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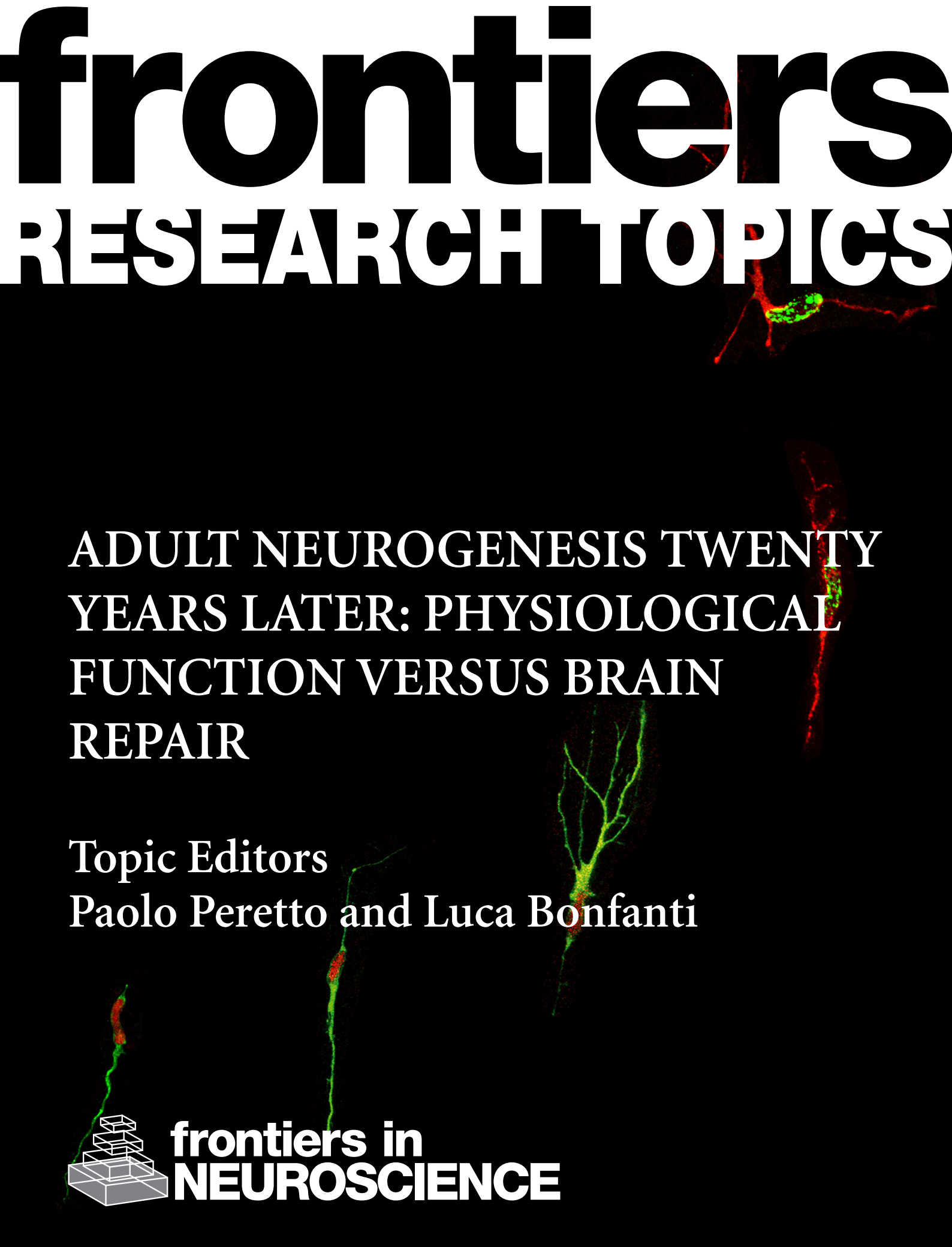

ADULT NEUROGENESIS TWENTY YEARS LATER: PHYSIOLOGICAL FUNCTION VERSUS BRAIN REPAIR

Topic Editors
Paolo Peretto and Luca Bonfanti

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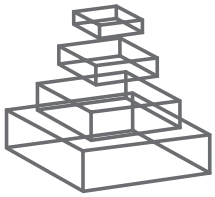

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ADULT NEUROGENESIS TWENTY YEARS LATER: PHYSIOLOGICAL FUNCTION VERSUS BRAIN REPAIR

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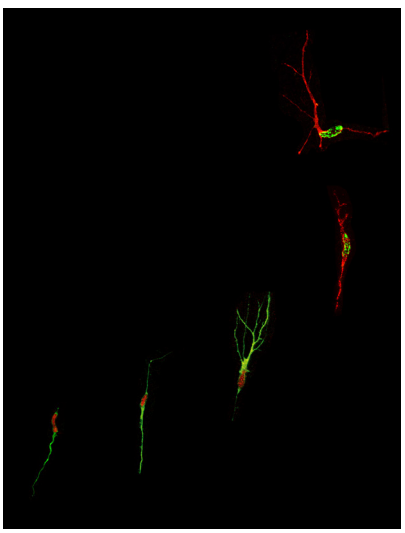

Different developmental stages of newly generated neurons in the peripuberal and adult rabbit cerebellar cortex.

The discovery that mammalian brains contain neural stem cells which perform adult neurogenesis - the production and integration of new neurons into mature neural circuits - has provided a fully new vision of neural plasticity. On a theoretical basis, this achievement opened new perspectives for therapeutic approaches in restorative and regenerative neurology. Nevertheless, in spite of striking advancement concerning the molecular and cellular mechanisms which allow and regulate the neurogenic process, its exploitation in mammals for brain repair strategies remains unsolved. In non-mammalian vertebrates, adult neurogenesis also contributes to brain repair/regeneration. In mammals, neural stem cells do respond to pathological conditions in the so called "reactive neurogenesis", yet without substantial regenerative outcome. Why, even in the presence of stem cells in the brain, we lack an effective reparative outcome in terms of regenerative neurology, and which factors hamper the attainment of this goal? Essentially, what remains unanswered is the question whether (and how) physiological functions of adult neurogenesis in mammals can be exploited for brain repair purposes.

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Adult neurogenesis and its promise as a hope for brain repair

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After the pioneer report by Joseph Altman of adult neurogenesis (AN) in mammals in 1962, the phenomenon of AN was "rediscovered" some 20 years later, first in songbirds and then in mammals. Since the 1990s, interest in AN was fueled by the hope that it could lead to the treatment of neurological deficits by grafting these neurons or their progenitors into brain areas affected by disease or injury. Unfortunately, after 20 years of intense research efforts there is no clear indication that AN can be harnessed for the repair of brain circuits. We argue that the exuberant optimism regarding the potential application of AN for brain repair was misguided by the belief that neurons and their precursors had extensive developmental plasticity. Many of the experiments investigating the potential of AN for brain repair were inspired by the idea that neuronal precursors would be able to adapt, and easily change their developmental fate to replace the lost neurons. However, research during the last 20 years has shown that, in most cases, the fate of neurons is strongly determined and that it rarely changes. Understanding the mechanisms that control neural cell fate may allow for the engineering of adult stem cells so that they can give rise to neurons with properties appropriate for the host circuit to be repaired. The lack of phenotypic flexibility of neuronal progenitors may eventually prove to be advantageous, as this may provide a high degree of predictability (and safety) in the properties of reprogrammed cells. We suggest that AN is still a useful model to understand how neurons integrate into adult brain circuits, and that brain repair will require a thorough understanding of the genetic programs that control neuronal fate and neuronal migration.

A cut in the skin is repaired within a few days, and a broken bone heals in a few weeks. In contrast, damage to the nervous system results in deficits that are only partially reversible. This limited ability for functional recovery led to the widely held belief that the brain and spinal cord are not able to regenerate. A direct challenge to this assumption was launched by the pioneer discovery by Joseph Altman who first described neurogenesis in the brain of adult rats (Altman, 1962). Using radioactively-tagged thymidine, Altman suggested that new neurons were added into several regions of the adult rat brain, including into the olfactory bulb (OB) and dentate gyrus (DG) (Altman and Das, 1965).

The initial discovery of Altman was mostly ignored for two decades, until Goldman and Nottebohm reported neurogenesis in the brain of adult canaries (Goldman and Nottebohm, 1983). However, the impact of this discovery was also limited, because many considered AN in birds an oddity that could not occur in mammals. So, despite a few occasional studies confirming the addition of new neurons into the brain of adult mammals in the 1970s and 1980s, the phenomenon of mammalian AN was outside of mainstream neuroscience for many years.

Interest in mammalian brain repair and AN gained momentum in the early 1990' with the discovery that the adult mouse brain contained stem cells that could be induced to proliferate *in vitro* by the addition of growth factors (Reynolds and Weiss, 1992). These stem cells grew to form aggregates of cells called "neurospheres" and differentiated into neurons and glia. The discovery of neurospheres galvanized the field of brain repair and for the first time provided researchers with a robust *in vitro* system to produce new neurons from adult mammalian brains in large quantities and revitalized the interest in neuronal transplantation as a brain repair strategy. The goal of neuronal transplantation is to add neurons into the brain to repair brain lesions. In AN, new neurons are spontaneously added into the functioning circuits of a mature brain. Thus, neuronal transplantation and AN inform each other reciprocally. Grafting different cell types into the brain provided novel insights into the factors regulating survival and integration of new neurons into brain circuits (Stenevi et al., 1976). Moreover, the techniques of neuronal transplantation enabled some key findings in AN. However, despite this progress, the clinical applications of grafting neurons have not materialized yet, and remains an experimental method for research.

In parallel with the progress in neuronal stem cell research and neuronal transplantation, important advances were made on the question of neurogenesis in the adult mammalian brain. In the early 1990s it was confirmed that there were two main areas of the rodent brain that received new neurons, the OB and the hippocampal DG. The progenitors of new neurons are located in the subventricular

zone (SVZ) and in the subgranular zone (SGZ) of the DG. The neuroblasts generated in the SVZ migrate long distances to reach the adult OB and differentiate into neurons (Lois and Alvarez-Buylla, 1994), suggesting that grafted neurons or progenitors could also have the potential to migrate throughout the brain. However, it was later found that only a specific type of neuronal progenitors derived from the medial ganglionic evidence (MGE) had the ability to disperse broadly (Wichterle et al., 1999).

Interestingly, the original reports by Altman suggested that new neurons may be added to brain regions such as the cerebral cortex and thalamus (Altman, 1962). The late 1990s saw a resurgence in the possibility that neurons are added outside of the OB and DG (for example into the neocortex, striatum or hippocampal CA1), both spontaneously or after lesions. Many of these reports have been surrounded by controversy, and the generally accepted view is that AN is mostly limited to the OB and DG. The possibility remains that low levels of AN exist in other brain regions (Ernst et al., 2014) and that under certain circumstances young neurons could be recruited into regions outside of the OB and DG.

The spectacular advances in animal cloning in the late 1990s, pioneered by the generation of Dolly the sheep, gave new impetus to the question of cellular phenotypic flexibility (Wilmut et al., 1997). If a fibroblast form the mammary gland could be reprogrammed to produce a whole sheep, perhaps a neuronal stem cell could be reprogrammed to produce any lost neuron in the brain. In the late 1990s several studies suggested that transplanted stem cells have great phenotypic flexibility. For example, it was reported that blood stem cells appeared to have the potential to become neurons in the brain. Most of these cases of "transdifferentiation" were likely due to fusion of the grafted cells and resident cells. Recent experiments rather indicate that neuronal stem cells are predetermined to generate specific neuronal types, and that altering the environment in which those progenitors differentiate does not change the type of neurons that they produce (Kelsch et al., 2007; Merkle et al., 2007).

The perspective gained from these last 20 years suggests that adult mammalian neurogenesis is mostly confined to the OB and DG, and that neuronal progenitors appear to have very limited (if any) phenotypic flexibility. In view of these constraints, it seems clear why the initial hopes for the therapeutic potentials of AN have not materialized. Next, we would like to suggest strategies so that AN could be harnessed for brain repair.

PROSPECTS OF ADULT NEUROGENESIS FOR BRAIN REPAIR: SUGGESTIONS

There are two main types of neurons produced in the mammalian brain during AN: granule neurons in the OB and the DG. In addition, other neuronal types are also produced in the OB and the DG, but in much smaller numbers (such as periglomerular cells for the OB).

Adult neurogenesis may be useful to replace the granule cells in the OB and DG in situations where these cells are lost to injury or disease. However, it is unlikely that grafting these cells or their progenitors could have any beneficial effects in any other brain regions, for two main reasons: First, transplantation of these cells leads to a mass of clumped cells at the graft site, and the vast majority of cells fail to migrate to colonize the surrounding parenchyma. If cells cannot disperse through the brain, they cannot reach the sites where they are needed, and thus, they cannot replace the neurons lost to disease or injury. Second, the proper function of brain circuits requires that neurons with defined properties perform specific functions. Both adult-born granule cells in the OB and DG have highly specialized properties. There is no evidence that grafting of progenitors can lead to a spontaneous reprogramming of their phenotypes. Thus, it is difficult to imagine how grafting cells with predetermined properties tailored for the function of a highly specialized circuit may lead to functional restoration of a completely different circuit in another part of the brain.

In summary, we believe that the therapeutic potential of the endogenous, unmanipulated neuronal progenitors in the mammalian brain may not be promising. At the same time, we believe that AN offers an outstanding opportunity to learn principles about how new neurons integrate into mature brain circuits, and that these principles can guide the use of engineered stem cells for brain repair.

We would like to offer some specific suggestions that we believe could be useful for the design of therapeutic strategies for brain repair:

Determination of cell fate: The appropriate function of a circuit requires neurons with specialized properties and defined patterns of connections. To maximize the chances that a new neuron will restore the function of a damaged circuit, it will be crucial to define the mechanisms by which a stem cell can be directed to a specific fate so that the new neuron has properties as similar as possible as those of the lost neurons. Identifying the genetic programs that control neuronal differentiation will eventually allow for the generation of neuronal populations with well-defined identity. However, for some applications the requirements for specific neuronal identity may not be so crucial. For example, for neurological disorders due to hyperactivity (e.g., epilepsy), the addition of inhibitory neurons that could simply reduce the overall levels of activity could be sufficient to ameliorate the outcome of the disease.

Migratory ability: One of the main hurdles for neuronal replacement therapies is due to the very limited ability of grafted neurons to migrate through the adult brain so that they can reach the sites where neurons have been lost. Currently, the only neuronal progenitors that can migrate extensively through the adult brain are those from the embryonic medial ganglionic eminence (MGE). Unfortunately, the MGE progenitors only give rise to inhibitory interneurons. Transplanting interneurons could be a useful intervention to control neurological diseases characterized by hyperactivity, such as epilepsy. However, it is unlikely that transplanting MGE progenitors could restore function in the most common neurological disorders, such as Alzheimer's, Parkinson's or stroke, which are characterized by neuronal loss. There are 2 avenues that would be worth exploring regarding these issues. First, we should investigate the molecular mechanisms that enable MGE progenitors to disperse widely through the brain.

Perhaps delivering the genes that confer the migratory ability of MGE cells into neuronal stem cells for other cell types (e.g., stem cells for projection neurons) would enable them to disperse through the brain. Second, MGE cells could be engineered so that they retain their migratory ability, but can be tailored to switch their cell fate, and thus, they could give rise to other neuronal types after they complete their migration.

Integration into brain circuits: After young neurons have completed their migration they have to form appropriate synaptic connections with the pre-existing neurons. Perhaps this could the most challenging of all aspects of brain repair. The mechanisms that control synapse formation, selection, and maintenance are only partially understood, and these are highly complex processes. Moreover, after brain lesions new neurons will face damaged circuits, thus increasing the difficulty of making the correct synaptic connections. Again, AN could offer crucial clues about how this process could be recapitulated with grafted neuronal progenitors/stem cells. Two areas of research could be fruitful to enable the appropriate wiring of young neurons into adult circuits.

- We still do not have a good understanding of the role of electrical activity on the formation and maintenance of synapses as new neurons integrate into brain circuits during AN. For example, it is not clear what are the respective roles of the activity of the pre-existing neurons vs. the cell-autonomous activity of the new neurons on the integration of the young neurons (Lin et al., 2010). Investigating these issues could offer important clues to design strategies to optimize the integration of grafted young neurons into the brain.
- Second, identifying molecular pathways that regulate the formation of specific synapses between neurons could lead to a strategy where these molecules are expressed in the young neurons to "force" the formation of synapses.

Fifty years after the discovery of AN the field is realizing that some of the initial hopes to use this process for brain repair were naively optimistic. The assembly of brain circuits is an extremely complicated process, and it is not realistic to hope that young neurons would "know what to do" when faced with a damaged brain to restore its structure and function. Dispersion through the adult brain is a major bottleneck that has to be resolved to achieve efficient brain repair. We now know that there are strong genetic determinants that define the identity of the progeny produced by neuronal progenitors, and that young neurons (endogenous or grafted) do not have the ability to "adapt" their identities to the needs of the circuit (Kelsch et al., 2007; Merkle et al., 2007). Finally, we need to understand the principles that guide the formation of synapses between young and pre-existing neurons to enable the appropriate wiring of the circuit. Using neuronal replacement to achieve brain appears a daunting prospect, but we know that addition of new neurons into pre-existing circuits is exactly what happens during AN. Adult-born neurons may not be useful to repair the brain, but learning from AN should guide our attempts to use engineered stem cells to achieve this goal.

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Notching up neural stem cell homogeneity in homeostasis and disease

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Adult neural stem cells (NSCs) are perceived as a homogeneous population of cells that divide infrequently and are capable of multi-lineage differentiation. However, recent data revealed that independent stem cell lineages act in parallel to maintain neurogenesis and provide a cellular source for tissue repair. In addition, even within the same lineage, the stem and progenitor cells are strikingly heterogeneous including NSCs that are dormant or mitotically active. We will discuss these different NSC populations and activity states with relation to their role in neurogenesis and regeneration but also how these different stem cells respond to aging. NSCs depend on Notch signaling for their maintenance. While Notch-dependence is a common feature among NSC populations, we will discuss how differences in Notch signaling might contribute to adult NSC heterogeneity. Understanding the fate of multiple NSC populations with distinct functions has implications for the mechanisms of aging and regeneration.

Keywords: Notch signaling, subventricular zone, hippocampal dentate gyrus, neurogenesis, aging

INTRODUCTION

Neural stem cells (NSCs) remain neurogenic during postnatal life within two regions of the mammalian brain, the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the wall of the lateral ventricles. The discovery of adult neurogenesis in mammals opened new avenues for research in regenerative medicine, with a goal of manipulating endogenous NSCs for brain repair (Gage and Temple, 2013). Over the last twenty years we have made considerable progress in our understanding of adult neurogenesis in homeostasis and disease (Ming and Song, 2011). However, several gaps remain in our knowledge that have to be filled before the potential of adult NSCs for regeneration can be fully exploited.

One such gap, and a field of active research, relates to the presence of heterogeneous subpopulations of NSCs within the same neurogenic niche (Alvarez-Buylla et al., 2008; Bonaguidi et al., 2012). The properties, functions, and lineage relationships between these subpopulations are still ill defined. Notch signaling is one molecular pathway involved in the regulation of NSC maintenance, proliferation, quiescence, and cell fate decisions. We propose that Notch signaling strength and diversity of downstream targets might, in concert with other molecular pathways, niche signals, and intrinsic factors, contribute to establishing adult NSC heterogeneity.

THE ADULT NEUROGENIC LINEAGE IN SHORT

Work from the 90's and early 2000's discovered the sequential developmental stages that cells undergo during lineage progression within the adult neurogenic niches (Alvarez-Buylla et al., 2001; Kempermann et al., 2004). Adult NSCs share ultrastructural and antigenic features with astrocytes (Kriegstein and Alvarez-Buylla, 2009). Most adult NSCs are quiescent or intermittently dividing, which explains their resistance to antimitotic drug and high dose tritiated-thymidine treatment (Morshead et al., 1994; Doetsch et al., 1999; Seri et al., 2001). In the SVZ, NSCs that proliferate likely undergo asymmetric cell divisions to self-renew and generate committed intermediate progenitors (IPs). These mitotically active IPs generate neuroblasts that migrate to the olfactory bulb (OB) where they mature into multiple types of neurons (Alvarez-Buylla et al., 2008). In the SGZ, NSCs also generate IPs that pass through a series of maturation stages before they differentiate into neuroblasts and subsequently granule neurons of the DG. This early work already implied some degree of heterogeneity in adult NSC and IP populations (Kempermann et al., 2004), suggesting that progression through the neurogenic lineage may be a more complex process than the simple NSC to IP to neuroblast transition. Later work added new insights into this emerging complexity and even proposed the coexistence of independent stem cell lineages acting in parallel.

