993). Macrophages and inflammation in the central nervous system. *Trends Neurosci.* 16, 268–273. doi: 10.1016/0166-2236(93)90180-t

Pow, D. V., Perry, V. H., Morris, J. F., and Gordon, S. (1989). Microglia in the neurohypophysis associate with and endocytose terminal portions of neurosecretory neurons. *Neuroscience* 33, 567–578. doi: 10.1016/0306-4522(89)90409-0

Presta, M., Urbinati, C., Dell'era, P., Lauro, G. M., Sogos, V., Balaci, L., et al. (1995). Expression of basic fibroblast growth factor and its receptors in human fetal microglia cells. *Int. J. Dev. Neurosci.* 13, 29–39. doi: 10.1016/0736-5748(94)00065-b

Prinz, M., and Mildner, A. (2011). Microglia in the CNS: immigrants from another world. *Glia* 59, 177–187. doi: 10.1002/glia.21104

Rochefort, N., Quenech'du, N., Watroba, L., Mallat, M., Giaume, C., and Milleret, C. (2002). Microglia and astrocytes may participate in the shaping of visual callosal projections during postnatal development. *J. Physiol. Paris.* 96, 183–192. doi: 10.1016/s0928-4257(02)00005-0

Ryu, K. Y., Cho, G. S., Piao, H. Z., and Kim, W. K. (2012). Role of TGF-beta in survival of phagocytizing microglia: autocrine suppression of TNF-alpha production andoxidative stress. *Exp. Neurobiol.* 21, 151–157. doi: 10.5607/en.2012.21.4.151

Saijo, K., and Glass, C. K. (2011). Microglial cell origin and phenotypes in health and disease. *Nat. Rev. Immunol.* 11, 775–787. doi: 10.1038/nri3086

Schmechel, D. E., and Rakic, P. (1979). Arrested proliferation of radial glial cells during midgestation in rhesus monkey. *Nature* 277, 303–305. doi: 10.1038/277303a0

Sequerra, E. B., Costa, M. R., Menezes, J. R. L., and Hedin-Pereira, C. (2013). Adult neural stem cells: plastic or restricted neuronal fates? *Development* 140, 3303–3309. doi: 10.1242/dev.093096

Sequerra, E. B., Miyakoshi, L. M., Fróes, M. M., Menezes, J. R. L., and Hedin-Pereira, C. (2010). Generation of glutamatergic neurons from postnatal and adult subventricular zone with pyramidal-like morphology. *Cereb. Cortex* 20, 2583–2591. doi: 10.1093/cercor/bhq006

Seri, B., García-Verdugo, J. M., Collado-Morente, L., McEwen, B. S., and Alvarez-Buylla, A. (2004). Cell types, lineage and architecture of the germinal zone in the adult dentate gyrus. *J. Comp. Neurol.* 478, 359–378. doi: 10.1002/cne.20288

Shigemoto-Mogami, Y., Hoshikawa, K., Goldman, J. E., Sekino, Y., and Sato, K. (2014). Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone. *J. Neurosci.* 34, 2231–2243. doi: 10.1523/jneurosci.1619-13.2014

Shin, W. H., Lee, D. Y., Park, K. W., Kim, S. U., Yang, M. S., Joe, E. H., et al. (2004). Microglia expressing interleukin-13 undergo cell death and contribute to neuronal survival in vivo. *Glia* 46, 142–152. doi: 10.1002/glia.10357

Sierra, A., Encinas, J. M., Deudero, J. J., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L. S., et al. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell* 7, 483–495. doi: 10.1016/j.stem.2010.08.014

Streit, W. J. (2001). Microglia and macrophages in the developing CNS. *Neurotoxicology* 22, 619–624. doi: 10.1016/s0161-813x(01)00033-x

Su, P., Zhang, J., Zhao, F., Aschner, M., Chen, J., and Luo, W. (2014). The interaction between microglia and neural stem/precursor cells. *Brain Res. Bull.* 109, 32–38. doi: 10.1016/j.brainresbull.2014.09.005

Tang, X., Falls, D. L., Li, X., Lane, T., and Luskin, M. B. (2007). Antigen-retrieval procedure for bromodeoxyuridine immunolabeling with concurrent labeling of nuclear DNA and antigens damaged by HCl pretreatment. *J. Neurosci.* 27, 5837–5844. doi: 10.1523/jneurosci.5048-06.2007

Tavazoie, M., Van der Veken, L., Silva-Vargas, V., Louissaint, M., Colonna, L., Zaidi, B., et al. (2008). A specialized vascular niche for adult neural stem cells. *Cell Stem Cell* 3, 279–288. doi: 10.1016/j.stem.2008.07.025

Thanos, S., Fischer, D., Pavlidis, M., Heiduschka, P., and Bodeutsch, N. (2000). Glioanatomy assessed by cell-cell interactions and phagocytotic labelling. *J. Neurosci. Methods* 103, 39–50. doi: 10.1016/s0165-0270(00)00294-6

Tsuda, M., Masuda, T., Tozaki-Saitoh, H., and Inoue, K. (2013). Microglial regulation of neuropathic pain. *J. Pharmacol. Sci.* 121, 89–94. doi: 10.1254/jphs.12r14cp

Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., et al. (2013). Layer V cortical neurons require microglia support for survival during postnatal development. *Nat. Neurosci.* 16, 543–551. doi: 10.1038/nn.3358

Ventura, R. E., and Goldman, J. E. (2007). Dorsal radial glia generate olfactory bulb interneurons in the postnatal murine brain. *J. Neurosci.* 27, 4297–4302. doi: 10.1523/jneurosci.0399-07.2007

Vilhardt, F. (2005). Microglia: phagocyte and glia cell. *Int. J. Biochem. Cell Biol.* 37, 17–21. doi: 10.1016/j.biocel.2004.06.010

Voigt, T. (1989). Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. *J. Comp. Neurol.* 289, 74–88. doi: 10.1002/cne.902890106

Walton, N. M., Sutter, B. M., Laywell, E. D., Levkoff, L. H., Kearns, S. M., Marshall, G. P., et al. (2006). Microglia instruct subventricular zone neurogenesis. *Glia* 54, 815–825. doi: 10.1002/glia.20419

Yu, Y., and Ye, R. D. (2014). Microglial aβ receptors in Alzheimer's disease. *Cell. Mol. Neurobiol.* 35, 71–83. doi: 10.1007/s10571-014-0101-6

Ziv, Y., Ron, N., Butovsky, O., Landa, G., Sudai, E., Greenberg, N., et al. (2006). Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat. Neurosci.* 9, 268–275. doi: 10.1038/nn1629

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 December 2014; accepted: 21 January 2015; published online: 17 February 2015.

Citation: Xavier AL, Lima FRS, Nedergaard M and Menezes JRL (2015) Ontogeny of CX3CR1-EGFP expressing cells unveil microglia as an integral component of the postnatal subventricular zone. Front. Cell. Neurosci. ***9****:37. doi: 10.3389/fncel.2015.00037*

This article was submitted to the journal Frontiers in Cellular Neuroscience.