ADULT NEURAL STEM AND PROGENITOR CELL HETEROGENEITY WITHIN THE SAME LINEAGE

The ability of adult NSCs to survive antimitotic drug treatment and regenerate the neurogenic lineage suggested that they rarely divide (Doetsch et al., 1999; Seri et al., 2001). This relative quiescence of NSCs is potentially linked to their potential for long-term maintenance over the lifespan of an organism (Kippin et al., 2005; Porlan et al., 2013). Indeed, quiescence of adult NSCs is actively promoted through several mechanisms including BMP and Notch signaling (Imayoshi et al., 2010; Mira et al., 2010), neurotransmitters (Berg et al., 2013), cell adhesion molecules (Kokovay et al., 2012), cell-cycle inhibitors (Kippin et al., 2005), and intrinsic factors (Martynoga et al., 2013). However, quiescence is not a prerequisite for somatic stem cell identity, and proliferating stem cell exist alongside more quiescent stem cell pools in several organs (Li and Clevers, 2010). A similar situation

of dual stem cell populations may also exist in the neurogenic niches of the adult mammalian brain. A small but detectable fraction of adult NSCs proliferate quite rapidly in both the SVZ and SGZ (Encinas et al., 2011; Ponti et al., 2013). Even though NSCs can shuttle between active and quiescent states (Lugert et al., 2010; Bonaguidi et al., 2011), not all NSCs that proliferate re-enter a quiescent state, but some divide repeatedly (Lugert et al., 2010; Encinas et al., 2011; Ponti et al., 2013). Similarly, although quiescent NSCs enter cell-cycle to generate active NSCs (Bonaguidi et al., 2011; Encinas et al., 2011) and their exit from the quiescent state is augmented in response to neurogenic stimuli and during regeneration (Morshead et al., 1994; Doetsch et al., 1999; Lugert et al., 2010; Lopez-Juarez et al., 2013), it remains unclear what proportion of NSCs can be activated during homeostatic neurogenesis. Thus, it is possible that active and quiescent NSCs represent distinct pools where transition between the two is limited and tightly controlled, at least in the absence of injury, rather than all adult NSCs being equal in their ability to switch from quiescence to activity and back.

Discrimination of quiescent from activated NSC populations and niche astrocytes has proven difficult, but progress has been made toward this end in recent years (Pastrana et al., 2009; Beckervordersandforth et al., 2010; Mira et al., 2010; Ferron et al., 2011). Active NSCs within the SVZ express the epidermal growth factor receptor, respond to EGF with increased proliferation, and are eliminated by antimitotic drug treatment, similar to IPs, but retain expression of astrocytic and radial glia markers (Pastrana et al., 2009; Giachino et al., 2014). In the adult DG, a non-radial morphology correlates with the active state (Suh et al., 2007; Lugert et al., 2010), but molecular markers that demarcate quiescent and active NSC populations have still to be defined. Interestingly, genetic lineage tracing using promoters that potentially over-represent either quiescent or active cells suggest differences in the contribution of these populations to long-term neurogenesis (DeCarolis et al., 2013). Identifying markers that discriminate between NSCs with distinct proliferative activities is needed to develop genetic tools for independent and simultaneous lineage tracing of active and quiescent NSC pools, and determine their respective lifespan and inter-conversion *in vivo*. Lineage tracing approaches with inducible-cre/flp technologies and intersectional analyses combining promoters of cell-cycle marker genes with NSC marker genes may be useful to this end.

Heterogeneity in the adult neurogenic lineage is not restricted to NSCs. In the SVZ, a proportion of Ascl1+ IPs expresses radial glia markers whereas others do not (Pastrana et al., 2009; Giachino et al., 2014). In the SGZ, a combination of Nestin, Ascl1, Tbr2, and Doublecortin expression defines at least three stages of committed progenitors (Kempermann et al., 2004; Hodge et al., 2008). It is still unclear if marker heterogeneity of IPs reflects differences in their self-renewal abilities, but it is interesting that differences in cell-cycle dynamics of IP populations in the SVZ have been recently reported (Ponti et al., 2013). Moreover, late IPs and neuroblasts make up a large fraction of proliferating cells in both neurogenic niches (Hodge et al., 2008; Lugert et al., 2012; Ponti et al., 2013), proposing that substantial lineage amplification occurs right before cell-cycle exit and terminal differentiation.

HETEROGENEOUS NSC LINEAGES ACTING IN PARALLEL

Adult NSCs are capable of multi-lineage differentiation and able to clonally generate multiple neural lineages under appropriate conditions *in vitro* (Suh et al., 2007; Pastrana et al., 2011). Moreover, they show some degree of lineage plasticity upon heterotopic or heterochronic transplantation into neurogenic niches distinct to their origin (Suhonen et al., 1996; Sequerra et al., 2010). Indeed, elegant lineage tracing experiments demonstrated that DG NSCs are bi-potent at the single cell level and self-renew *in vivo* (Suh et al., 2007; Bonaguidi et al., 2011). SVZ NSCs are able to generate neurons, astrocytes and oligodendrocytes at the population level *in vivo* (Alvarez-Buylla et al., 2001; Menn et al., 2006; Benner et al., 2013). However, and apparently in contrast to these findings, other fate-mapping and transplantation studies demonstrated that SVZ NSCs are heterogeneous and their spatial distribution correlates with cell fate (Kelsch et al., 2007; Merkle et al., 2007). NSCs show a preference toward the production of distinct types of neurons depending on their dorsal-ventral position within the adult SVZ, and this differentiation bias seems to be intrinsic as it is maintained after *in vitro* passage and heterotopic transplantation (Merkle et al., 2007). In the same line, NSCs cultured in the absence of growth factors are able to generate either neurons or oligodendrocytes, in addition to astrocytes, but not both neurons and oligodendrocytes within the same lineage tree (Ortega et al., 2013). NSCs with oligodendrocytic potential are enriched in the dorsal and anterior SVZ (Gritti et al., 2002; Ortega et al., 2013). Genetic fate mapping indicates that these heterogeneous NSC populations in the adult SVZ are remnants of distinct germinative niches of the embryonic forebrain and derive from Emx1, Dbx1, Gsh2, Nkx2.1 or Nkx6.2 expressing lineages (Kohwi et al., 2007; Ventura and Goldman, 2007; Young et al., 2007; Lopez-Juarez et al., 2013; Merkle et al., 2014). These data suggest that the SVZ is arranged as a mosaic that is established during embryogenesis and the location within the niche can predict the type of progeny that NSCs give rise to. Whether this heterogeneity of lineages is intrinsically determined or regulated by extrinsic cues at distinct locations within the niche, or a combination of both, is still a matter of debate (Sequerra et al., 2013). The fact that manipulation of morphogen signals skews NSC differentiation potential *in vivo* suggest that the local environment plays a role in regional specification of adult NSCs (Ihrie et al., 2011). Moreover, pathological conditions stimulate NSCs to produce progenies that are different from the ones generated during homeostasis and these can migrate toward non-canonical locations, suggesting that cell fate is not fixed (Kernie and Parent, 2010; Ohira, 2011). Defining the degree of lineage plasticity of adult NSCs and the signals that can override their intrinsic programming has important implications for developing cell replacement strategies based on the mobilization of endogenous cells.

CONTRIBUTION OF NOTCH SIGNALING TO HETEROGENEITY OF THE ADULT NSC POOL

Notch signaling is a key mediator of NSC maintenance in the developing and adult brains (Ables et al., 2011; Pierfelice et al., 2011). Notchs are transmembrane receptors and undergo a series of proteolytic cleavages that liberate the Notch intracellular domain (NICD) upon binding to their ligands. The canonical

Notch signal links the NICD to the nuclear CSL (RBP-J in mice) transcriptional complex (Mumm and Kopan, 2000). The activity of Notch target genes of the *Hes*/*Hey* family is fundamental for maintaining NSCs in an undifferentiated state, suppressing the expression of proneural genes including Ascl1 (Louvi and Artavanis-Tsakonas, 2006). Inhibition of Notch or RBP-J in adult NSCs results in NSC loss and impaired neurogenesis (Breunig et al., 2007; Andreu-Agullo et al., 2009; Ables et al., 2010; Aguirre et al., 2010; Chapouton et al., 2010; Ehm et al., 2010; Imayoshi et al., 2010; Lugert et al., 2010; Imayoshi and Kageyama, 2011). Canonical Notch signaling activity and *Hes5* expression in particular distinguish NSCs from IPs in the developing and adult brain (Basak and Taylor, 2007; Mizutani et al., 2007; Andreu-Agullo et al., 2009; Imayoshi et al., 2010; Lugert et al., 2010; Giachino et al., 2014). It is unclear if Notch signaling contributes to adult NSC heterogeneity, but studies revealing that impairing distinct Notch pathway components affects distinct stages of the neurogenic lineage suggest that this might be the case. Notch1 promotes adult NSC proliferation whilst maintaining the undifferentiated state, proposing a role of the pathway in the maintenance of the active NSC subpopulation (Nyfeler et al., 2005; Androutsellis-Theotokis et al., 2006; Breunig et al., 2007; Ables et al., 2010; Aguirre et al., 2010; Basak et al., 2012). In contrast, activation of the RBP-J mediated canonical Notch pathway, potentially promoted by Dll1 ligand expressed by proliferating NSCs and IPs, preserves the quiescent NSC pool (Carlen et al., 2009; Ehm et al., 2010; Imayoshi et al., 2010; Basak et al., 2012; Kawaguchi et al., 2013). These data suggest that Notch can differentially regulate active and quiescent NSC populations in a context-dependent manner. Given that *Hes5* is expressed in both dividing and dormant adult NSCs, Notch is unlikely to be the only key to NSC heterogeneity (Lugert et al., 2010; Giachino et al., 2014). However, differences in the strength, dynamics, and targets of the pathway may contribute to heterogeneity and explain the discrepancies observed in mouse mutant phenotypes. Low levels of Notch activation (NICD) for instance promote proliferation of embryonic neural progenitors, whereas high levels lead to growth arrest *in vitro* (Guentchev and McKay, 2006). Therefore, one possibility is that the differential actions of Notch on proliferation vs. quiescence of adult NSCs are a function of dose. Another possibility is that oscillatory vs. sustained expression of Notch targets differentiate active and quiescent NSC pools. In mouse embryonic NSCs and progenitors, levels of Hes1/Hes5 oscillate in anti-phase with their repressed target Ascl1, and this oscillatory expression promotes proliferation (Imayoshi et al., 2013). In contrast, sustained Hes1 expression inhibits NSC proliferation and neurogenesis in the developing central nervous system (Baek et al., 2006). It is currently not known if the expression of *Hes* genes is sustained or oscillatory in adult NSCs, and the available tools are not adequate to address this issue *in vivo*. However, oscillatory expression of Notch targets together with low refractory expression of Ascl1 in adult NSCs that are in the cell-cycle would be compatible with data showing that the *Ascl1::CreER* locus can be used to lineage trace a subpopulation of neurogenic NSCs *in vivo* (Kim et al., 2011). Conversely, quiescent adult NSCs express high levels of Id proteins (Nam and Benezra, 2009), which can interfere with the negative autoregulation of *Hes1* and therefore modulate its oscillatory expression (Bai et al., 2007). These findings prompt to speculate that *Hes* genes are persistently expressed at high levels in quiescent NSCs.

Another possible scenario to explain the multifaceted functions of Notch in adult NSCs would be that distinct pathway components are differentially engaged in Notch signaling activity in different cell subpopulations. It has been proposed that Notch1 is required to maintain the active adult NSC pool but is dispensable during quiescence (Ables et al., 2010; Basak et al., 2012). This suggests that other members of the Notch family could provide a maintenance signal for quiescent NSCs and compensate for the absence of Notch1 in this population (Basak et al., 2012). Heterogeneity of Notch activity is related to cellular diversity in the neurogenic niches of the adult zebrafish brain, where Notch3 gates NSC activation whereas Notch1b maintains activated progenitors (Alunni et al., 2013). Interestingly, Notch3 restricts stem cell activation in muscle and mammary, while Notch1 is associated with proliferation (Carlson et al., 2008; Kitamoto and Hanaoka, 2010; Bjornson et al., 2012; Mourikis et al., 2012; Lafkas et al., 2013). It is tempting to speculate that distinct Notch receptors differentially regulate quiescent and active stem cell subpopulations in the adult mammalian brain. In addition to NSC heterogeneity within the same lineage, independent NSC lineages are fated to generate distinct OB neurons in the SVZ. Lineage tracing experiments with *Hes5::CreER* transgenic mice demonstrated that NSCs with active canonical Notch signaling generate multiple neuron subtypes in the OB (Giachino et al., 2014) implying that all adult NSC lineages require Notch for maintenance. However, this does not exclude that different receptors or receptor combinations mediate Notch activity within the NSCs of each lineage. Interestingly, genetic fate mapping of cells expressing individual Notchs revealed multiple lineages in bone marrow and mammary gland (Lafkas et al., 2013; Oh et al., 2013; Sale et al., 2013).

Not only the activity of Notch paralogues, but also combinations of different Notch target genes in NSC subpopulations may be a source of heterogeneity. NSCs in the developing neural tube of the mouse are heterogeneous based on their expression of *Hes* family genes, with cohorts of radial glia cells expressing either *Hes1* or *Hes5*, or both (Basak and Taylor, 2007; Nelson et al., 2013). Brain lipid binding protein (BLBP), a direct target of Notch signaling (Anthony et al., 2005), is also expressed heterogeneously by radial glia (Hartfuss et al., 2001). Recently, it was shown that different combinations of *Her/Hes* family genes correlate with the proliferation rate of NSCs and progenitors in the adult zebrafish (Chapouton et al., 2011). In the same line, BLBP expression positively correlates with proliferation of *Hes5*-expressing NSCs in the adult mouse SVZ, whereas most quiescent NSCs express *Hes5* but not BLBP (Giachino et al., 2014). How combinatorial or selective expression of Notch targets regulates the fate and activity of adult NSC subpopulations deserves closer scrutiny in the future.

IMPLICATIONS OF ADULT NSC HETEROGENEITY FOR AGING AND REGENERATION

The adult mammalian neurogenic niches show a remarkable capacity for self-repair and remodeling in response to lesion (Doetsch et al., 1999; Seri et al., 2001; Kuo et al., 2006; Nomura et al., 2010). However, neurogenesis declines with age in both

the SVZ and SGZ (Lazarov et al., 2010) and most neuron subtypes produced in the OB are equally affected (Shook et al., 2012). Understanding the basis of this overall age-related decline in neurogenesis, and the mechanisms controlling self-renewal over the life on an individual, is fundamental to exploit the regenerative potential of adult NSCs.

One cause of reduced neurogenesis during aging is the impairment of the NSC compartment. An open question is whether this impairment reflects depletion or increased quiescence of NSCs (Hattiangady and Shetty, 2008; Lugert et al., 2010; Encinas et al., 2011; Shook et al., 2012; Giachino et al., 2014). This issue remains debated as most studies did not take into account the differential effects that aging may have on NSC subpopulations. One possibility is that active NSCs are more susceptible to imbalances between maintenance and differentiation signals that may occur during aging. These imbalances affect self-renewal, and therefore active NSCs are preferentially lost compared to quiescent populations (Lugert et al., 2010; Encinas et al., 2011; Giachino et al., 2014). Furthermore, some quiescent NSCs may activate to replenish an exhausted active NSC pool and thereby also be lost during aging. This is supported by findings that quiescent NSCs are reduced with time following depletion of the active NSC pool either by genetic means (Basak et al., 2012), or chronic treatment with antimitotic drugs (Doetsch et al., 1999). However, a variable but substantial proportion of NSCs is preserved in both the SGZ and SVZ of old mice, but these are dormant rather than senescent and can be reactivated (Jin et al., 2003; Hattiangady and Shetty, 2008; Lugert et al., 2010; Giachino et al., 2014). It will be important to genetically trace the progeny of distinct NSC populations during aging to assess differences in their lifespan, lineage plasticity and regenerative potential. Understanding the mechanisms responsible for NSC dormancy in the mouse neurogenic niches could help to understand why most NSCs in the human brain stop generating neurons after birth (Sanai et al., 2011).

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Cell cycle activity of neural precursors in the diseased mammalian brain

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Basic research during embryonic development has led to the identification of general principles governing cell cycle progression, proliferation and differentiation of mammalian neural stem cells (NSC). These findings were recently translated to the adult brain in an attempt to identify the overall principles governing stemness in the two contexts and allowing us to manipulate the expansion of NSC for regenerative therapies. However, and despite a huge literature on embryonic neural precursors, very little is known about cell cycle parameters of adult neural, or any other somatic, stem cell. In this review, we briefly discuss the long journey of NSC research from embryonic development to adult homeostasis, aging and therapy with a specific focus on their quiescence and cell cycle length in physiological conditions and neurological disorders. Particular attention is given to a new important player in the field, oligodendrocyte progenitors, while discussing the limitation hampering further development in this challenging area.

Keywords: cell cycle, neural stem cells, oligodendrocyte precursor cell (OPC), neurodegenerative diseases, adult neurogenesis

INTRODUCTION

The study of the cell cycle is one of the most prolific areas in developmental neuroscience with hundreds of publications spanning half a century and contributing new methodologies, basic knowledge and a deeper understanding of brain development and evolution (Fujita, 1962; Schultze and Korr, 1981; Takahashi et al., 1995; Dehay and Kennedy, 2007; Salomoni and Calegari, 2010; Borrell and Calegari, in press).

Cell cycle regulation in itself is a huge field and several reviews already discussed its molecular control during brain development and adulthood (Dehay and Kennedy, 2007; Salomoni and Calegari, 2010; Beukelaers et al., 2011b). As one factor fuelling interest in this area, short cell cycles were found to correlate with a higher proliferative potential of neural precursors at the cellular and tissue level and across phylogeny (Borrell and Calegari, in press). This correlation led to functional manipulations showing that the proliferative potential of neural precursors is increased by shortening their cell cycle while, conversely, lengthening it leads to differentiation and neurogenesis (Calegari and Huttner, 2003; Lange et al., 2009; Pilaz et al., 2009; Artegiani et al., 2011; Beukelaers et al., 2011a).

Considering that the first calculation of the cell cycle during development coincided with the first report on adult neurogenesis five decades ago (Altman, 1962; Fujita, 1962) and that immense efforts are currently invested worldwide in stem cell research and regenerative medicine, it comes as a surprise that cell cycle studies during adulthood, contrary to development, are extremely limited with only a handful addressing the diseased brain. Here we summarize our knowledge on cell cycle parameters of adult neural precursors in physiological and pathological conditions with particular attention to a new player in biomedicine, oligodendrocyte progenitors. This is important to identify potential correlations of biological significance and to identify our gaps in knowledge that the field should address in the years to come.

NEUROGENIC PRECURSORS

CELL CYCLE IN PHYSIOLOGICAL CONDITIONS

Mammalian NSC generate neurons and glia throughout life within two restricted areas: the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles (Zhao et al., 2008; Kriegstein and Alvarez-Buylla, 2009). In both niches a pool of NSC, progenitors and neuroblasts coexist in a dynamic system in which the production of neurons is regulated by intrinsic and extrinsic factors (Lois and Alvarez-Buylla, 1994; Cameron and McKay, 2001). Similarly to their embryonic precursors (Merkle et al., 2004; Li et al., 2013), adult NSC maintain a radial morphology (Doetsch et al., 1999; Seri et al., 2001), contact blood vessels (Palmer et al., 2000; Tavazoie et al., 2008) and share common markers (Kriegstein and Alvarez-Buylla, 2009). However, in contrast to embryonic development no unique marker has been identified that exclusively labels one, but not others, precursor types (Ming and Song, 2011). In addition, no positive marker of quiescent cells is available to date. These limitations, together with the fact that a significant proportion of NSC are quiescent, makes it remarkably difficult to assess cell cycle parameters during adulthood.

In the SGZ, dividing NSC (type 1) give rise to intermediate progenitors (type 2) that in turn generate neuroblasts (type 3) producing granule neurons (Seri et al., 2001; Kempermann et al., 2004). The significance of adult hippocampal neurogenesis is not fully understood but evidence points to a role in learning and memory (Kempermann, 2008; Deng et al., 2010). With regard to the lineage of hippocampal NSC, studies have calculated that type 1 cells undergo 3–4 asymmetric divisions before becoming

postmitotic astrocytes (Encinas et al., 2011) while others have concluded that at least some type 1 cells can self-renew unlimited times throughout life (Bonaguidi et al., 2011). Despite this controversy, studies attempting to measure the cell cycle in the adult hippocampus found that cycling NSC divide every about 1 day (Lugert et al., 2010; Encinas et al., 2011; Brandt et al., 2012) with S being the most variable phase among progenitors (Brandt et al., 2012). In particular, type 1 cells complete the cell cycle in 23 h with an S-phase of 10 h (Brandt et al., 2012). Subsequently, type 2 cells lengthen to 27 h while type 3 shorten again to 23 h. Although G1, G2, and M were not individually measured, cell cycle differences were found to be almost exclusively due to S-phase (**Table 1**) (Brandt et al., 2012).

The SVZ is the second and most proliferative neurogenic niche of the adult mammalian brain (Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011). Here, NSC (B cells) have an apical process intercalating between ependymal cells and contacting the ventricle (Mirzadeh et al., 2008) and a basal process contacting blood vessels (Shen et al., 2008). B cells give rise to amplifying progenitors (C cells) that generate migrating neuroblasts (A cells) dividing along the SVZ and rostral migratory stream toward the olfactory bulb where they ultimately differentiate into neurons (Petreanu and Alvarez-Buylla, 2002; Ming and Song, 2011). At any given time, B cells represent 10% of all cycling cells (Doetsch et al., 2002) with a similar proportion being cycling as opposed to quiescent (Ponti et al., 2013). B cells complete the cell cycle in 18 h and G1 and S in 8 and 5 h, respectively (**Table 1**) (Ponti et al., 2013). Regarding C cells, these represent over 60% of proliferating cells (Doetsch et al., 2002) with nearly 90% of them cycling at any given time (Ponti et al., 2013). C cells are proposed to divide symmetrically 2–3 times before generating A cells and have more heterogeneous cell cycles of 17–22 h, a longer S-phase of 12–15 h and a remarkably short G1 of 2 h (Ponti et al., 2013). Finally, A cells account for 26% of dividing cells in the SVZ (Doetsch et al., 2002), have a cell cycle similar to C cells with perhaps a longer G1 (2–5 h) and shorter S of 10 h (**Table 1**) (Ponti et al., 2013).

Altogether, cell cycle differences within neurogenic niches seem minor with the only consistent change being a lengthening of S-phase from NSC to progenitors and shortening from progenitors to neuroblasts. Not only is the significance of such changes unknown but no parallelism is evident between embryonic and adulthood precursors because in the former S-phase was found to be longer in NSC than in progenitors (Arai et al., 2011) and G1 during adulthood was found to length from C to A cells but not from B to C cells (Ponti et al., 2013).

CELL CYCLE IN PATHOLOGICAL CONDITIONS

Neural progenitors increase their proliferation, meant both as exiting quiescence and shortening the cell cycle, under pathological conditions in both neurogenic niches (Dash et al., 2001; Arvidsson et al., 2002). Most studies focused on the SVZ where neural precursors change their migration and are redirected to the injured area to acquire the phenotype of local cells (Arvidsson et al., 2002), thus, making the SVZ a potential target of therapy. Increased proliferation and altered migration were found in rodent models of multiple sclerosis (Rasmussen et al., 2011; Mecha et al., 2013), Huntington's disease (Tattersfield et al., 2004), Parkinson (Aponso et al., 2008) and stroke (Thored et al., 2006), the latter of which was also shown in humans (Jin et al., 2006; Minger et al., 2007). Among these diseases, the neurogenic response triggered by stroke is the most prominent and best characterized.

Stroke is a cerebrovascular accident resulting in a permanent damage and second leading cause of death worldwide (WHO, 2013). Two days after striatal stroke, SVZ precursors shorten the cell cycle form 19 to 12 h due to a shorter G1 from 13 to 8 h and S from 5 to 2 h (**Table 1**) (Zhang et al., 2006, 2008). Subsequently, cell cycle progressively lengthens reaching normal values 14 days after stroke. Interestingly, the proportion of proliferative, as opposed to differentiative, divisions increases (from 10 to 50%) during the period of short cell cycles and, conversely, decreases (back to 10%) during the long ones (Zhang et al., 2008) although it is important to note that in these studies precursor types were not distinguished and that these values refer to the total SVZ population. Nevertheless, stroke results in an increasing cohort of neuroblasts migrating from the SVZ toward the striatum with a peak at day 14 (Zhang et al., 2004) and lasting for at least 4 months (Arvidsson et al., 2002; Thored et al., 2006; Yamashita et al., 2006). Most of these newborn neurons undergo apoptosis but those that survive functionally integrate (Yamashita et al., 2006; Hou et al., 2008) with evidence indicating that this endogenous neurogenesis can contribute to functional recovery after stroke since, for instance, ablation of neural precursors impairs recovery (Jin et al., 2010; Sun et al., 2013). Yet, it still has to be shown whether an artificial increase in endogenous neurogenesis would favor brain function.

Altogether, cell cycle parameters of precursor cells after stroke recapitulate embryonic development in the sense that short cell cycles are coupled to proliferation and long cell cycles to differentiation. In this context, cell cycle re-entry is likely instrumental to guide the stroke-induced neurogenic response and, in fact, activation of cell cycle regulators is known to occur in both rodents and humans (Love, 2003; Rashidian et al., 2007). Yet, this response in postmitotic neurons is likely to induce apoptosis rather than cell cycle re-entry (Rashidian et al., 2007). This is supported by the fact that injecting a cdk inhibitor in the ischemic area reduces apoptosis and extension of the ischemic core (Osuga et al., 2000) suggesting that cell cycle regulators have different effects in postmitotic neurons or precursors, which should be considered in manipulations aimed to improve recovery. In this context, increasing the endogenous pool of neurogenic precursors by manipulating their cell cycle seems a promising approach to therapy. Moreover, other targets have recently emerged including ependymal cells (Carlen et al., 2009) and glial precursors (Zhang et al., 2011). To our knowledge cell cycle of ependymal cells during neurodegeneration has not been assessed while some groups are now pioneering the study of gliogenesis.

GLIOGENIC PRECURSORS

CELL CYCLE IN PHYSIOLOGICAL CONDITIONS

Astrocytes and oligodendrocytes are the most abundant cell type of the adult brain with the latter gaining more interest for their role and potential use during brain recovery (Richardson et al., 2011; Tsai et al., 2012).

Table 1 | Cell cycle parameters of neurogenic and oligodendrogenic precursors.

precursors	CNS area	cell or tissue type	GF (%)	G1	S	G2+M	cell cycle
neurogenic precursors	hippocampus	type 1	-	-	10 h	-	23 h
		type 2	-	-	14 h	-	27 h
		type 3	-	-	10 h	-	23 h
	SVZ	B cells	9	8 h	5 h	6 h	18 h
		C cells	87	2 h	12-15 h	4 h	17-22 h
		A cells	-	2-5 h	9-11 h	4 h	17-20 h
		total cells	20 / 24	13 h / 8 h	4-5 h / 2 h	2 h / 2 h	19 h / 12 h
oligodendrogenic precursors	brain	white matter	30-99	-	0.8 d	-	10 d
		grey matter	50-98	-	1 d	4 d*	36 d
	spinal cord	white matter	99	-	1.1 d	-	15 d
		grey matter	99	-	0.8 d	-	27 d

*Left to right: precursor types in different areas of the CNS and individual cell types or tissues are indicated with proportion of cycling cells (growth fraction, GF), length of individual phases and total cell cycle in hours (h) or days (d). Split cells indicate values calculated in physiological (left) or pathological (right) conditions. OPC values were acquired at the age of approximately 2 months. *Value calculated from (Simon et al., 2011; Young et al., 2013).*

Oligodendrocyte progenitor cells (OPC) play pivotal roles in CNS development (Richardson et al., 2006) and adulthood where they represent the most abundant and homogeneously distributed cycling cell population of the CNS (Richardson et al., 2011). Oligodendrocytes during development are generated from different regions in consecutive waves but it is unknown whether each population has any specific role in brain function (Kessaris et al., 2006). Adult OPC represent 8 and 2% of the white and gray matter, respectively (Dawson, 2003; Rivers et al., 2008) with resident and migrating OPC in the SVZ and septum giving rise to mature myelinating oligodendrocytes in physiological and pathological conditions (Menn et al., 2006). Maturation of OPC involves changes in morphology and marker expression including Pdgfra, Ng2 for OPC and Olig2 and Sox10 for the whole lineage (Fumagalli et al., 2011). OPC are reactive to neurotransmitters (Bergles et al., 2000; Stevens et al., 2002) and display highly dynamic behavior with regard to migration, filopodia extension, proliferation, differentiation and reaction to injury (Hughes et al., 2013).

Starting at postnatal day 7 and during adulthood, approximately 50–80% of OPC in the whole brain were described as cycling based on BrdU incorporation (Rivers et al., 2008; Psachoulia et al., 2009; Simon et al., 2011). Another report based on EdU however calculated a growth fraction of about 99% (Young et al., 2013) but this thymidine analog has raised concern with regard to toxicity (Ponti et al., 2013). Cell cycle length of OPC differs among brain regions and is significantly longer than that of neurogenic precursors. In the white matter, in particular corpus callosum, OPC cell cycle linearly increases from 2 days at 1 week postnatal to a plateau of 150 days at 8 months (Rivers et al., 2008; Psachoulia et al., 2009; Young et al., 2013) with the spinal cord white matter yielding comparable results (**Table 1**) (Young et al., 2013). In the cortical gray matter cell cycle length was estimated to be about 37 days at 2 months (Simon et al., 2011; Young et al., 2013) with S/G2/M of 5 days (Simon et al., 2011). Cell cycle in the cortex also showed a decrease similar to the corpus callosum with the important difference that a plateau is not reached and cell cycle increases to up to 340 days at 18 months (Psachoulia et al., 2009; Young et al., 2013). This almost linear relationship between age and cell cycle implies a lengthening by about 16 h every day starting at birth (Young et al., 2013), that is, every cell cycle is two thirds longer than the previous one. Finally, cell cycle in the gray matter of the spinal cord is significantly shorter than in the cortex with OPC dividing every 8 or 27 days at 3 weeks or 2 months postnatal, respectively (**Table 1**) (Young et al., 2013). With regard to the proportion of OPC that divide to proliferate as opposed to generate mature oligodendrocytes, different studies led to different estimations while consistently reporting higher

values for the white matter both in the brain and spinal cord with both declining during aging (Rivers et al., 2008; Psachoulia et al., 2009; Kang et al., 2010; Simon et al., 2011; Zhu et al., 2011; Young et al., 2013).

In conclusion, OPC exhibit a remarkably longer cell cycle than neurogenic precursors, which to our opinion reflects long periods of quiescence followed by re-entry in a cell cycle that is a fraction of the total inter-mitotic time. Moreover, the cell cycle of OPC differs between gray and white matter, which is possibly explained by region-specific differences as revealed by transplantation experiments (Vigano et al., 2013).

CELL CYCLE IN PATHOLOGICAL CONDITIONS

Accumulating evidence indicates that OPC play key roles during brain injury (Nguyen et al., 2006; Huang et al., 2011; Zhang et al., 2013). Demyelination in multiple sclerosis leads to impaired saltatory signal conduction and loss of axon integrity (Huang et al., 2011). OPC react by migrating into the lesion and differentiate in mature myelinating oligodendrocytes and Schwann cells (Zawadzka et al., 2010). This reaction enhances recovery and is known to decrease with age making it a potential target for regenerative therapies (Nguyen et al., 2006; Zawadzka et al., 2010; Huang et al., 2011; Deshmukh et al., 2013).

Stab wound in the cortex increases proliferation in the whole brain with a five-fold higher response in the ipsilateral compared to contralateral hemisphere 3 days post injury (Simon et al., 2011). In particular, at 1 week 74% of OPC cycle suggesting that cells enter the cell cycle from quiescence and, concomitantly, shorten the G1-phase of their cell cycle (Simon et al., 2011).

Cerebral ischemia has a strong impact on oligodendrocytes since they lack the ability to proliferate and, once damaged, to myelinate axons (McTigue and Tripathi, 2008). After stroke resident and SVZ-derived OPC start to proliferate and migrate to the penumbra where they differentiate into mature oligodendrocytes that myelinate newly sprouted axons thus enhancing neuronal survival and short-term synaptic plasticity (Zhang and Chopp, 2009; Ueno et al., 2012; Zhang et al., 2013) and preclinical studies showed improved healing of stroke after pharmaceutically enhanced oligodendrogenesis (Zhang et al., 2013).

Most studies have focused on neuronal aspects of brain recovery and the role of other cell types awaits further investigation. Only recently studies started to focus on cell cycle parameters of OPC and other cell types such as astrocytes and pericytes playing critical roles in disease including glial scar formation and inflammation (Goritz et al., 2011; Lambertsen et al., 2012).

DISCUSSION

Decades of cell cycle measurements during development have been instrumental to understand and manipulate the contribution of neural precursors in the mammalian brain (Fujita, 1962; Schultze and Korr, 1981; Takahashi et al., 1995; Dehay and Kennedy, 2007; Salomoni and Calegari, 2010; Borrell and Calegari, in press). Studies during adulthood have just begun and parallelisms between the two contexts are hard to identify due to our limited understanding of adult lineages and difficulties in assessing cell cycle and quiescence. Notably, during development progenitors have longer cell cycles than stem cells (Borrell and Calegari, in press). Yet, differences of greater significance were found by comparing cells undergoing proliferative vs. differentiative division within these two populations (Calegari et al., 2005; Arai et al., 2011). Hence, analyses of, say, type 1/B vs. 2/C cells can only reveal part of the truth with identification of proliferative vs. differentiative precursors within each type being perhaps more important. Moreover, independently from physiological correlations between cell cycle and stemness, it is clear that artificial manipulations can still be effective in increasing stem cell expansion since these can override physiological processes as indicated by studies on NSC and OPC (Artegiani et al., 2011; Beukelaers et al., 2011a; Caillava et al., 2011; Nobs et al., 2013).

It is premature to know whether manipulation of neural precursors will ever allow practical and efficient means toward effective therapy, but the history of cell cycle measurements during development suggests that this may pave a promising road. To achieving this goal, technical limitations need first to be overcome including the identification of markers for the relevant cell types, establishing behavioral tests reflecting functional recovery rather than compensatory learning (Hicks et al., 2009) and animal models of disease faithfully recapitulating the human condition. As one example of the latter, models of stroke often involve the striatum whereas human ischemia mainly affects cortical areas while the few that involve the striatum cause mild deficits (Delavaran et al., 2013). Moreover, modeling disease is often done in young mice while most neurodegenerative diseases are relevant during aging, which has major effects on cell cycle and neurogenesis (Artegiani and Calegari, 2012). We envision that improvements in these aspects of biomedical research will have the greatest impact in the field and hope that this review will help readers to identify, hence overcome, some of our current limitations.

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Restricted nature of adult neural stem cells: re-evaluation of their potential for brain repair

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Neural stem cells (NSCs) in the walls of the lateral ventricles continue to produce new neurons and oligodendrocytes throughout life. The identification of NSCs, long-range neuronal migration, and the integration of new neurons into fully formed mature neural circuits—all in the juvenile or adult brain—has dramatically changed concepts in neurodevelopment and suggests new strategies for brain repair. Yet, the latter has to be seen in perspective: NSCs in the adult are heterogeneous and highly regionally specified; young neurons derived from these primary progenitors migrate and integrate in specific brain regions. Neurogenesis appears to have a function in brain plasticity rather than brain repair. If similar processes could be induced in regions of the brain that are normally not a target of new neurons, therapeutic neuronal replacement may one day reinstate neural circuit plasticity and possibly repair broken neural circuits.

Keywords: adult neural stem cells, V-SVZ, brain repair, circuit plasticity, specification

For many years, it was believed that once development is completed, no new nerve cells are added to the central nervous system. This view imposed a severe limitation on our thinking of possible mechanisms for neuronal replacement and brain repair. This dogma began to change with observations made by Joseph Altman in the 1960's: $[H]^3$-thymidine labeled progenitors gave rise to cells in several brain regions that had the morphology of neurons under the light microscope (Altman, 1962). However, the identity of these labeled cells remained highly controversial for many years (see: Rakic, 2002). Conclusive evidence for adult neurogenesis in homeotherms came from the work of Fernando Nottebohm and colleagues in the 1980's using electrophysiology, electron microscopy, and tracer methods. This work in adult songbirds, where seasonal changes in the size of song control nuclei had been previously documented, showed that new neurons continually replace older cells that have died (see: Nottebohm, 2002). Subsequent studies in mammals demonstrated that neurons are continually added to the adult olfactory bulb (OB) and the dentate gyrus during juvenile and adult life (for review see: Ihrie and Alvarez-Buylla, 2011; Gage and Temple, 2013).

New OB neurons are born postnatally within an extensive germinal zone lining the walls of the lateral ventricles. The ventricular-subventricular zone (V-SVZ) is the largest germinal niche in the adult mammalian brain. This germinal layer has been most extensively studied in rodents (Fuentealba et al., 2012; Tong and Alvarez-Buylla, 2014). Primary progenitor cells, which are frequently referred to as NSCs, generate new neurons and oligodendrocytes in the juvenile and adult brain. From the early observations in mammals and songbirds, to the more recent work in the mammalian hippocampus and the V-SVZ, adult neurogenesis has been proclaimed to offer a new hope for brain repair (Nottebohm, 1985; Gage and Temple, 2013). Certainly, the discovery of adult neurogenesis demonstrates that neuronal birth, migration over extremely long distances, and the integration of these cells into established brain circuitry *is indeed all possible*. However, as we discuss below, the NSCs, progenitors, and signaling that allow for new neurons to be born and the mechanisms that permit young neurons to migrate and integrate within the adult brain, do not seem to be intended for brain repair (summarized in **Table 1**). Instead, most of the evidence suggests that adult neurogenesis enables constant modification of neural circuits—likely related to unique forms of brain plasticity in which some key neurons are eliminated and replaced. Here we focus most of our discussion on the V-SVZ where NSCs, their lineages, and the migration of young neurons have been extensively characterized.

In the adult rodent V-SVZ a subpopulation of progenitors with astroglial characteristics, known as B1 cells, function as the NSCs. B1 cells generate young neurons that migrate long distances via the rostral migratory stream (RMS) to the OB where they differentiate into local circuit GABAergic interneurons. For potential brain repair, the understanding of the plasticity of these primary progenitors is key. If these cells are capable of generating a wide diversity of neuronal cell types they could potentially be directed to form nerve cells lost in a disease or following trauma. Earlier work suggested that NSCs could be plastic and that the environment played a key role in directing their differentiation into specific neuronal cell types (Fallon et al., 2000; Shihabuddin

Table 1 | Adult Neurogenesis in the V-SVZ: Challenges for Brain Repair.

Adult V-SVZ neurogenesis	Circuit plasticity	Brain repair?
Cell type specification	• Primary progenitors are regionally specified (Merkle et al., 2007) • Generation of GABAergic interneurons • Replacement of specific subsets of older neurons in the OB	• No confirmed evidence for differentiation into cell types required for replacement of lost neurons in different brain regions (Herrera et al., 1999; Raedt et al., 2009)
Migration of young neurons	• Mostly restricted to the V-SVZ and RMS, directed toward the OB (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996)	• Requires exiting the V-SVZ and RMS • Requires migration and integration into the lesion site
New neuron integration	• Integration into defined circuits • Specialized synaptic contacts with mitral and tufted projection neurons (Whitman and Greer, 2009)	• Requires integration and survival of young neurons in environments that are normally non-neurogenic • Synaptic contacts with different neuronal partners depending on the types of circuit damaged
In context of human brain	• Mostly restricted to infants (Sanai et al., 2004, 2011; Bergmann et al., 2012)	• Limited evidence of neurogenesis under pathological conditions in the adult or aged human brain

et al., 2000). It was suggested that NSCs isolated from one brain region could function as those in another (e.g., hippocampal NSCs transplanted into the V-SVZ) (Brustle and McKay, 1996; Suhonen et al., 1996), whereas more recent work suggests that V-SVZ cells cannot acquire cortical, striatal or hippocampal properties following transplantation (Herrera et al., 1999; Raedt et al., 2009). Additional evidence indicates that NSCs under normal physiological conditions are highly specialized and regionally specified in a cell-autonomous manner to produce specific types of neurons destined for unique circuits within particular brain regions (Merkle et al., 2007). This limitation may be overcome by the trans-differentiation of the precursors using transcription factors (Chen et al., 2012), but in their default state their differentiation seems to be limited.

The specification of the neuroepithelium occurs early in development, restricting the potency of NSCs in the forebrain (Rubenstein, 2011) as well as the spinal cord (Jessell et al., 2011). In the developing murine embryonic brain early progenitors produce striatal, cortical, and septal neurons—possibly orchestrated by combinations of several transcription factors (Rubenstein, 2011)—but later progenitors fail to generate earlier fates (Shen et al., 2006). Whereas depending on their position within the VZ germinal zone embryonic NSCs generate both glutamatergic and GABAergic neurons for different forebrain regions, adult V-SVZ NSCs mostly generate GABAergic neurons destined for the OB. In the adult V-SVZ, NSCs are not only restricted to differentiate into OB interneurons but are heterogeneous and regionally specified (**Figure 1**). Adenovirus-mediated labeling of NSCs in different locations of the lateral, medial, and pallial wall has shown that dorsal NSCs generate mostly superficial granule cells (GCs) and dopaminergic tyrosine hydroxylase (TH)-positive (but not calbindin-positive) periglomerular cells (PGCs), while ventral NSCs produce deep GCs and calbindin-positive (but not TH-positive) PGCs and NSCs in the medial wall give rise to calretinin-positive PGCs and GCs. This positional specification seems to be primarily cell intrinsic, since labeled progenitors maintained their original phenotype *in vitro* and upon heterotopic grafting within the niche (Merkle et al., 2007). The positional identity of adult NSCs is likely inherited from the early embryonic differential expression of combinations of transcription factors by neuroepithelial and radial glia cells (for review see Kriegstein and Alvarez-Buylla, 2009; Rubenstein, 2011). Furthermore, transplantation of V-SVZ NSCs to non-neurogenic regions (outside the germinal niche) of the adult brain under normal physiological conditions is not permissive for neurogenesis; V-SVZ cells grafted into the cortex or striatum fail to migrate and produce few, if any, neurons (Herrera et al., 1999; Seidenfaden et al., 2006). For now it seems that NSCs and progenitor cells in the V-SVZ are highly restricted in their potential; this may 1 day be overcome by reprogramming.

An important obstacle for brain repair in the juvenile or adult brain is the long distances that frequently separate endogenous germinal niches, or sites of transplantation of progenitor cells, from the sites where new neurons would be required. Damaged areas are also frequently distributed over large volumes of brain tissue. Repair, therefore, requires long migrations of precursor cells through the complex postnatal brain parenchyma. NSCs (B1 and radial glia cells) are largely fixed to specific locations in the neuroepithelium. In contrast, young neurons derived from these specified locations need to migrate to their final destination, often very long distances. Consistently, neuroblasts derived from the adult V-SVZ, have an extraordinary capacity for long-range migration within the adult brain parenchyma (Lois and Alvarez-Buylla, 1994). In the context of brain repair, this migratory capacity could potentially allow young neurons to penetrate deep into a lesion and replace neurons where they are needed. However, under normal conditions, neuroblasts derived from the V-SVZ migrate within distinct corridors within the SVZ and the RMS (Doetsch and Alvarez-Buylla, 1996). This constrained migration mediated by chemotactic factors, glial ensheathment, and a vascular scaffold (Nguyen-Ba-Charvet et al., 2004; Sawamoto et al., 2006; Whitman et al., 2009; Kaneko et al., 2010) ensures the

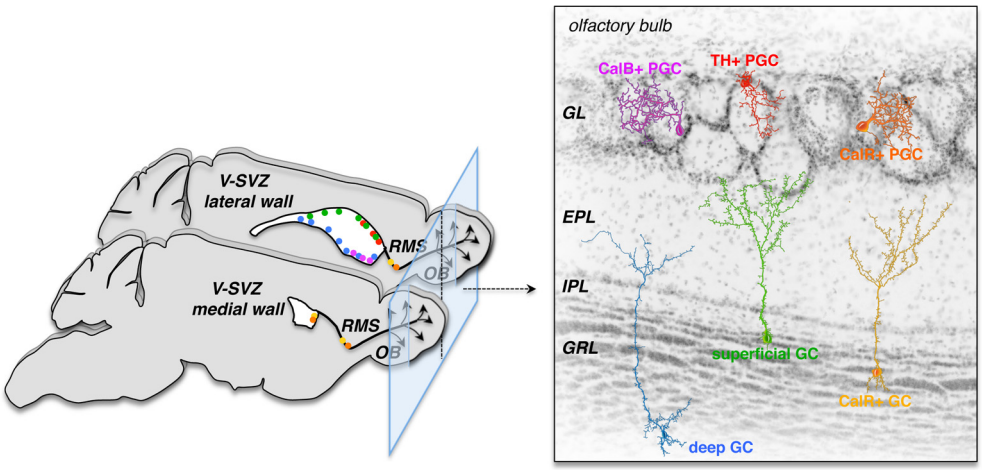

FIGURE 1 | V-SVZ NSCs are regionally specified and generate unique subtypes of OB interneurons depending on their location. This specified nature of primary progenitors sets limits to their direct use for brain repair; yet future strategies may exploit reprogramming to induce these NSCs to produce specific neuronal subsets required for brain repair. Left panel: the color dots in the medial (bottom) and lateral (top) walls of the lateral ventricles on the two sagittal views of the left mouse brain depict regions were specific subsets of olfactory bulb (OB) interneuron subtypes (right panel) are born. CalB, Calbindin; CalR, Calretinin; EPL, external plexiform layer; IPL, internal plexiform layer; GC, granule cell; GL, glomerular layer; GRL, granule cell layer; OB, olfactory bulb; PGC, periglomerular cell; RMS, rostral migratory stream; TH, tyrosine hydroxylase; V-SVZ, ventricular-subventricular zone (modified from Alvarez-Buylla et al., 2013).

delivery of these young neurons to the OB, where they then disperse radially into specific layers (Whitman and Greer, 2009; Ihrie and Alvarez-Buylla, 2011). Interestingly, there is some evidence that neuroblasts are able to leave the V-SVZ and RMS to invade the adjacent striatum. For example, following ischemia-induced activation of guidance molecules (e.g., CXCR4-SDF1) neuroblasts migrate toward the ischemic site but the ectopic migration may be due to aberrant expression of substrate and guidance molecules within the context of the lesions. It is also possible that the physical damage from these lesions could interfere with the normal migratory path, facilitating the derailment of neuroblasts into adjoining territories while they maintain their intrinsic differentiation potential and thus are rather misdirected OB interneurons (Liu et al., 2009). Although it has been suggested that few young neurons migrating toward the site of lesion can survive and possibly integrate (Arvidsson et al., 2002; Kokaia et al., 2006; Yamashita et al., 2006; Hou et al., 2008), most do not become mature neurons and do not survive long-term (Osman et al., 2011; Cui et al., 2013). Others have reported that intraventricular growth factor infusion may be necessary to promote regeneration (Kolb et al., 2007). In contrast to neurons, there is better evidence that oligodendrocyte progenitors derived from the V-SVZ can migrate into a demyelinated lesioned site and differentiate into new oligodendrocytes (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Menn et al., 2006). Upon brain injury some V-SVZ cells may also produce astrocytes that migrate to the injury site (Benner et al., 2013). Therefore, migration through the adult brain is limited to very specific paths and to specific subtypes of neurons and glial cells.

The ability to become synaptically incorporated into fully formed adult brain circuits is an essential characteristic of new neurons formed in the V-SVZ. While synaptic incorporation is a highly desired property for putative new neurons in the context of brain repair, these cells specifically integrate into the circuits of the OB. In the OB they mediate inhibition of mitral and tufted projection neurons and it is thought that their continual replacement into adulthood is associated to olfactory discrimination and plasticity (Gheusi et al., 2000; Cecchi et al., 2001; Sakamoto et al., 2011). Plasticity-related functions for adult-born neurons have also been proposed in songbirds (Alvarez-Buylla et al., 1990; Scharff et al., 2000) and in the rodent hippocampus (Kempermann, 2012; Gage and Temple, 2013). Therefore, the available evidence strongly suggests that the integration of newly formed neurons occurs within very specific circuits where ongoing plasticity requires new nerve cells. It is tempting to speculate that diseased or injured brain circuits could similarly present a permissive environment for the recruitment of new neurons, but this remains to be demonstrated. If the young neurons that are normally produced in the adult brain are so highly tuned to specific brain circuits, an important limitation might be their competence to differentiate into neuronal cell types that can migrate and integrate to reconstitute function in damaged circuits. Neuronal progenitors capable of differentiation, migration and integration within the environment of neurodegeneration or trauma may exist, but these cells do not seem to be the ones normally produced in the adult brain.

The above shows that the birth, migration, and integration of young neurons is fashioned for specific brain circuits with the demand for a special form of plasticity in the adult brain. This sets limits to the use of adult NSCs in brain repair. Species-specific differences in adult neurogenesis may also determine the potential use of NSCs in brain repair. For example, it has been known for sometime, that reptiles and amphibians can regenerate neuronal populations that are not repaired in mammals or

birds (Polenov and Chetverukhin, 1993; Font et al., 2001; Garcia-Verdugo et al., 2002; Chapouton et al., 2007). However, there are also important differences among mammals. The human V-SVZ differs in its cytoarchitecture compared to the rodent germinal zone. While the human V-SVZ is also lined with a monolayer of ependymal cells at the apical side of the walls of the lateral ventricles, it basally consists of a hypocellular gap, an astrocytic ribbon that contains neural progenitor cells, and a transition zone into the parenchyma. In infant humans the V-SVZ is an important source of new neurons not only for the OB, but also for specific subregions of the anterior prefrontal cortex (Sanai et al., 2004, 2011; Yang et al., 2011). Unlike in rodents where neurogenesis persists throughout the animal's lifespan—though it is drastically reduced during aging—only very few migrating young neurons are observed in the adolescents or in adult human brains. Consistently, radiocarbon birth dating suggests that the vast majority of neurons in the OB are as old as the person, implying they are born during early development (Bergmann et al., 2012). Those neurons that are added to specific brain regions during early childhood could be key in understanding critical-period plasticity and postnatal developmental deficits. In the hippocampus, the story may be somewhat different when compared to the OB. It remains controversial how many new neurons continue to be added in the dentate gyrus in the adult human brain. Some studies suggest the presence of newly born hippocampal neurons in adults and a surprisingly stable birth rate from adolescence to aging (Eriksson et al., 1998; Spalding et al., 2013), yet other studies reveal very few cells expressing markers of young neurons in the hippocampus after birth (Knoth et al., 2010). In adult humans, in addition to the intrinsic limitations imposed by adult NSC specification, the possible low number of NSCs or their long-term quiescence pose further constraints, which may limit their potential use in cell replacement therapies. Even if the activation of resident quiescent NSCs—if they exist—is possible, the migration, survival, and integration into specific brain regions may be a greater challenge given the large size of the human brain.

While we have made significant progress in understanding the identity and lineages of adult NSCs, there is no evidence that these cells can repair neural circuits or replace diverse neuronal cell types. The function of adult neurogenesis remains unknown, but significant evidence points to processes of brain plasticity rather than brain repair. Regions of the postnatal brain that continue to receive new neurons may be plastic throughout life; they may not be constrained by defined critical-periods of plasticity that ends as their neuronal components mature (Southwell et al., 2010). The addition of new neurons may be seen as a constant infusion of youth into mature neural circuits. The more directed use of neuronal replacement for brain repair will likely require very specific types of progenitor cells that can navigate and appropriately integrate into target regions. Most likely this will involve the reprograming of embryonic or adult brain cells for the production of those specific neuronal cell types that can integrate and repair damaged neural circuits. Neural progenitor cells from the mouse embryonic MGE for example have been shown to migrate long distances upon transplantation and can contribute to neuronal replacement, although only with the generation of interneurons, not excitatory neurons (Wichterle et al., 1999; Alvarez-Dolado et al., 2006; Southwell et al., 2010). Human fetal cells transplanted into stroke-lesioned mice migrate long distances and differentiated into mature neurons in the lesioned striatum or cortex (Kelly et al., 2004; Darsalia et al., 2007). Also further efforts in the field of iPSC-derived neural progenitors may lead to neuronal replacement strategies. We also cannot exclude that very specific subsets of the heterogeneous population of endogenous adult V-SVZ NSCs may harbor the potential to function as precursors for neurons in different brain regions; this requires detailed analysis of the potential of individual NSCs located in different regions of the adult V-SVZ.

The identification of NSCs among astroglia and the abundance of glial cells suggest that the adult brain may contain a large reservoir of cells that could be target for such reprograming. Efforts along these lines have already begun (Guo et al., 2013; Niu et al., 2013), but further understanding of astrocyte heterogeneity is required (Hochstim et al., 2008; Tsai et al., 2012).

In the present perspective article we have focused mostly on adult neurogenesis in the V-SVZ, as this is the most extensive germinal region of the adult mammalian brain. Similar concepts of specification, confined migration, and integration probably also apply to neurogenesis in the adult hippocampus and avian forebrain (Scharff, 2000; Nottebohm and Liu, 2010; Ming and Song, 2011; Gage and Temple, 2013). This very high level of specification may suggest, to some, that adult neurogenesis is irrelevant to brain repair. However, this is not the case: one important lesson learned from studies of adult neurogenesis is that processes considered impossible three decades ago do indeed take place in the adult brain; young neurons can be produced and they can migrate, and most importantly integrate, into adult brain circuits. Their seamless migration and integration, whether in the song control circuits of birds, or in the OB and hippocampus of adult mammals, still has much to teach us about how to accomplish similar neuronal replacement for neurons lost during neurodegeneration or trauma.

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Is integration and survival of newborn neurons the bottleneck for effective neural repair by endogenous neural precursor cells?

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After two decades of research the existence of adult neural precursor cells and the phenomenon of adult neurogenesis is well established. However, there has been little or no effective harnessing of these endogenous cells to promote functional neuronal replacement following neural injury or disease. Neural precursor cells can respond to neural damage by proliferating, migrating to the site of injury, and differentiating into neuronal or glial lineages. However, after a month or so, very few or no newborn neurons can be detected, suggesting that even though neuroblasts are generated, they generally fail to survive as mature neurons and contribute to the local circuitry. Is this lack of survival and integration one of the major bottlenecks that inhibits effective neuronal replacement and subsequent repair of the nervous system following injury or disease? In this perspective article the possibility that this bottleneck can be targeted to enhance the integration and subsequent survival of newborn neurons will be explored and will suggest some possible mechanisms that may need to be modulated for this to occur.

Keywords: adult neurogenesis, neural stem cells, neural repair, SVZ, SGZ, hippocampus, olfactory bulb, neurite outgrowth

INTRODUCTION

Two decades of research has demonstrated that a surprisingly wide variety of factors can influence adult neural precursor cell biology (Christie and Turnley, 2012). This includes extrinsic factors, such as growth factors, cytokines, chemokines, neurotrophins, steroids and extracellular matrix molecules as well as cell intrinsic factors such as transcription factors and signal transduction pathway regulators (Christie and Turnley, 2012; Christie et al., 2013a). In general, endogenous adult neural precursor cells can be quite easily induced to proliferate and migrate, and depending on the context, differentiate into neuronal or glial cell types. However, fewer factors have been identified that induce newborn neurons to integrate into the local circuitry and survive more than a few weeks after their birth. Indeed at least 50% of newborn neurons fail to survive longer than a month or two after their generation (Petreanu and Alvarez-Buylla, 2002; Dayer et al., 2003). This makes sense under normal physiological conditions, where newborn neurons replenish local neurons lost due to normal turnover, to homeostatically maintain neuron numbers (Valley et al., 2009). Addition of newborn neurons to existing circuitry has specific functional outcomes. In the olfactory bulb, addition of new neurons is required for short-term olfactory memory, perceptual learning, and for innate olfactory responses (Breton-Provencher et al., 2009; Moreno et al., 2009; Sakamoto et al., 2011). In the hippocampus, adult neurogenesis plays roles in anxiety and affective behaviors, cognition and spatial memory (Ming and Song, 2011), and is proposed to be vital for forgetting of hippocampal-dependent short-term memories (Frankland et al., 2013). However, in instances of larger neuronal loss, such as following injury or disease, this failure of newborn neurons to increase their integration and survival in conjunction with increases in proliferation and redirected migration means that the full potential of adult neural progenitor cells (NPCs) to repair the damage may not be realized. This perspective article will explore some of the mechanisms and factors that may be targeted to enhance newborn neuron survival, summarized in **Table 1**.

SOURCE OF NEURAL STEM/PROGENITOR CELLS—SUBVENTRICULAR ZONE vs. HIPPOCAMPUS

There are two principle sources of neural progenitor cells in the adult brain: the subventricular zone (SVZ) lining the lateral walls of the lateral ventricles, which supplies new neurons to the olfactory bulb and the subgranular zone (SGZ) of the hippocampal dentate gyrus, which produces new hippocampal granule cell neurons. Under normal physiological conditions, both of these NPC populations primarily generate neurons, however there are differences in the neuroblasts and neurons they produce and their response to neural damage. SVZ-derived cells must migrate a long distance along the rostral migratory stream to reach the olfactory bulb, while hippocampal-derived cells only migrate a short distance from the SGZ into the granule cell layer directly adjacent. In response to neural damage, both populations increase their proliferation but apparently only SVZ-derived cells migrate to ectopic sites of damage (Ming and Song, 2011), while hippocampal cells remain within the granule cell layer. The differentiation fate of SVZ-derived cells is also more easily switched to a glial fate in response to damage and the subsequent production of

Table 1 | Factors that regulate or may be targeted to promote survival of adult newborn neurons.

Factor	Role	References
EXOGENOUS		
Neurotransmitters—GABA and glutamate	Activity induced survival and synaptic integration	Gascon et al., 2006; Ge et al., 2006; Platel et al., 2010; Kim et al., 2012; Chancey et al., 2013
Neurotrophins—BDNF	Enhances neurite outgrowth, dendritic arborization, and spine density	Miyamoto et al., 2006; Cheung et al., 2007; Chan et al., 2008; Gao and Chen, 2009; Gao et al., 2009; Bergami et al., 2013
ENDOGENOUS		
Rho GTPases	Cytoskeletal reorganization—dendrite/axon outgrowth, dendritic spine formation—regulation of plasticity induced survival	Nikolic, 2002; Keung et al., 2011; Christie et al., 2013b; Vadodaria et al., 2013
	NPC migration	Leong et al., 2011
	Extant neuron survival in stroke and Parkinson's disease models	Lemmens et al., 2013; Rodriguez-Perez et al., 2013
SOCS2	Regulation of growth factor signaling and neurite outgrowth	Goldshmit et al., 2004a,b; Basrai et al., 2013
TRANSCRIPTION FACTORS		
zif268/egr1, KLF9, NeuroD1, cAMP response element, ATF5, miR-132	Regulation of neuronal morphology and maturation	Giachino et al., 2005; Gao et al., 2009; Jagasia et al., 2009; Scobie et al., 2009; Pathania et al., 2012; Wang et al., 2012; Veyrac et al., 2013
p63	Anti-apoptotic	Cancino et al., 2013
NFATc4	Mediates BDNF-induced survival	Quadrato et al., 2012
OTHER POTENTIAL MODULATORS		
Ephs/ephrins	Regulation of axonal and dendritic sprouting, synaptic plasticity	Goldshmit et al., 2011; Overman et al., 2012; Spanevello et al., 2013
Peri-neuronal nets	Inhibits synaptic plasticity; degradation promotes plasticity	Kwok et al., 2011; Wang et al., 2011
Environmental enrichment/ Forced use	Enhances synaptic plasticity	Rochefort et al., 2002; Miwa and Storm, 2005; Yamaguchi and Mori, 2005; Alonso et al., 2006; Mandairon et al., 2006; Overman et al., 2012

chemokines and cytokines, while SGZ cell fate remains largely neuronal depending on type of damage (Christie and Turnley, 2012). However the end stage of neuronal integration and survival does not appear to differ greatly, with the majority of neurons failing to survive in both cases.

ADULT NEWBORN NEURONAL SURVIVAL UNDER NORMAL PHYSIOLOGICAL CONDITIONS

INTEGRATION OF NEWBORN NEURONS INTO EXISTING LOCAL CIRCUITRY

While there are homeostatic mechanisms in place to regulate the integration and subsequent survival of newborn neurons into local circuits under normal physiological conditions, this does not mean to imply that this process is always held at a constant level. Indeed, the numbers of newborn neurons that survive and integrate can be varied by altered levels of circuit activity/plasticity. In the olfactory bulb, alteration of local activity by complex odor environments or odor discrimination learning increases the survival of newborn neurons (Rochefort et al., 2002; Miwa and Storm, 2005; Alonso et al., 2006; Mandairon et al., 2006), while conversely at the critical time of neuronal integration, sensory deprivation and hence decreased circuit activity results in increased newborn neuron death (Yamaguchi and Mori, 2005). Similarly, numerous activity and environment-induced alterations in adult hippocampal neurogenesis have been described, including exercise, environmental enrichment and learning increasing newborn neuron numbers, with stress, steroids, and depression decreasing them. However, with some exceptions, these factors alter final mature newborn numbers by increasing or decreasing proliferation of neural precursor cells and hence production of new neurons, rather than acting primarily at the integration and survival stage. It seems that the newborn hippocampal neurons, during the critical few weeks after their generation, require activity in the form of effortful learning, to promote their survival (Shors et al., 2012).

So, how do newborn neurons integrate into the extant local circuitry? While the mechanisms are not yet well understood it seems that newly generated neurons compete for synaptic space,

with only those that make successful connections surviving. The newborn neurons must compete not only with each other but also with older neurons to integrate into the circuitry. It should be noted that the regions into which newborn neurons integrate are actually open to the competition. In other words, they are very plastic regions of the CNS that unlike other CNS regions, are primed to allow new neurons to be added to the circuitry. This is highlighted in neural precursor cell transplantation studies, whereby new neurons were produced and survived if transplanted into neurogenic regions but not into non-neurogenic regions (Suhonen et al., 1996; Shihabuddin et al., 2000). While there are similarities in regulation of neuronal survival and integration of SVZ- and SGZ-derived neurons under normal physiological conditions, there are quite important differences following injury. This includes their ability to migrate to distant sites of damage and differences into which they integrate. The SVZ-derived cells will migrate to sites of damage in non-neurogenic regions, e.g., cortex or striatum and therefore need to try to integrate and survive in areas that are usually non-receptive to such processes. SGZ-derived cells remain in a neurogenic environment, although injury-induced plasticity may be increased (Perederiy et al., 2013). In the injured non-neurogenic CNS there is a variable degree of rewiring and plasticity occurring, which may change the normally non-neurogenic environment to a partially neurogenic environment in some instances and which may account for the various reports of newborn neurons at sites of injury or disease (Christie and Turnley, 2012). So, what can be taken from our knowledge of factors that regulate normal physiological adult newborn neuron integration and survival and be applied to the injured regions of the CNS?

FACTORS THAT ENHANCE NEWBORN NEURON INTEGRATION AND SURVIVAL UNDER NORMAL PHYSIOLOGICAL CONDITIONS

Compared to our knowledge of factors that promote adult neural precursor cell proliferation or differentiation, there is comparatively little known about factors that promote newborn neuron survival and integration *per se*. Broadly speaking, what information we do have centers on ways to alter plasticity—particularly at the synaptic or cell morphological level (dendrite and axon morphology). These may be categorized into factors that regulate circuit/neuronal activity (e.g., neurotransmitters) and factors that regulate cellular morphology (e.g., cytoskeletal regulators, neurotrophins), as well as less-defined but otherwise acknowledged general regulators of neural plasticity, such as environmental enrichment and use/activity induced plasticity.

These different signals converge on a range of transcription factors, the roles, and regulation of which are beginning to be elucidated (Christie et al., 2013a). These include transcription factors that regulate neurochemical and/or morphological maturation, such the inducible immediate early gene zif268/egr1 (Veyrac et al., 2013), KLF9 (Scobie et al., 2009), NeuroD1 (Gao et al., 2009), the cAMP response element (Giachino et al., 2005; Jagasia et al., 2009) and ATF5 (Wang et al., 2012), as well as the microRNA miR-132 (Pathania et al., 2012). The anti-apoptotic p63 (Cancino et al., 2013) and NFATc4, which mediates BDNF-dependent survival (Quadrato et al., 2012), also promote newborn neuron survival.

Neurotransmitters

Early in the life of a newborn neuron, before integration has taken place, the cells are responsive to non-synapse-mediated GABA stimulation. After further maturation, the cells become synoptically responsive to GABA with concomitant responsiveness to glutamate (Gascon et al., 2006; Ge et al., 2006; Kim et al., 2012; Chancey et al., 2013). It has been proposed that this responsiveness may allow synaptic integration into local circuitry to occur in a process whereby a highly motile filopodia of a newborn neuron contacts a pre-existing synapse, possibly in response to excess neurotransmitter release. This connection then matures, becomes stabilized and the filopodium develops into a mature dendritic spine. Astrocyte-released glutamatergic activation of neuroblast NMDA receptors has been shown to be required for synaptic integration (Platel et al., 2010), while the level of cell-intrinsic excitability also modulates survival (Lin et al., 2010). For such an interaction to be most effective, there likely needs to be a substantial degree of synaptic and dendritic remodeling occurring, as in the plastic neurogenic regions of the CNS, which requires substantial cytoskeletal rearrangement and regulation. Enhancing plasticity by enhancing cytoskeletal rearrangement provides another potential target for increasing the likelihood of integration and survival of newborn neurons.

Cytoskeletal rearrangement as a potential target for enhancing newborn neuron integration into local circuitry—modulating the RhoA family of small GTPases

While a multitude of factors have effects on cytoskeletal rearrangement, most of these converge on the family of small Rho GTPases, RhoA, Rac1, and cdc42. The Rho GTPases are regulators of neurite outgrowth, with RhoA activation inhibiting and Rac1 activation promoting neurite outgrowth and dendritic spine formation (Nikolic, 2002). Enhancement of a newborn neuron's ability to produce dendrites, axons, or dendritic spines may give it a competitive advantage, while increasing synaptic remodeling or turnover of existing local circuitry could also increase the chances of newborn neuron integration. A number of recent studies implicate these molecules in regulation of adult newborn hippocampal neuron maturation and survival, as recently reviewed (Vadodaria and Jessberger, 2013).

Conceptually, Rac1 may promote neuronal survival and integration while RhoA may oppose it, given the promotion and inhibition of neurite outgrowth and spine formation respectively. This indeed appears to be the case. Deletion or inhibition of Rac1 or cdc42 blocked dendrite and spine formation of adult hippocampal newborn neurons, although there were no effects on neuron survival (Vadodaria et al., 2013). Conversely, constitutive activation of RhoA decreased the percentage of newborn neurons (Keung et al., 2011), while inhibition of RhoA signaling specifically enhanced the survival of newborn hippocampal neurons, which correlated with enhanced spatial memory (Christie et al., 2013b). Rho kinase inhibition also promotes ectopic migration of SVZ-derived neural precursor cells and subsequent neuron survival (Leong et al., 2011). Therefore, activation of Rac1 or cdc42 or inhibition of RhoA signaling pathways may be of potential therapeutic effectiveness at promoting survival and integration of newborn neurons. Results are yet to be reported on

effects on neurogenesis, however use of the Rho kinase inhibitor HA1077 (Fasudil) promotes extant neuron survival, regeneration and functional improvement, in Parkinson's disease and stroke models (Lemmens et al., 2013; Rodriguez-Perez et al., 2013).

FACTORS THAT REGULATE NEURONAL MORPHOLOGY AND SURVIVAL—OTHER POTENTIAL TARGETS TO ENHANCE SURVIVAL AND INTEGRATION OF NEWBORN ADULT NEURONS

While administration of Fasudil appeared to have few side effects, not many studies have been performed at present and so the possibility exists that problems may emerge. A number of other factors are known to regulate neuronal morphology and also play a role in neurogenesis and newborn neuron survival. These may, at least in part, signal via Rho GTPases to perform their function, but given the broad expression of Rho GTPases, in some instances it may be more prudent to target molecules that have a more limited expression profile, to avoid potential side effects. These have been recently reviewed (Vadodaria and Jessberger, 2013) so only a subset will be discussed briefly here.

SOCS2

An example of such a molecule is the intracellular regulator of cytokine and growth factor signaling, Suppressor of Cytokine Signaling-2 (SOCS2). Although best known as a negative feedback regulator of growth hormone signaling (Metcalf et al., 2000), it has a broader range of molecules it interacts with, including EGF and Epo (Basrai et al., 2013; Goldshmit et al., 2004a,b). Overexpression of SOCS2 enhances neurite length and branching, which may in part be due to activation of Rac1 and inhibition of Rho kinase (Goldshmit et al., 2004a,b). During development, SOCS2 is expressed widely throughout the developing brain and regulates developmental neurogenesis (Polizzotto et al., 2000; Turnley et al., 2002). In the adult its expression is primarily in the dentate gyrus and hippocampal CA3 region, suggesting it plays a role in adult neurogenesis. Overexpression of SOCS2 increased numbers of newborn adult hippocampal neurons without affecting their differentiation or proliferation of neural precursor cells (Ransome and Turnley, 2008). These results are in keeping with the idea that enhancing neurite outgrowth and complexity may enhance survival and integration of newborn neurons.

Neurotrophins

Although many growth factors have been shown to play a role in some aspect of adult neurogenesis, often promoting proliferation and survival of neural precursor cells (Christie and Turnley, 2012), few have been shown to play a role in the specific stage of neuronal maturation, integration and survival. The neurotrophin brain-derived neurotrophic factor, BDNF, is one such factor. BDNF has been shown to regulate neuronal morphology via activation of the Rho GTPases Rac1 and cdc42 (Miyamoto et al., 2006; Cheung et al., 2007). It is important for neurite outgrowth, dendritic arborization and spine density of adult hippocampal neurons, but not proliferation or cell fate specification (Chan et al., 2008; Gao and Chen, 2009; Gao et al., 2009; Bergami et al., 2013) and deletion of TrkB decreases survival and induces an anxiety-like phenotype (Bergami et al., 2008). Further, in the absence of hippocampal BDNF, there is increased death of adult newborn hippocampal neurons following traumatic brain injury (Gao and Chen, 2009).

OTHER MODULATORS OF NEURAL PLASTICITY THAT MAY BE HARNESSED TO PROMOTE SURVIVAL AND INTEGRATION

Normal physiological levels of neurogenesis, newborn neuron integration, and survival are enhanced by activity-induced plasticity. This can be applied to the therapeutic scenario, where environmental enrichment, exercise, or forced use can induce circuit sprouting and plasticity and lead to functional improvement. While this has been examined more in terms of axonal regeneration, such as by inhibition of repulsive axon guidance molecules, it is also applicable to enhancement of neuron integration into damaged circuitry. For example, blocking of members of the Eph/ephrin family promotes axonal and dendritic sprouting/regeneration and enhances functional recovery (Goldshmit et al., 2011; Overman et al., 2012; Spanevello et al., 2013). This sprouting/plasticity can be further enhanced with forced use of the affected limb (i.e., enhancing plasticity) in a stroke model (Overman et al., 2012). Altering the expression of different Ephs or ephrins has positive or negative effects on neurogenesis (Ricard et al., 2006; Chumley et al., 2007; Jiao et al., 2008; Hara et al., 2010; Murai et al., 2010; Ashton et al., 2012). It is likely that their neuronal progeny also express these molecules, suggesting that blocking them or modulating their signaling pathways (again, converging on Rho GTPases) may enhance newborn neuron survival and integration into damaged but sprouting local circuitry. Indeed, deletion of ephrinB3 leads to enhanced neurogenesis around the lesion site in a stroke model, however it also resulted in more severe ischemic damage (Doeppner et al., 2011). Targeting of other repulsive guidance molecules, such as Semaphorins, Slits, and DCC may also be advantageous (Christie and Turnley, 2012).

Another factor that inhibits neural regeneration and limits access to neuronal circuits is the peri-neuronal net (Kwok et al., 2011). This is made up of extracellular matrix, such as proteoglycans, surrounding neuronal cell bodies, and their processes. Degradation of the nets, for example using chondroitinases, enhances sprouting, plasticity, and functional improvement after injury (Wang et al., 2011). Given that breakdown of the peri-neuronal nets makes it easier for synaptic plasticity to occur, it may also allow easier access and integration of newborn neurons, although this has yet to be examined.

COMBINATION OF NEWBORN NEURON SURVIVAL PROMOTING AGENTS WITH NEUROPROTECTIVE, PROLIFERATIVE, OR PLASTICITY INDUCING AGENTS

While a number of factors have been described above that have the potential to enhance newborn neuron integration and survival in an injury or disease setting, we cannot forget that the damaged CNS environment is much different to the normal physiological neurogenic niches where neurogenesis normally takes place. On the plus side, the injured CNS undergoes a certain amount of sprouting and local circuit rearrangement, which may open up the opportunity for newborn neurons to compete for synaptic space along with sprouting extant neurons, unlike

the comparatively non-plastic normal CNS parenchyma. On the minus side, the damaged CNS environment is quite an inhibitory environment, such that newborn neuroblasts may need to be protected so that they can differentiate and become mature neurons, rather than dying of other causes along the way. Neuroprotective or anti-inflammatory strategies probably also need to be included with therapy to improve newborn neuron maturation, integration, or plasticity. There are a number of promising molecules in this regard, such as erythropoietin (Epo) which is neuroprotective and enhances the numbers of neural precursor cells and neurons following neural injury (Shingo et al., 2001; Wang et al., 2004; Lu et al., 2005; Tsai et al., 2006; Byts and Siren, 2009; Zhang et al., 2012). It has shown promise in a large range of pre-clinical studies and is currently undergoing clinical trial for a range of conditions. Combinatorial studies now need to be performed, with for example Epo and inhibition of Rho kinase, to determine whether the outcome is further improved, and numbers of surviving and integrated newborn neurons is enhanced more than either factor alone.

CONCLUSIONS

There is a high rate of failure of newborn adult neurons to integrate into local circuitry and survive under physiological conditions and an even higher failure rate in injury or disease conditions. We propose that provision of factors that enhance the competitiveness of newborn neurons to integrate into circuitry, either by affecting the newborn neurons themselves or by affecting plasticity of the local circuitry, will improve this bottleneck of low newborn neuron survival, leading to improved functional outcome following neural damage. Further, it is likely that such treatments will be more effective if combined with treatments that will make the injured CNS a less toxic environment, by use of inflammatory modulators or neuroprotective agents. After two decades of research we know what needs to be done and we now have several avenues of approach to be tested that may actually allow the therapeutic promise of adult neural stem cells to be realized.

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Functional neurogenesis in the adult hippocampus: then and now

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INTRODUCTION

After two decades of research, the neurosciences have come a long way from accepting that neural stem/progenitor cells (NSPCs) generate new neurons in the adult mammalian hippocampus to unraveling the functional role of adult-born neurons in cognition and emotional control. The finding that new neurons are born and become integrated into a mature circuitry throughout life has challenged and subsequently reshaped our understanding of neural plasticity in the adult mammalian brain. It is now widely accepted that neurogenesis in the adult central nervous system occurs in multiple brain regions within the rodent brain, including the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG). Since the discovery of ongoing neurogenesis in the adult brain, the field has been addressing questions regarding the cellular identity of adult NSPCs, the molecular pathways regulating maturation and integration of newborn neurons into preexisting circuitries, and how new neurons contribute to adult brain function. Technological advances over the last two decades such as targeted modulation (loss- and gain-of-function) of adult neurogenesis and refinements in behavioral testing paradigms have enabled us to begin addressing these questions directly. Here we give a brief overview of old and new studies examining the function of adult hippocampal neurogenesis (AHN) in the context of evolving technology, which has exponentially expanded our understanding of the neurogenic process in the adult mammalian brain.

EARLY STUDIES: FROM CORRELATION TO CAUSALITY

Early on, the field went through a phase of correlating levels of AHN with performance in behavioral tests of hippocampus-dependent learning and memory, and affective behavior. Manipulations that increase AHN such as environmental enrichment, physical activity, and also treatment with certain antidepressants were found to enhance performance in spatial navigation tasks (e.g., Morris water maze; MWM) and in tests of anxiety-related behaviors (forced swim test, elevated plus maze) (Kim et al., 2012). Conversely, stress, aging, and inflammation, all of which negatively affect AHN, resulted in decreased performance in tasks of spatial navigation and emotion-related behaviors (Kim et al., 2012). Although correlative, these data generated in the late 1990s and early 2000s, suggested a role for AHN in hippocampus-dependent processes of cognition and emotion. The first studies attempting to show causal relationship between AHN and hippocampus-dependent behavior were published in the early 2000s, using the antimitotic drug methylazoxymethanol acetate (MAM) and focal irradiation of the hippocampus to ablate AHN. MAM-treated and focally irradiated mice showed impairments in hippocampus-dependent trace-conditioning and certain forms of long-term spatial memory (Shors et al., 2001; Snyder et al., 2005; Deng et al., 2009), suggesting that AHN was required for particular aspects of learning and memory. However, seemingly inconsistent findings from multiple studies with confounding variables such as incomplete elimination of neurogenesis and unwanted off-target effects (such as irradiation-induced inflammation) impeded a precise understanding of the contribution of AHN to hippocampal function (Deng et al., 2010).

FUNCTIONAL HIPPOCAMPAL NEUROGENESIS AND EVOLVING METHODOLOGY

Significant advances in conditional gene targeting allowing the generation of transgenic mice and virus-based approaches enabled the selective targeting of adult hippocampal NSPCs and their neuronal progeny, and revealed not only the molecular pathways important for the different stages of neurogenesis, but also specific behavioral correlates of altered AHN (Saxe et al., 2006; Dupret et al., 2008; Jessberger et al., 2009; Deng et al., 2010; Ming and Song, 2011). Commonly used approaches include the expression of cell death-inducing genes (such as diphtheria toxin or its receptor and thymidine kinase that kills dividing cells upon ganciclovir injections), overexpression of pro-apoptotic genes (such as Bax), and expression of light-sensitive ion channels (such as channelrhodopsins enabling conditional depolarization or hyperpolarization of newborn neurons) in

NSPCs and/or their neuronal progeny (Deng et al., 2010). Fewer methods have been utilized to genetically boost neurogenesis. One elegant approach has been to utilize transgenic mice where the pro-apoptotic gene BAX was conditionally deleted in nestin-expressing NSPCs (iBAX^{nestin}), resulting in substantially enhanced levels of AHN (Sahay et al., 2011a). As compared to previous cytostatic drug- and irradiation-based strategies, these techniques improved temporal and tissue-specific control for ablating the desired neuronal population. Studies utilizing these strategies in combination with an array of behavioral tests have revealed novel roles for AHN. Together with correlational studies, genetic, and pharmacological approaches to manipulate levels of AHN are currently being used to understand the functional significance of AHN. Spatial discrimination tasks such as feared context, radial-arm maze, modified MWM, and the two-choice discrimination task have been utilized to test for a function of newborn neurons (Saxe et al., 2007; Clelland et al., 2009; Deng et al., 2009; Sahay et al., 2011a; Nakashiba et al., 2012) and there is now sufficient evidence suggesting that AHN plays a crucial role in the pattern separation functions of the DG (Treves et al., 2008; Yassa and Stark, 2011). The two-choice discrimination task where mice must discriminate between spatially proximate stimuli may become one of the behavioral tasks of choice (Clelland et al., 2009; Mctighe et al., 2009). Complementary approaches to knockdown AHN revealed selective deficits in this task and the radial arm maze. On the other hand, boosting AHN by genetically enhancing newborn neuron survival (using iBax^{nestin}) enhances discrimination between similar contexts in a contextual fear-conditioning task (Sahay et al., 2011a). Notably, AHN becomes critical only when contexts/patterns become more similar and therefore more difficult to discriminate during recall; thus, AHN seems to be dispensable for discriminating between highly dissimilar contexts/patterns but crucial for computing and discerning highly similar input patterns. Transgenic strategies enabling selective ablation of young and adult-born DG neurons vs. mature DG granule neurons in combination with modifications of the MWM show that in the absence of mature neurons, separation between similar spatial contexts is enhanced, whereas, "completing" a pattern with only a subset of the cues is impaired (Nakashiba et al., 2012). These results highlight an interesting interplay between "newborn" and "old" neuronal populations, suggesting different yet complementary functions of pattern "separation" vs. "completion," respectively. Collectively, studies from multiple labs provide evidence of a strong link between AHN and proposed pattern separation functions of the DG (Sahay et al., 2011b). Furthermore, recent data using novel transgenic mice and virus-based approaches (e.g., optogenetics and TK-based approaches) support the hypothesis that new neurons are particularly important for memory encoding and retrieval during a critical period 4–8 weeks after new neurons are born (Deng et al., 2009; Gu et al., 2012).

Recent reports also support a role for AHN in emotional control and affective behavior. These studies benefitted not only from novel methods to ablate AHN, but also refinements in testing paradigms for specific aspects of emotion-related behaviors (Samuels and Hen, 2011; Kheirbek et al., 2012). Particularly, irradiated and transgenic mice with diminished AHN exhibit signs of heightened stress response as observed in the food avoidance test (after acute stress), increased despair-like behavior in the forced swim test, and increased anhedonia in sucrose preference tests (Snyder et al., 2011). These deficits may be in part due to a dysfunctional regulation of the hypothalamic-pituitary-adrenal (HPA) axis that may lead to a disproportionate response to stress-inducing stimuli in mice with impaired AHN (Snyder et al., 2011). Interestingly, although ablation of AHN led to a heightened stress response along with behavioral correlates of depression-like behaviors, increasing neurogenesis by itself does not appear to be sufficient for promoting anxiolytic or antidepressant-like behaviors in the iBax mice (Sahay et al., 2011a). However, this may be due to a "ceiling" effect or due to limitations of current testing paradigms for examining "gain of function" in emotion-related behaviors.

FUNCTIONAL AHN: OPEN QUESTIONS

Accumulating evidence over the last years has clearly demonstrated a role for AHN in hippocampus-dependent cognition and emotional control. However, it is currently unclear *how* exactly newborn neurons shape the DG circuitry and mediate DG-dependent pattern separation. A large number of open questions remain: how are individual patterns represented in the DG (Deng et al., 2013)? How does the hippocampal circuit "change" with the addition of each pattern-associated cohort of newborn neurons? How does top-down or cortical input regulate AHN and its function in learning new information? How much do newborn young neurons contribute to memory engrams in the DG? How do adult-born hippocampal neurons regulate the HPA axis, which contributes to the neurogenesis-associated regulation of anxiety-related behaviors? Do distinct subsets of newborn neurons contribute to pattern separation vs. emotional regulation role of the DG? Other questions pertain to the relevance of varying levels of AHN, basally, by environmental stimuli, and in the context of disease: How do variations in AHN contribute to individuality in exploratory behavior and could this be extended to humans (Freund et al., 2013)? How does aging regulate AHN and can boosting AHN alleviate age-related decline in aspects of cognition? Can AHN be harnessed for endogenous brain repair and restoration of neuronal function in diseases that is associated with diminished or altered AHN, such as major depression, epilepsy, Alzheimer's disease, and Parkinson's disease? Interestingly, recent findings that levels of hippocampal neurogenesis remain substantial even through the fifth decade of life in the adult human brain, opens up possibilities for doing functional studies in humans related to AHN (Spalding et al., 2013), for example by combining non-invasive imaging strategies together with DG-dependent behavioral paradigms (Brickman et al., 2011; Yassa and Stark, 2011; Dery et al., 2013). With the development of novel genetic tools there is great hope for answering these questions, however, it also seems plausible that we need to develop more refined and sensitive testing paradigms to closely examine AHN-dependent behaviors. In addition, it

is clear that most genetic approaches are only suitable for studies using mice, limiting the possibility to use different species to broaden the relevance of the obtained findings. Thus, developing novel methods to measure and /or manipulate AHN in primates and even humans will be important to move the field toward biomedical relevance.

Be that as it may, the finding that the adult mammalian brain continuously generates new neurons throughout life has contributed significantly to our understanding of brain functioning and recent technological advances provide further impetus for studying the function of AHN in health and disease.

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How neurogenesis finds its place in a hardwired sensory system

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So far most studies on adult neurogenesis aimed to unravel mechanisms and molecules regulating the integration of newly generated neurons in the mature brain parenchyma. The exceedingly abundant amount of results that followed, rather than being beneficial in the perspective of brain repair, provided a clear evidence that adult neurogenesis constitutes a necessary feature to the correct functioning of the hosting brain regions. In particular, the rodent olfactory system represents a privileged model to study how neuronal plasticity and neurogenesis interact with sensory functions. Until recently, the vomeronasal system (VNS) has been commonly described as being specialized in the detection of innate chemosignals. Accordingly, its circuitry has been considered necessarily stable, if not hard-wired, in order to allow stereotyped behavioral responses. However, both first and second order projections of the rodent VNS continuously change their synaptic connectivity due to ongoing postnatal and adult neurogenesis. How the functional integrity of a neuronal circuit is maintained while newborn neurons are continuously added—or lost—is a fundamental question for both basic and applied neuroscience. The VNS is proposed as an alternative model to answer such question. Hereby the underlying motivations will be reviewed.

Keywords: accessory olfactory bulb, AOB, vomeronasal, VNO, neurogenesis, pheromones, plasticity, innate

INTRODUCTION

The idea of a mature brain as an organ with limited growth, cell renewal and rewiring has considerably changed since pioneer studies on adult neurogenesis (Altman and Das, 1966; Altman, 1969; Graziadei and Monti-Graziadei, 1979; Lledo and Gheusi, 2006; Kempermann, 2012). In the adult mammalian brain, neural progenitors are present in the subventricular zone (SVZ) of the lateral ventricles and the hippocampal subgranular zone (SGZ) where they give rise respectively to Dlx2/5/6-derived GABAergic olfactory bulb interneurons and glutamatergic granule cells of the dentate gyrus (DG) of the hippocampus (Doetsch et al., 1999; Seri et al., 2001; Kriegstein and Alvarez-Buylla, 2009). Admittedly, neurogenesis in other adult brain regions is generally believed to be very limited under physiological conditions (Nishiyama et al., 1996; Horner et al., 2000; Dawson et al., 2003; Luzzati et al., 2006, 2011; Bonfanti and Peretto, 2011), although it could be induced after injury (Ramaswamy et al., 2005; Gould, 2007; Yu et al., 2008; Kernie and Parent, 2010; Saha et al., 2013) or as a consequence of tissue inflammation and degeneration (Buffo et al., 2008; Ohira et al., 2010; Luzzati et al., 2011; Belarbi and Rosi, 2013). Nowadays several approaches have been developed to maintain and manipulate pluripotent stem cells *in-vitro* in the perspective of brain repair (Takahashi and Yamanaka, 2006; Yamanaka and Blau, 2010). Particularly the rodent olfactory bulb (OB) has been widely studied to clarify the logic of neuronal stem-cell biology in the SVZ opening new venues to brain-repair strategies, cell transplants techniques in disease models and other translational approaches (Gage and Temple, 2013).

However, the development of clinical translations cannot stand aside the basic research, focused in this case on the physiologic function of the neurogenic regions *in-vivo* (see for critical reviews on this point Lau et al., 2008; Lindvall and Kokaia, 2010).

In addition, studying OB neurogenesis may yield new insights in the biology of olfaction, being olfactory sensory activity and behaviors easy readouts of any experimental manipulation in rodents.

Understanding how the environment affects newborn neurons integration into mature networks, and consequently normal brain function, are certainly meaningful aims to define the boundaries between physiology and pathology in translational neuroscience. The restoration of brain connectivity after trauma or the comprehension of the etiology of major brain disorders may certainly move forward and undoubtedly more clinically oriented approaches would benefit from the unbiased attempts of basic research to address these issues (Fang and Casadevall, 2010;

Abbreviations: aAOB, anterior accessory olfactory bulb; AOB, accessory olfactory bulb; CR, calretinin; DCX, doublecortin; DG, dentate gyrus; ECL, external cellular layer; EGC, external granule cell; ET, external tufted; GAD, glutamic acid decarboxylase; GC, granule cell; Gl, glomerular layer; HMW, high molecular weight; ICL, internal cellular layer; IGC, internal granule cell; LMW, low molecular weight; LOT, lateral olfactory tract; MACs, main accessory cell; MOB, main olfactory bulb; MOE, main olfactory epithelium; MOS, main olfactory system; NSE, non-sensory epithelium; OB, olfactory bulb; OSNs, olfactory sensory neurons; pAOB, posterior accessory olfactory bulb; PC, principal cell; PG, periglomerular cell; PV, parvalbumin; SA, short axon cell; SGZ, subgranular zone; SVZ, subventricualr zone; TH, tyrosine hydroxylase; VNO, vomeronasal organ; VNS, vomeronasal system; VSNs, vomeronasal sensory neurons.

Enserink, 2013). In the present manuscript a special attention will be given to neurogenesis in a particular olfactory subsystem—namely the accessory/vomeronasal system (VNS)—due to the fact that, despite its behavioral relevance in rodent sociality, it received so far a minor deal of attention. The main point hereby stressed concerns the unclear relationship between form—plastic and changing—and function—presumably stable, innate—in the VNS. Due to its distinct peculiarities, compared to the rest of the olfactory system, the VNS offers an unparalleled opportunity to analyze how newborn neurons constantly integrate into mature circuits without interfering with the physiological behavioral and endocrine development. Recent findings on neurogenesis in the vomeronasal organ (VNO) and accessory olfactory bulb (AOB) will be listed and discussed with a particular emphasis on the AOB, since it represents the first central brain region of this olfactory pathway.

THE VOMERONASAL SYSTEM AS A MODEL TO STUDY ADULT NEUROGENESIS

Neurogenesis in the OB has been studied predominantly in the main olfactory pathway. The neurons constantly replaced in the main olfactory bulb (MOB) are GABAergic local interneurons (periglomerular, PGs, and granule cells, GCs) mainly derived from the Dlx2 subpallial domain in the SVZ (Puelles et al., 2000; Alvarez-Buylla and Garcia-Verdugo, 2002; Lledo and Gheusi, 2006; Whitman and Greer, 2009). These cells play a key role in regulating MOB input and output activity (Spors et al., 2012), and they have been proved to actively contribute to olfactory processing (Mandairon et al., 2011; Alonso et al., 2012) given their activity dependent survival and functional recruitment (Magavi et al., 2005; Mouret et al., 2008; Sultan et al., 2011a,b). In most of these reports the role of newborn neurons in the context of olfactory discrimination, short and long-term olfactory memory has been analyzed using synthetic odor compounds or artificial behavioral tasks. These paradigms are well suited to answer specific questions about the logic of sensory transduction (e.g., tuning, discrimination, detection threshold). However, framing the same analysis within the contexts of reproduction and sociality may be more informative to clarify whether neurogenesis itself is necessary or not to the mature brain. Indeed reproduction and sexual selection constitute a powerful evolutionary force and therefore the primary drive for any functional adaptation of a brain circuit. So far only few recent studies correlated MOB neurogenesis, with the regulation of social behavior in mice (see for example Larsen et al., 2008; Kageyama et al., 2012; Monteiro et al., 2013). The functional studies on the role of neurogenesis in the VNS are considerably fewer despite the major contribution of the VNS in rodent sociality (Tirindelli et al., 2009; Mucignat-Caretta, 2010; Chamero et al., 2012; Ibarra-Soria et al., 2013). Moreover the presence of neurogenesis in the AOB has been largely ignored, if not denied (Mak et al., 2007). However, neurogenesis occurs postnatally both at the VNS periphery, in the VNO, and more centrally, in the AOB (VNO: Barber and Raisman, 1978; Graziadei and Monti-Graziadei, 1979; Jia and Halpern, 1998; Giacobini et al., 2000; Martinez-Marcos et al., 2005; Weiler, 2005; Brann and Firestein, 2010; Enomoto et al., 2011; AOB: Bonfanti et al., 1997; Martínez-Marcos et al., 2001; Peretto et al., 2001; Huang and Bittman, 2002; Oboti et al., 2009, 2011; **Figure 1**). Interestingly cell survival in the AOB is higher after sensory stimulation around weaning and puberty onset (ca. 4 weeks in mice) when, after gonadal and endocrine maturation, social and reproductive behaviors become more clearly manifest (Oboti et al., 2011). Concurrently, despite the VNO seems to be already functional at birth (Coppola and O'Connell, 1989), the process of wiring and synaptogenesis of the VNO-AOB circuit has been shown to extend postnatally and to reach maturity only around the third postnatal week (Horowitz et al., 1999).

This seems to suggest the occurrence of a post-pubertal functional tuning of the VNS circuitry through neurogenesis, plasticity and constant rewiring, which goes beyond an early postnatal maturation of the system, similarly to the main olfactory system (**Figure 1**; Bonfanti and Peretto, 2011; Lepousez et al., 2013).

The reason why the VNS has been neglected by more recent studies on olfactory neurogenesis is possibly due to two main reasons. Firstly, the VNS is absent in humans, therefore limiting the interest in extending the study of olfactory neurogenesis to this system in rodents. Secondly, this sensory system has been traditionally associated to *pheromone* detection, innate signal processing and stereotyped endocrine responses, for which plasticity, neurogenesis, and rewiring are apparently not necessary. Nonetheless, regardless of any homologies in the mammalian olfactory systems, we undoubtedly share with rodents and other species the functions that this sensory pathway specifically regulates, when present (**Figure 2**). Therefore, one of the main reasons why neurogenesis in the rodent VNS deserves more attention is related to understanding the neural bases of mammalian neuroendocrine and behavioral development and how they are affected by environmental cues. Ultimately, the rodent VNS is a suitable and simple model to tackle wider issues related to other mammals in general, humans included.

The aim of the following paragraphs is to evaluate different aspects of VNS neurogenesis ranging from the phenotypes of newly generated neurons to their functional impact on specific circuits. The comparative description accompanying each of these points aims to open new questions for a wide range of approaches. These entailing the developmental, circuit, and system biology of olfaction. Aside, emerges the interesting—yet unanswered—question of how this olfactory subsystem acquires its function in the precise way it does, while its circuits are constantly changing. Rather than supporting the hardwired nature of this process, the evidences here reported suggest a necessary role for neurogenesis, neuronal plasticity, and environmental adaptation for its accomplishment.

NEUROGENESIS IN THE VOMERONASAL ORGAN

Olfactory sensory neurons (OSNs) are directly exposed to the environment to detect chemical stimuli through membrane bound receptors on their cilia (MOS) or microvilli (VNS). In the olfactory epithelia, ciliated and microvillus neurons, supporting cells and ensheating glial cells are constantly renewed during pre- and postnatal development (Murdoch and Roskams, 2008) by neural stem cells deriving from both neural crest and olfactory placode precursor lineages (Katoh et al., 2011; Heron et al., 2013; Suzuki et al., 2013). Due to this peripheral localization,

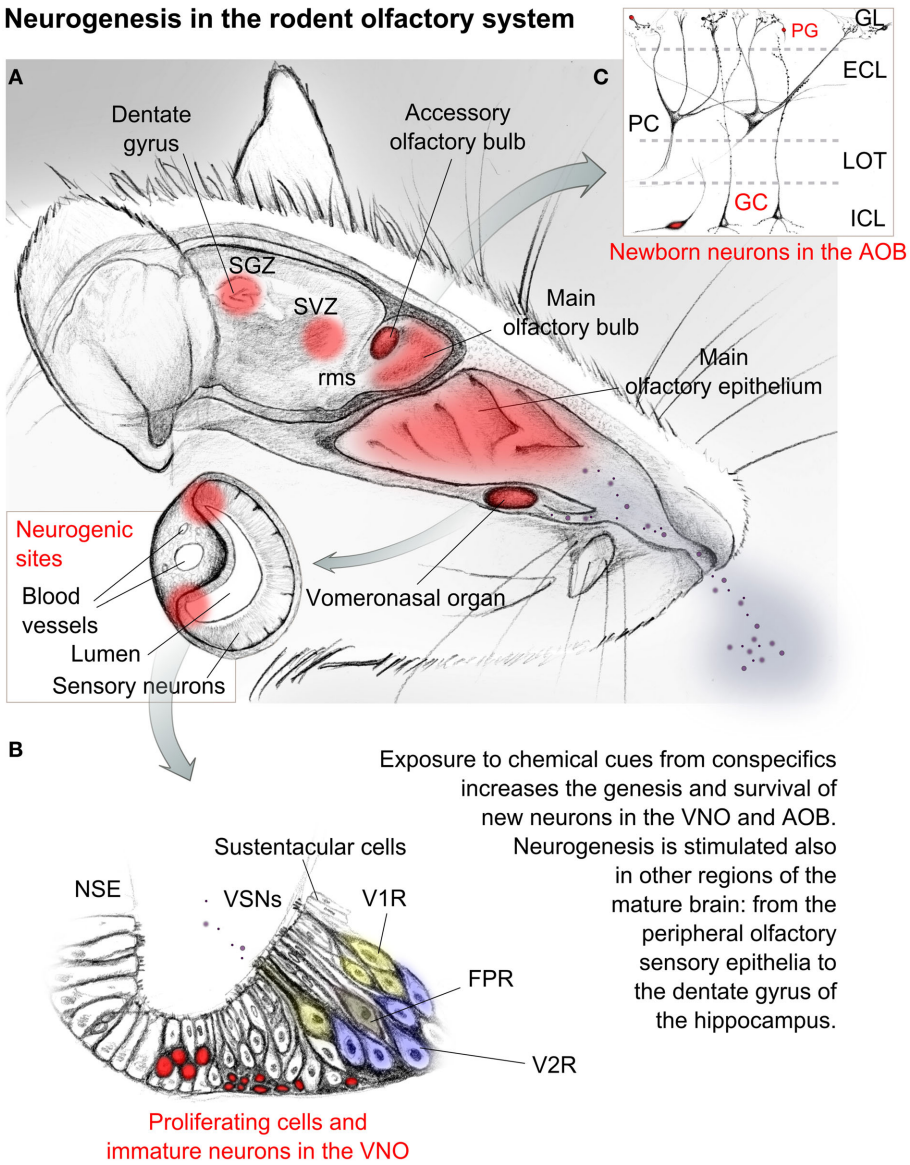

FIGURE 1 | Sketched representation of the mouse olfactory system. Central and peripheral neurogenic regions are evidenced in red. **(A)** Social odors and chemical cues are detected through the main olfactory epithelium and the vomeronasal organ, which is enclosed in a bony capsule opened rostrally toward the nasal cavity. A highly vascularized cavernous tissue flanking the organ allows tissue contraction and therefore the access of mucuous fluids transporting chemical cues toward the sensory epithelium. **(B)** Enlarged view of the vomeronasal sensory epithelium and its cell types. Proliferating cells are localized at the lateral and basal margins of the matured sensory epithelium. Sensory neurons here located send axonal projections to the accessory olfactory bulb. **(C)** Simplified anatomy of the accessory olfactory bulb cellular layers. Cells evidenced in red in **(B,C)** represent immature or regenerating neurons. Abbreviations: SGZ, subgranular zone; SVZ, subventricular zone; rms, rostral migratory stream; GL, glomerular layer; PG, periglomerular cell; ECL, external cellular layer; ICL, internal cellular layer; LOT, lateral olfactory tract; PC, principal cell; GC, granule cell; V1R, vomeronasal receptor neuron type1; V2R, vomeronasal receptor neuron type2; FPR, formyl peptide receptor neuron; VSNs, vomeronasal sensory neurons; NSE, non-sensory epithelium.

OSN renewal has been generally associated to tissue growth (during development), homeostasis and repair (during adulthood) as gene expression patterns are maintained very similar (Heron et al., 2013). In the VNO, as in the MOE, the proliferation of different subsets of neuronal progenitors gives rise to OMP-positive mature receptor neurons (Murdoch and Roskams, 2008; Enomoto et al., 2011) but begins slightly later. In the rat olfactory epithelium OMP starts to be expressed at E14, while in the VN epithelium it occurs only at P2 (Kulkarni-Narla et al., 1997). In the mouse OMP is expressed in vomeronasal sensory neurons (VSNs) a few days earlier, during the last week of gestation (Tarozzo et al., 1998). These data indicate that VSNs are not fully developed at birth as most of their maturation begins and occurs postnatally.

Mature VSNs can be divided in three main families, depending on the receptors expressed and the ligands they have been

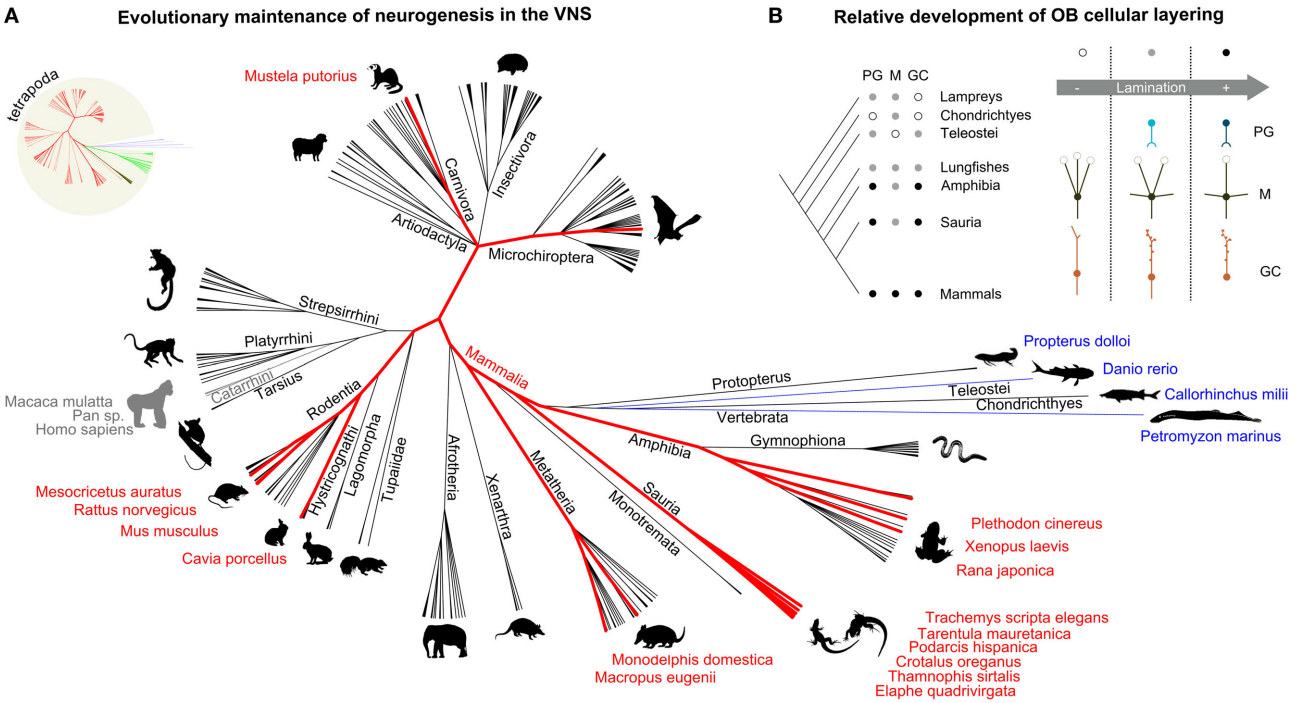

FIGURE 2 | Neuronal plasticity in the vomeronasal system is a conserved trait across several vertebrate species. The increased organizational complexity of the olfactory systems is plausibly related to their protracted development through postnatal neurogenesis. **(A)** Cladogram representing the main vertebrate taxa in which the presence of a vomeronasal system has been reported (black lines). Taxa indicated in blue possess the cellular and molecular elements of the VNS, without a defined structural organization. Old world monkeys are indicated in gray, as reference, although they generally do not possess a functional VNS. The species (and related taxa) in which neurogenesis in the VNS has been reported are highlighted in red. **(B)** Features of the vertebrate olfactory systems include: more defined cellular layering (white: low, gray: moderate, black: high lamination), presence of periglomerular cells, PG (white: absent, gray: ambiguous, black: present), development of mitral cell secondary dendrites, M (white: absent, only multiglomerular primary dendrites, gray: presence of both multiglomerular primary dendrites and secondary dendrites, black: monoglomerular primary dendrites and secondary dendrites), loss of granule cell axon, GC (white: smooth dendrites, axon, gray: spiny dendrites, axon, black: spiny dendrites, no axon). Sources: (Meisami and Bhatnagar, 1998; Eisthen, 2000; Halpern and Martinez-Marcos, 2003; Grus and Zhang, 2008; Eisthen and Polese, 2009; Mucignat-Caretta, 2010); NCBI.

reported to detect: V1Rs, activated preferentially by low molecular weight (LMW) hormone metabolites and other small molecules possibly contained in urine and bodily secretions; V2Rs, activated mainly by high molecular weight (HMW) peptidic compounds such as lipocalins, or smaller MHC peptides; formyl peptide receptors (FPRs), involved in the immune cell response to infections (Chamero et al., 2012; **Figure 1B**). V1R expressing neurons populate the apical portion of the sensory epithelium, V2Rs are located more basally while FPRs are more heterogeneously distributed (Rivière et al., 2009).

The earlier steps of VSNs differentiation are regulated by the proneural bHLH genes Mash1 and Neurogenin1, the former maintaining stem-cell progenitors, the second determining their multipotency (Cau et al., 2002), which is further regulated by the gene Ctip2 (Enomoto et al., 2011). Accordingly, loss of Ctip2 shifts the V1R/V2R differentiation ratio toward the V1R phenotype, suggesting a pivotal role in VSNs maturation and the possibility that the V2R-lineage entails both V1R and V2R committed neurons (Enomoto et al., 2011). The molecular mechanisms specifying the FPR lineage are not known.

VSNs proliferation is not homogeneous across the sensory epithelium but seems to be increasingly more localized at its margins, as development proceeds (Barber and Raisman, 1978; Giacobini et al., 2000; Martinez-Marcos et al., 2005; de la Rosa-Prieto et al., 2011). Although immature VSNs show limited migratory capabilities during adulthood (Martinez-Marcos et al., 2005; de la Rosa-Prieto et al., 2011), their proliferation increases until 2 months of age, in mice (Weiler, 2005; Brann and Firestein, 2010). Newborn VSNs are produced in clusters giving rise to patterned waves of migrating neurons (as observable by DCX immunohistochemistry). It would be interesting to clarify whether neurons expressing receptors of the same family are simultaneously generated at a given time. However, BrdU experiments suggested that both V1Rs and V2Rs are produced at the same pace in physiological conditions (de la Rosa-Prieto et al., 2010). Interestingly, postnatal development and growth seems to be present also in the non-sensory epithelium (NSE, **Figure 1B**) of the VNO (Garrosa et al., 1998; Elgayar et al., 2013).

Despite the vast number of MOE receptor genes (ca 1500 in the mouse) each OSN expresses only one of them. In the VNO this rule is not followed since each VSN may express more than one receptor gene (Martini et al., 2001; Silvotti et al., 2007; Ishii and Mombaerts, 2011). Interestingly, the phenotypic identity of newborn VSNs can be affected by histone

modifications following application of urine ligands *in-vitro* (Xia et al., 2010). While application of HDAC inhibitors to cultured VSNs decreases the expression of immature neuronal marker Nestin, and increases the expression of markers of differentiation such as Map2, Neuro D1-D2, and V2R genes (Xia et al., 2010). It remains to be clarified whether these effects, represent a generalized adaptive response of the VNO epithelium to sensory stimulation or if it constitutes a mechanism to selectively specific subsets of newborn VSNs, as shown in the MOE (Watt et al., 2004; Dias and Ressler, 2013). Both simple parsimony and recent evidences suggest that this latter might be the case (Broad and Keverne, 2012). However, other factors such as odor exposure (Xia et al., 2006), hormonal changes (Kaba et al., 1988; Paternostro and Meisami, 1996) and sensory activity (Hovis et al., 2012) contribute to postnatal VNO neurogenesis and VNO-AOB rewiring indicating the persistence of constant adjustments in this circuit. Altogether these results strongly suggest that VNO neurogenesis do not serves mere tissue homeostasis and repair, but actively contributes to the functional tuning of the organ during postnatal development. A possibility still largely unexplored.

NEUROGENESIS IN THE ACCESSORY OLFACTORY BULB, A COMPARATIVE NOTE

Afferent axons from VSNs reach the brain at the level of the dorsal part of the OB. Here they form glomerular-like structures with the apical dendrites of a separate group of projection neurons which constitute the accessory olfactory bulb (AOB; **Figures 1A,C**). As for the MOB, their activity is regulated by inhibitory glomerular and granule cells (**Figure 1C**). Neurogenesis in the AOB involves mainly these two cell types (Oboti et al., 2009). Despite earlier doubts on its presence, several lines of evidence support the idea that adult neurogenesis represents a constitutive feature of the AOB. It can be found in adult mice of both genders (Oboti et al., 2009; Nunez-Parra et al., 2011), in adult rats (Peretto et al., 2001) and rabbits (personal observation). In addition, AOB neurogenesis has been reported not only in mammals (Altman and Das, 1966; Altman, 1969; Hinds, 1968a,b; Kaplan and Hinds, 1977; Bayer, 1983; Kaplan, 1985; Kishi, 1987), but also in other vertebrate species such as amphibians (Fritz et al., 1996), and reptiles (Garcia-Verdugo et al., 1989; Pérez-Cañellas and García-Verdugo, 1996; Pérez-Cañellas et al., 1997) indicating that it represents a conserved trait across different taxa (**Figure 2A**), rather than a parallel convergence. Neuronal plasticity in the olfactory system occurs independently of the presence of a discernible VNS, as in primates, fishes, cetaceans, and birds for example (García-Verdugo et al., 2002; Mucignat-Caretta, 2010); **Figure 2A**). In addition, taxa in which some of the typical cellular and molecular elements of the VNS are present, although with different levels of organization (as anurans, lungfishes, sea lampreys, teleosts, and cartilaginous fishes; Eisthen and Wyatt, 2006; **Figure 2A**), retain neuronal plasticity and neurogenesis in the primary olfactory structures during post-hatching and more mature stages. This indicates that a plastic VNS may not be an apomorphic (underived) trait of terrestrial vertebrates (**Figure 2A**; **Table 1**). In bat species, even though the VNS is not always developed, the presence of immature neurons typically expressing doublecortin (DCX) has been noted in the AOB (Amrein I., personal communication).

In mice, rats, rabbits and guinea pigs SVZ neuronal progenitors give rise to neuroblasts integrating in the AOB mainly as granule cells localized in the inner granule cell layer, below the lateral olfactory tract. The presence of newborn neurons in periglomerular layer is very limited, possibly reflecting a much slower turn-over rate of PGs (Martínez-Marcos et al., 2001; Peretto et al., 2001; Oboti et al., 2009; Nunez-Parra et al., 2011). A limited number of cells can be found in the plexiform layer between the GC and PG layers, where principal cells (PC) are located (Oboti et al., 2009; PCs are homologous to MOB mitral cells), possibly representing other cell types such as external granule cells and dwarf cells (Larriva-Sahd, 2008). The increasing importance of local interneurons for OB signal elaboration, reflected by the increased complexity in OB structural lamination (Eisthen and Polese, 2009; **Figure 2B**), implies a possible correlation with the maintenance of their turnover. Overall, OB neurogenesis may represent a necessary and conserved feature of the olfactory pathways, reminiscent of the higher neuronal plasticity showed by the paleocortex, to which it belongs (see **Table 1** for a list of representative studies explicitly focused on VNO or AOB postnatal development and neurogenesis). In the following paragraphs, attention will be given to the anatomy of the AOB, the phenotypes of AOB newborn cells and the possible role in its circuitry considering the present knowledge about its role in the VNS.

NEUROGENESIS IN THE TWO ACCESSORY OLFACTORY BULB SUBREGIONS

Both the glomerular and principal cell (PC) layer of the AOB look clearly partitioned by the segregated V1R/Gai2 and V2R/Gao afferent fibers. Axonal projections from these two neuronal populations establish synaptic contact with either the anterior (aAOB) or posterior (pAOB) AOB, respectively. This separation is visible in the PC layer neuropil (*linea alba*, Larriva-Sahd, 2008). In rodents the V1R and V2R pathways have been shown to selectively respond to low molecular weight organic molecules (Leinders-Zufall et al., 2000; Sugai et al., 2006) and high molecular weight compounds of peptidic nature, respectively (Leinders-Zufall et al., 2004, 2009; Kimoto et al., 2005). Accordingly to this functional dichotomy, differences in c-Fos expression patterns in the two AOB regions have been observed after exposure to gender related odors in male and female mice (Kumar et al., 1999; Halem et al., 2001). Interestingly, in mice and rats (Peretto et al., 2001; Oboti et al., 2009, but not in opossums Martínez-Marcos et al., 2001), newborn cells reaching the AOB in physiological conditions (no odor exposures) seem to be unequally distributed along the rostro-caudal axis. This may reflect a differential rate of development of the two AOB sub-regions or alternatively be related to the V1R/V2R functions being subjected to differential adaptive pressures in a given eco-ethological niche (Suárez et al., 2011a,b). Accordingly, recently a dual embryonic origin of the AOB has been proposed (Huilgol et al., 2013). In this study, Huilgol and coauthors showed that PCs in the pAOB derive from the thalamic eminences at the diencephalic/telencephalic boundary (DTB) from Lhx5 expressing neurons, as part of the amygdala,

Table 1 | List of representative studies explicitly focused on VNO and AOB neurogenesis.

Species	VNO	AOB
Mouse (*Mus musculus*)	Barber and Raisman, 1978 Monti-Graziadei, 1992 Cappello et al., 1999 Giacobini et al., 2000 Weiler, 2005 Martinez-Marcos et al., 2005 Murdoch and Roskams, 2008 Brann and Firestein, 2010 Enomoto et al., 2011	Hinds, 1968a,b Bonfanti et al., 1997 Oboti et al., 2009–2011 Veyrac and Bakker, 2011 Sakamoto et al., 2011 Nunez-Parra et al., 2011
Rat (*Rattus norvegicus*)	Monti-Graziadei, 1992 Weiler et al., 1999a–2005 Inamura et al., 2000 Martínez-Marcos et al., 2000 Matsuoka et al., 2002	Altman and Das, 1966 Altman, 1969 Kaplan and Hinds, 1977 Kishi, 1987 Bayer, 1983 Peretto et al., 2001 Corona et al., 2011 Portillo et al., 2012
Rabbit (*Oryctolagus cuniculus*)	Othman, 2011	Personal observation
Guinea pig (*Cavia porcellus*)		Personal observation
Hamster (*Mesocricetus auratus*)	Ichikawa et al., 1998 Taniguchi and Taniguchi, 2008	Huang and Bittman, 2002
Opossum (*Monodelphis domestica*)	Jia and Halpern, 1998	Shapiro et al., 1997
	Shapiro et al., 1997	Martínez-Marcos et al., 2001
Wallaby (*Macropus eugenii*)	Ashwell et al., 2008	Ashwell et al., 2008
Ferret (*Mustela putorius furo*)	Weiler et al., 1999b	
Bat (various spp.)		Amrein et al., 2007 (OB)
Garter snake (*Tamnophis sirtalis*)	Wang and Halpern, 1982	
Striped snake (*Elaphe quadrivirgata*)	Kondoh et al., 2012	
Wall lizard (Podarcis hispanica)		Garcia-Verdugo et al., 1989 Sampedro et al., 2008 Font et al., 2012
Red-eared slider (*Trachemys scripta elegans*)		Pérez-Cañellas et al., 1997
Gecko (*Tarentola mauritanica*)		Pérez-Cañellas and García-Verdugo, 1996
Clawed frog (*Xenopus laevis*)		
No	Hansen et al., 1998 Higgs and Burd, 2001	Fritz et al., 1996
Yes	Endo et al., 2011	
Salamander (*Plethodon cinereus*)	Dawley et al., 2000	
	Dawley and Crowder, 1995	
Japanese brown frog (*Rana japonica*)	Taniguchi et al., 1996	
Zebra fish (Danio rerio)		Byrd and Brunjes, 2001 *(OB)* Adolf et al., 2006 (OB)

Species in which neurogenesis in the VNS components of the olfactory system could be present are indicated italics.

BST and Cajal-Retzius neurons (Huilgol et al., 2013), while the aAOB PCs share common origin with MOB mitral cells, as indicated by Tbx21 expression in the OB primordium (Huilgol et al., 2013). The DTB is evolutionary conserved in amphibians and mammals indicating that the pAOB may be a residue of the earliest sensory systems originating from the thalamic eminences and controlling olfactory responses in amphibians (Krug et al., 1993; Huilgol et al., 2013). However, the apparent morphology of AOB

granule cell layer does not reveal a similar dichotomy, as AOB granule cells connectivity may be more ambiguous (Larriva-Sahd, 2008). Despite the different origin of pAOB projection neurons, immature GABAergic interneurons of both the aAOB and pAOB may include cells derived from the same Dlx2/5/6, Emx1, and Meis2 lineages in the SVZ (Kohwi et al., 2007; Agoston et al., 2013). Overall this suggests the existence of different regulatory mechanisms locally specifying the phenotype of cells belonging to the same neuronal lineage but integrating in different circuits (aAOB vs. pAOB, but also AOB vs. MOB). Further understanding of the underlying mechanisms would extend our knowledge of the morphological and functional adaptations newborn neurons may be capable of. In addition, given the functional segregation of the VNS circuits (V1R-aAOB, V2R-pAOB), different levels of neuronal plasticity and neurogenesis may reflect a different degree of adaptability to the diversity of chemical stimuli each subsystem elaborates. However, despite the differences these features may have a similar functional relevance for their proper function.

PHENOTYPES OF NEWBORN NEURONS IN THE ACCESSORY OLFACTORY BULB

New neurons migrating from the SVZ through the rostral migratory stream, reach all AOB layers: glomerular (Gl), external (ECL), and internal (ICL) cellular layers (Larriva-Sahd, 2008). At present no detailed analysis has been made to identify these cell types. Moreover, the presence in the MOB of newly generated tbr2-derived glutamatergic cells (Brill et al., 2009) and GABAergic-serotonergic (Inta et al., 2008) interneurons has been recently proven, while in the AOB it remains to be verified.

In the MOB, the cell types forming the glomerulus (juxtaglomerular cells) are classified in periglomerular (PG), short axon (SA) and external tufted (ET) cells, based on their neurochemistry, morphology, and connectivity. Juxtaglomerular cells can be divided in two main GABAergic chemotypes based on the expression of different isoforms of the GABA synthesis enzyme—GAD-65, GAD-67—together with other markers such as dopamine (DA), or its synthesis enzyme tyrosine hydroxylase (TH), calbindin, calretinin, and others (Shipley et al., 2004). Virtually all dopaminergic neurons express GAD-67, while little if no overlap is present between the GAD-65 and the TH sub-populations (Kiyokage et al., 2010). As typical SA cells, TH-GAD-67 neurons innervate multiple glomeruli while GAD-65 neurons are mostly monoglomerular with only few secondary processes directed to other glomerular formations (Aungst et al., 2003; Kiyokage et al., 2010).

In the AOB glomerular layer scarce if not absent GAD-67 staining has been reported together with almost complete lack of TH expression (Mugnaini et al., 1984; Oboti et al., 2009). This possibly indicates a predominance in the AOB of the monoglomerular GAD-65 chemotype. However, newly generated cells with morphological features of both PG uniglomerular cells and SA multiglomerular cells have been identified in the AOB (Oboti et al., 2009) suggesting that the low levels of GAD-67 expression do not necessarily imply the absence of SA-like cells (Mugnaini et al., 1984; Larriva-Sahd, 2008).

In the MOB, dopaminergic PG cells are responsible of thresholding mitral cell firing in response to olfactory inputs (Pírez and Wachowiak, 2008). The lack of dopaminergic signaling in the AOB may imply a minor need for gain control of vomeronasal inputs on PCs being their firing threshold possibly determined by input coincidence from heterotypical glomerular afferents (Meeks et al., 2010).

Other inhibitory cells located more deeply in the AOB are external and internal granule cells, located above and below the lateral olfactory tract (LOT), respectively. Evidence showed the vast majority of newborn cells reaching the AOB is represented by internal granule cells (Oboti et al., 2009; Nunez-Parra et al., 2011). In the ICL main accessory cells are also present (MACs, Larriva-Sahd, 2008) and are distinguishable from granule cells by larger soma and nuclear size and by their sporadic presence in the LOT. Although newborn cells can be often found in the LOT, their nuclear size was always comparable to normal granule cells (external granule cells, in this case), thus limiting the likelihood for MACs to be regenerated during adulthood.

Recently, in the rat MOB the presence of newborn neurons in the external plexiform layer (EPL) has been proved (Yang, 2008). These neurons have been reported to be PV/CR expressing Van Gehuchten cells, multipolar cells and superficial SA cells (Yang, 2008). Since DCX- and BrdU-positive cells can be found in homologous locations in the AOB (ECL), the presence here of these cell types is possible but yet to be investigated.

It is not known whether SVZ-derived interneurons migrating to the AOB belong to the same lineage of those in the MOB. It is possible that genetically distinct populations of interneurons are heterogeneously distributed in the two OB sub-regions. Recently, viral fate-mapping experiments revealed the mosaic nature of the SVZ proliferative domains giving rise to different and heterogeneous pools of GABAergic interneurons (Merkle et al., 2007). However, upon inspection of the material used in this study, no apparent regionalization of either aAOB- or pAOB-committed progenitors was found (viral infected GFP+ cells were found in the AOB of mice injected at all SVZ levels, personal observation). Interestingly, although most of newborn AOB neurons labeled with BrdU coexpress NeuN at 4 weeks of age (80%) as in the MOB, the level of coexpression with other interneuronal markers is much lower (BrdU/GABA, BrDU/GAD-67, and BrdU/calretinin reach only about the 30%; Oboti et al., 2009) (MOB: BrdU/GAD67 is about 80% in the GrL and 30% in the GL; Parrish-Aungst et al., 2007). These results indicate that the phenotype of AOB newborn neurons is similar to the MOB but conserve some peculiarities specified either locally or in the SVZ. The different relative abundance of morpho- and chemo-types in this structure, renders the AOB an interesting circuit to study the differential role of a given cell type in different compartments of the bulbar circuitry. For example by studying PC (AOB mitral cell homolog) electrical responses to peripheral nerve stimulations it would be possible to clarify to which extent SA cells in the AOB may be dispensable—in case of their limited presence in this structure—for a certain olfactory coding task, or—by comparison—which specific function do they serve when present in other bulbar circuits.

SENSORY ACTIVITY-DEPENDENT SURVIVAL AND FUNCTION OF NEWBORN CELLS

As shown by olfactory enrichment or deprivation studies, the maturation and survival of newborn neurons in the MOB depends on sensory inputs (Cummings et al., 1997; Rochefort et al., 2002; Mandairon et al., 2006). Newborn neurons reach the MOB in massive waves but are gradually selected during integration into local circuits (Petreanu and Alvarez-Buylla, 2002) activated by sensory inputs (Magavi et al., 2005; Mouret et al., 2008; Sultan et al., 2011a,b). Importantly, loss or ablation of newborn neurons in the MOB can impair olfactory function (Breton-Provencher et al., 2009; Mandairon et al., 2011) since younger cells are preferentially involved in these circuits (Nissant et al., 2009; Alonso et al., 2012).

Although renewing at a slower rate (Oboti et al., 2009), newborn cells in the AOB are likely to similarly contribute to VNS function. As in the MOB, sensory activity increases the survival of newborn neurons in the AOB (Oboti et al., 2009, 2011; Nunez-Parra et al., 2011). This effect is mediated by chemical cues present in urine or bodily secretions (Nunez-Parra et al., 2011; Oboti et al., 2011), it is abolished after VNO genetic functional ablation in trpc2-ko mice (Oboti et al., 2011), it is persisting until 7 months of age (Nunez-Parra et al., 2011), and gives rise to neurons responding preferentially to experienced odor stimuli (Oboti et al., 2011).

The presence of gender related differences in AOB neurogenesis is controversial (no differences in CD1 mice Oboti et al., 2009, 2011; differences in B6 mice, Nunez-Parra et al., 2011). However, the effect of odor experience on AOB neurogenesis seems to be particularly evident in post-pubertal female mice after male odor stimulation (Oboti et al., 2009, 2011; Nunez-Parra et al., 2011). This seems particularly evident in the aAOB upon chronic exposure to low molecular weight (LMW) chemical cues present in male urine, which are mainly detected by the V1R neurons (Oboti et al., 2011). Larger protein compounds sensed through V2Rs are instead ineffective on neuronal survival in neither of the two AOB regions (Oboti et al., 2011). However, a lack of increase in surviving cells after sensory enrichment does not necessarily imply the absence of a sensory dependent functional recruitment of newborn elements, but only that the net amount of surviving cells remains stable. Considering that social odors are important primers on mice development and reproductive behavior, these findings suggest a possible role of AOB postnatal/adult neurogenesis in sensory processing in both genders. Accordingly, eliminating newborn cells in the whole bulb, AOB included, Sakamoto and colleagues showed for the first time an impairment in olfactory functions involving the VNS such as predator-odor avoidance, aggression and mounting in males (Sakamoto et al., 2011). A finding that has been extended to VNO-dependent mate recognition in females (Oboti et al., 2011). The effect of sensory inputs on AOB neurogenesis overall indicates that newborn neurons play an active and possibly relevant role on the vomeronasal circuitry during postnatal and adult life.

IMPACT OF NEWBORN NEURONS ON AOB CIRCUITS

A few comparative considerations with the MOB elementary functional unit—the olfactory column—can be insightful in defining the impact of newborn neurons on AOB network activity. The MOB olfactory column is considered equivalent to the cortical columns and barrels in the visual and somatosensory cortices (Shepherd, 2010). It comprises all OSNs projecting to a single glomerulus, all the mitral and tufted cells extending their dendrites to it and all the granule cells connected to these projection neurons. Granule cells can regulate mitral/tufted cell output providing self inhibition through dendrodendritic synapses on mitral cell lateral dendrites within the same column. In addition, they may exert lateral inhibition on adjacent columns by shunting the propagation of action potentials on distal lateral dendrites of extra-columnar mitral cells (Xiong and Chen, 2002). This implies a dual role of granule cells on mitral/tufted cell firing: through self-inhibition within the same column, granule cells may act synchronizing the firing rate of projection neurons belonging to different units while responding to the same sensory input (Dhawale et al., 2010); through lateral inhibition on extracolumnar mitral cells, granule cells may provide contrast enhancement between two different functional units (as other amacrine—axonless—cells in the retina for instance; Migliore and Shepherd, 2008). Both effects have been hypothesized to be relevant for olfactory discrimination (Migliore and Shepherd, 2008; Dhawale et al., 2010; see Lepousez et al., 2013 for a detailed review on this hypothesis). Given the apparent lack of columnar organization in the piriform cortex, this topological motif in the bulbar circuitry probably reflects its cortical like function and represents the modular unit encoding the diversity of olfactory inputs (Haberly, 2001; Migliore et al., 2007). Importantly, the constant re-adjustment of the synaptic inputs caused by renewal of both local interneurons and olfactory fibers has been associated with an optimization of this function (Alonso et al., 2006; Jones et al., 2008; Adam and Mizrahi, 2011).

The AOB appears structurally similar to the MOB, although it retains some peculiar features in both hodology and cell types. However, the occurrence of similar plastic events in both structures motivates the same reasoning done for the MOB. Olfactory glomeruli in the AOB are on average smaller than those in the MOB and appear to be clustered in pseudostratified formations. Contrarily to MOB glomeruli, they receive multiple inputs from different types of VSNs (Takami and Graziadei, 1991; Belluscio et al., 1999; Del Punta et al., 2002), with V1Rs projecting only to the aAOB and V2Rs to the pAOB. In addition, neurons expressing the same receptor/s in the VNO, may project to up to 20–30 different glomeruli (Belluscio et al., 1999), while same-receptor OSNs in the MOE project mainly to two symmetrical glomeruli in the MOB. This conserved pattern seems to underlie a higher degree of input convergence on MOB projection neurons and therefore functional specialization of each olfactory column in the MOB (Hildebrand and Shepherd, 1997; Su et al., 2009; Touhara and Vosshall, 2009) as mitral cells project to a single glomerulus, therefore receiving afferents from OSNs expressing the same receptor. Conversely, AOB PCs reach multiple glomeruli receiving inputs from different VSNs (a feature shared with the OB of fishes: Ngai et al., 1993; Speca et al., 1999). However, AOB projection neurons maintain V1R/V2R segregated apical dendritic arborizations depending on their location in the aAOB and pAOB

(Jia and Halpern, 1997). Nonetheless, a cross talk may exist between aAOB and pAOB principal cells via thinner lateral dendrites crossing the midline (Larriva-Sahd, 2008). As a result of their heterotypic connectivity, AOB PCs integrate inputs from different receptor types in the VNO and therefore different ligands. Slice recordings on *ex-vivo* VNO-AOB intact preparation showed that this is indeed the case (Meeks et al., 2010). Juxtaglomerular complexes in the AOB resemble the functional triads described in the MOB: PG and SA cells have inter- and intra-glomerular projections, external tufted cells contact a single glomerulus.

The limited extent by which AOB PGs are regenerated by SVZ-derived progenitors, together with the above mentioned lack of TH, could be explained by the lack of TH/GAD-67 cells in the AOB. Alternatively, since TH expression levels in PGs are traditionally used as a proxy for olfactory input (Nadi et al., 1981; Baker et al., 1983; Cho et al., 1996) and VNO activity is subordinated to initial odor detection by the MOE (Xu et al., 2005; Slotnick et al., 2010), the lack of TH and GAD-67 in the AOB could be just a consequence of the irregular nature of vomeronasal inputs. The expression patterns of other activity markers (such as cytochrome-c-oxydase or β-secretase-1) in the AOB glomerular layer resemble those in the MOB during sensory deprivation and therefore could support this hypothesis (Yan et al., 2007; He et al., 2014).

Conversely, granule cells in the AOB are the most represented cell type among newly generated neurons (Oboti et al., 2009; Nunez-Parra et al., 2011). They are typically located in the deep ICL (below the LOT) but also in the deeper portion of the ECL and in the LOT, just below PC somata. They project to PC dendrites belonging to the homologous region (aAOB or pAOB), but considering that PC axon collaterals cross repeatedly the two sub-regions, they could receive synaptic inputs from both. In addition their apical dendrites seem to reach the glomerular layer (Larriva-Sahd, 2008), although it is not clear whether they interact synaptically with the juxtaglomerular complex. Interestingly, while EGC dendritic processes appose on PC somata or proximal dendrites, those from IGC seem to localize preferentially on distal and apical processes, between glomeruli and PC somata (Larriva-Sahd, 2008), a feature confirmed by EM studies (Moriya-Ito et al., 2013). This distinction may imply a bias for AOB IGCs toward PC self-inhibition, instead of inter-columnar lateral inhibition. Eventually, since the vast majority of newly generated cells in the adult AOB are IGC, it is appealing to imagine neurogenesis in the AOB as a mechanism to shunt directly input signals from the VNO. Given the variable turnover rate of IGCs during postnatal development, this feature alone would be sufficient to justify changes in the response to vomeronasal sensory cues over time. In addition, given that both the survival and activation of newborn neurons is actively driven by vomeronasal sensory inputs, this selective shunting may contribute to encode stimulus familiarity. In general, a change in IGC turnover rate, together with other physiological changes, may set the timing for certain stimuli to be more or less effective as social signals or endocrine modulators (e.g., effect of male urine odors on female estrous varying depending on kinship or shared fostering). Ultimately, this could represent a possible answer to the question posed by the title of this manuscript.

Overall these observations suggest that newly generated GABAergic interneurons differently contribute to mature AOB circuits, if compared to the MOB. The different nature of GABAergic modulation of AOB output signals is further supported by the firing of AOB PCs, which appears to be longer sustained, if compared to MOB mitral cells (Meeks et al., 2010; Shpak et al., 2012). In addition, given PC heterogeneous glomerular connectivity and the convergence of their centripetal projections to more central targets (Salazar and Brennan, 2001), the information conveyed by their output signals is also different, and probably more complex. As a direct consequence and in the whole system perspective, the impact newborn IGCs have on PC output activity may definitely be higher than that of granule interneurons on MOB mitral cells.

CONCLUSIONS

Overall the considerations made in this manuscript are meant to underline that the VNS is not only constitutively plastic but also that this plasticity may constitute the basis for its peculiar function. Eventually the VNS circuitry cannot be considered hardwired but rather able to adjust its connectivity to environmental changes. If the function of the VNS described so far (see for critical views on this point Eisthen and Wyatt, 2006; Mucignat-Caretta et al., 2012) is the result of the interaction between plastic circuits and environmental stimuli, is definitely not known and certainly deserves further investigation. Plausibly neuronal plasticity and neurogenesis are indeed necessary to shape it and maintain it throughout postnatal life. Eventually different rates of neurogenesis can determine the extent by which VNS circuits adapt and tune to a given chemical environment, being it referred to social, reproductive, or aggressive/territorial behaviors. Even though the VNS is not simply the *pheromone-detector* in the nasal cavity of mammals or other vertebrates (Eisthen and Wyatt, 2006), it would be a challenge of future studies to test the impact of neuronal plasticity and neurogenesis on those functions, commonly associated to *pheromone* sensing. Indeed studying the molecular and genetic mechanisms underlying the neuroendocrine physiology of sociality may yield insights on the etiology of associated anomalies, even in those mammalian species, human included, in which this sensory pathway is not present. For this reason the rodent VNS may represent the unique opportunity to dissect this issue in an animal model in which these features strongly rely on its functional integrity and—plausibly—its capability of cell renewal through adult neurogenesis.

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The functional significance of newly born neurons integrated into olfactory bulb circuits

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The olfactory bulb (OB) is the first central processing center for olfactory information connecting with higher areas in the brain, and this neuronal circuitry mediates a variety of odor-evoked behavioral responses. In the adult mammalian brain, continuous neurogenesis occurs in two restricted regions, the subventricular zone (SVZ) of the lateral ventricle and the hippocampal dentate gyrus. New neurons born in the SVZ migrate through the rostral migratory stream and are integrated into the neuronal circuits of the OB throughout life. The significance of this continuous supply of new neurons in the OB has been implicated in plasticity and memory regulation. Two decades of huge investigation in adult neurogenesis revealed the biological importance of integration of new neurons into the olfactory circuits. In this review, we highlight the recent findings about the physiological functions of newly generated neurons in rodent OB circuits and then discuss the contribution of neurogenesis in the brain function. Finally, we introduce cutting edge technologies to monitor and manipulate the activity of new neurons.

Keywords: neurogenesis, main olfactory bulb, accessory olfactory bulb, granule cell, periglomerular cell, lateral inhibition, behavior, neural stem cell

INTRODUCTION

It was believed that the adult mammalian brain is incapable of producing new neurons. The prominent histologist Cajal proclaimed "Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated." (Ramon y Cajal, 1928). In 1960's, Altman and his colleague's pioneering study provided the first anatomical evidence of neurogenesis in the postnatal hippocampal region using a [H^3]-thymidine incorporation labeling (Altman and Das, 1965). These [H^3]-thymidine-labeling cells had neuronal morphology (Kaplan and Hinds, 1977). However, these findings were not accepted by Cajal's neuron doctrine that no new neurons are born in the adult brain. In 1980's, adult neurogenesis identified in songbird's brain was found to play a role in song learning (Goldman and Nottebohm, 1983). In 1990's, neural stem/progenitor cells were isolated from adult rodent brain, and adult neurogenesis was discovered in human hippocampus (Reynolds and Weiss, 1992; Eriksson et al., 1998). Since the discovery, adult neurogenesis has now become a well-accepted phenomenon including humans (Sanai et al., 2011; Bergmann et al., 2012; Spalding et al., 2013; Ernst et al., 2014).

In rodents, adult neurogenesis mainly occurs in two brain regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Kriegstein and Alvarez-Buylla, 2009; Suh et al., 2009; Aimone et al., 2011; Fuentealba et al., 2012). Adult neural stem/progenitor cells are regulated by many genes and signaling pathways (Kriegstein and Alvarez-Buylla, 2009; Suh et al., 2009). Neurons born in the SGZ migrate into the granule cell layer (GCL) and become granule cells of the DG, while neurons born in the SVZ migrate into the olfactory bulb (OB) through the rostral migratory stream (RMS), the pathway leading to the OB, and become local interneurons, granule cells (GCs) and periglomerular cells (PGCs) (Lledo et al., 2006; Ming and Song, 2011; Lepousez et al., 2013).

The olfactory system, which senses and processes odor information, is one of the oldest and important parts of the brain. Odor information is transferred to local neural circuits in the OB, and then conveyed to various regions of the olfactory cortex via principal neurons (mitral and tufted cells, hereafter referred to these neurons as M/T cells). Unlike most other central nervous

system areas, GABAergic inhibitory interneurons greatly outnumber principal neurons, suggesting that odor representations in the OB are shaped by local inhibitory circuits (Yokoi et al., 1995; Isaacson and Strowbridge, 1998; Egger and Urban, 2006). Furthermore, although most neurons comprising the mammalian central nervous system are produced during embryonic development, a large proportion of these interneurons in the OB are generated and continuously renewed throughout life. Why do such continuous neuronal addition and replacement occur in the OB? Two decades of huge investigation revealed the biological importance of integration of new neurons into the olfactory circuits (Lledo et al., 2006; Kelsch et al., 2010; Ming and Song, 2011; Lepousez et al., 2013).

In this review, we highlight recent findings about physiological features of new neurons in rodent OB circuits and then describe the role of new neurons in olfaction-associated behaviors. Finally, we introduce optical techniques to monitor and manipulate the activity of new neurons.

NEURONAL CIRCUIT OF THE OLFACTORY SYSTEM

The OB is the first relay station in the olfactory system that can process odor information (**Figure 1**). Odor information is detected by olfactory sensory neurons (OSNs). OSNs expressing the same odorant receptors project and converge their axons into the same glomeruli (Mori and Sakano, 2011). OSNs form excitatory synapses on primary dendrites of M/T cells. M/T cells project their axons to the olfactory cortex to covey odor information to higher brain areas in the forebrain. Mitral cells project their axons to nearly all areas of the olfactory cortex with a dispersed manner, while tufted cells target densely only to the anterior regions of the olfactory cortex (Ghosh et al., 2011; Miyamichi et al., 2011; Sosulski et al., 2011; Igarashi et al., 2012).

Synaptic connections in the external plexiform layer (EPL) of the OB are dominated by dendrodendritic reciprocal synapses between lateral dendrites of M/T cells and GCs, the latter being most numerous type of inhibitory interneurons in the OB. Unlike the neocortex, GABAergic inhibitory interneurons in the OB grealy outnumber principal neurons by 50–100:1 (Isaacson and Strowbridge, 1998; Egger and Urban, 2006). GCs form dendrodendritic synapses with M/T cells. In a dendrodendritic reciprocal synapse, both sides of the synapse are dendrites. M/T to GC is a glutamatergic excitatory synapse, while GC to M/T is a GABAergic inhibitory synapse (**Figure 1**). This large number of inhibitory synapses onto M/T cells may enable inhibitory circuits to refine odor representations. (Isaacson and Strowbridge, 1998; Egger and Urban, 2006; Lepousez and Lledo, 2013).

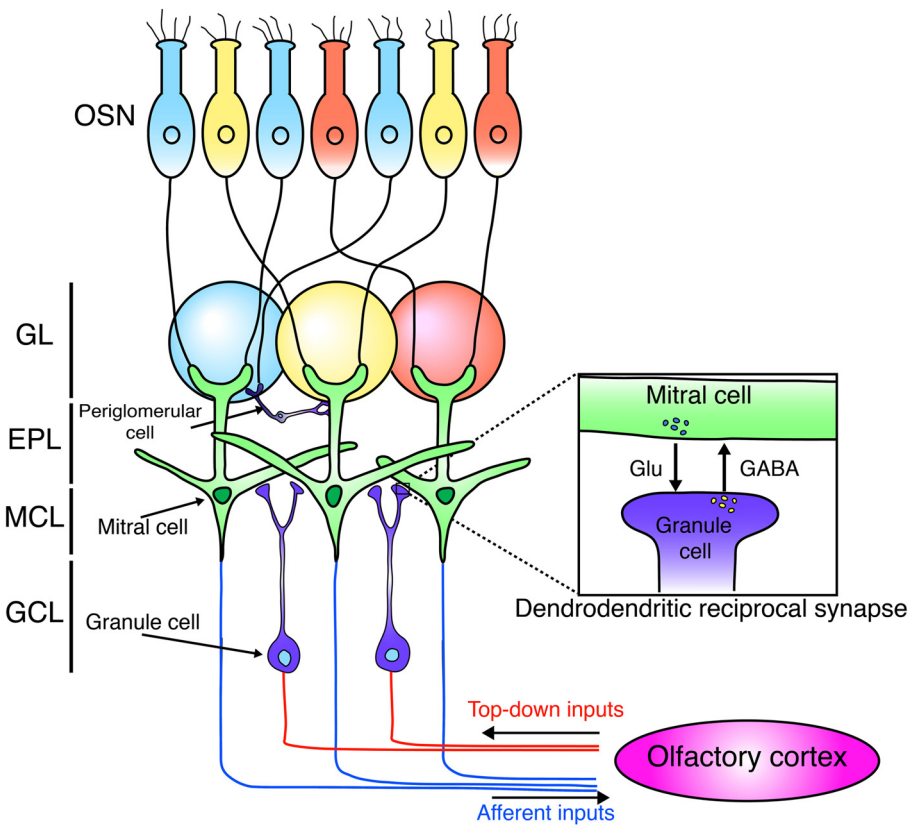

FIGURE 1 | Neural circuit of the olfactory bulb. Schematic diagram of the neuronal circuit of the olfactory bulb. OSNs expressing the same odorant receptors (blue, yellow, red) project and converge their axons into the same glomeruli. OSNs form excitatory synapses with mitral cells. Mitral cells project their axons to the olfactory cortex. Mitral cells form dendrodendritic synapses with granule cells. Granule cells receive centrifugal glutamatergic inputs from the olfactory cortex. OSN, olfactory sensory neuron; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer.

PGCs are another type of major GABAergic interneurons in the OB, and modulate the neural circuit in the glomerulus, consisting of terminals of the olfactory nerve and the dendrites of M/T. PGCs are subdivided into at least three subtypes based on immunoreactivity to calretinin (CalR), calbindin-28K (CalB), and tyrosine hydroxylase (TH) (Kosaka et al., 1998; Pressler and Strowbridge, 2006; Eyre et al., 2008, 2009; Kosaka and Kosaka, 2011). In mice, all three PGC subtypes seem to be GABA-expressing inhibitory neurons, but the definite functional roles of each PGC subtype in odor processing have not been well determined.

In addition to GCs and PGCs, numerous types of GABAergic interneurons have been identified in the OB (Pressler and Strowbridge, 2006; Batista-Brito et al., 2008; Eyre et al., 2008, 2009; Kosaka and Kosaka, 2011; Huang et al., 2013; Kato et al., 2013; Miyamichi et al., 2013), including deep short-axon cells, Blanes cells, and EPL interneurons. Although lineage and turnover analysis of these OB interneuronal populations has just been started (Batista-Brito et al., 2008; Bartolini et al., 2013), dynamic turnover of these interneurons by postnatal/adult neurogenesis may also contribute to the reorganization of OB circuitry.

UNIQUE FEATURES OF NEWLY GENERATED NEURONS

While M/T cells are generated only at an embryonic stage, GCs and PGCs are generated throughout life (Imayoshi et al., 2008; Imamura et al., 2011; Sakamoto et al., 2014). Long-term genetic labeling analysis revealed that the majority of GCs are replaced by newly generated neurons during adult life (Imayoshi et al., 2008). Newly generated GCs are preferentially located in a deep region, while pre-existing GCs are located in a superficial region in the GCL of the OB (Lemasson et al., 2005; Imayoshi et al., 2008; Sakamoto et al., 2014). Interestingly, it has been shown that outer/superficial GCs, whose dendrites preferentially target the superficial lamina of the EPL, establish synapses with tufted cells, whereas deep GCs mainly contact the dendrites of mitral cells in the deep lamina of the EPL (Mori et al., 1983; Orona et al., 1983; Shepherd and Greer, 2004; Imamura et al., 2006). Therefore, one attractive hypotheis is that these two GC subpopulations fundamentally modulate distinct neural circuits. This implies that the activity of tufted cells is under the preferential control of embryonic-born GCs (static, superficial layers), while postnatal-born GCs (turnover, deep layers) provide an inhibitory drive to both mitral and tufted cells.

In rodents, although numerous new neurons reach the OB each day (roughly one percent of the total OB GCs), only half of them are integrated into pre-existing neural circuits. The remains of them are eliminated by apoptosis during their maturation (Lledo et al., 2006). This "survival or death" depends on olfactory sensory experience. Sensory deprivation triggers a decrease in new GC survival, whereas olfactory enrichment and learning boost the survival of these neurons (Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002). Interestingly, day 14 to 28 after the generation is a critical period of newly born GCs when their survival is influenced by sensory experience (Yamaguchi and Mori, 2005). This time window overlaps with the period when newly generated neurons make synapses with pre-existing neurons, suggesting that synaptic inputs play a crucial role in the selection of adult born GCs (Kelsch et al., 2008; Yokoyama et al., 2011). Although the number of PGCs is one order smaller than that of GCs, new PGCs are also continuously produced throughout life (Ninkovic et al., 2007; Sakamoto et al., 2014). Like GCs, the survival of newly born PGCs is regulated in an activity-dependent manner. Sensory deprivation triggers a decrease in new PGCs' survival, whereas olfactory enrichment and learning boost the survival of adult generated PGCs (Rochefort et al., 2002; Alonso et al., 2006; Adam and Mizrahi, 2011; Sawada et al., 2011; Livneh and Mizrahi, 2012). A recent work also reported the generation of some glutamatergic short-axon cells at a very low proportion (Brill et al., 2009).

One recent elegant study provided direct evidence of the involvement of adult-born PGCs in olfactory sensory processing (Livneh et al., 2014). By using two-photon-targeted patch recordings, they showed that adult-born PGCs indeed respond to odor input. Interestingly, young adult-born neurons (2–4 weeks of age) have broader odor response profile than that of matured resident PGCs. Furthermore, sensory enrichment during developmental periods of adult-born neurons sharpens their odor response selectivity after maturation. These results indicated that continuous supply of these sensitive adult-born neurons into the olfactory circuit provides it with a mechanism of long-lasting plasticity (Livneh et al., 2014).

The OB receives input not only from OSNs but also from the olfactory cortex (**Figure 1**). This top-down input targets preferentially to the GCL and is important to shape the activity of M/T neurons (Manabe et al., 2011; Boyd et al., 2012; Markopoulos et al., 2012). In addition, recent studies showed that this cortical feedback is necessary for odor discrimination and food-intake (Nunez-Parra et al., 2013; Soria-Gomez et al., 2014). Furthermore, several studies showed the connectivity of newly generated neurons using monosynaptic rabies virus-based tracing system and revealed that newborn neurons in the OB receive glutamatergic inputs from neurons in the olfactory cortex (Arenkiel et al., 2011; Deshpande et al., 2013). Interestingly, new neurons exhibit more synaptic plasticity from centrifugal inputs than mature neurons do (Nissant et al., 2009). Furthermore, top-down inputs on the proximal dendrites of GCs also contribute to the survival/death of new neurons (Yamaguchi et al., 2013). Therefore, these observations imply that top-down glutamatergic input from the olfactory cortex to new GCs has a critical role in generating high plasticity in OB cirucuits.

Adult neurogenesis occurs in human brain as well as in rodents. Radiocarbon dating technologies revealed that adult neurogenesis in the OB is extremely limited though hippocampal neurogenesis occurs at a steady rate (Bergmann et al., 2012; Spalding et al., 2013). Surprisingly, new neurons born SVZ/lateral ventricles migrate and differentiate into striatum interneurons (Ernst et al., 2014). Furthermore, striatum neurogenesis is reduced in patients with Huntington's diseases. These results indicate that adult neurogenesis in humans has a unique pattern, and that these neurons derived from SVZ/lateral ventricles might be involved in brain functions such as cognition and motor coordination.

THE ROLES OF NEWLY GENERATED NEURONS FOR OLFACTION-RELATED BEHAVIORS

While the functional significance of continuous neurogenesis in hippocampus has been extensively studied (Deng et al., 2010; Aimone et al., 2011), the role of newly generated neurons in olfaction-related behaviors remains elusive. As mentioned above, newly born neurons form dendrodendritic synapses with M/T cells and control the activity of M/T cells to shape odor representation. Genetic ablation of newly born neurons in the OB impairs the structure and neural circuits in the OB (Imayoshi et al., 2008; Sakamoto et al., 2011). It was reported that newly generated GCs exhibit long-term synaptic plasticity, and that this ability is gradually lost as these neurons become mature, indicating that newly born GCs play a more important role in plastic change than mature GCs (Nissant et al., 2009). Importantly, electrophysiological recording revealed that ablation of adult born neurons impairs recurrent and lateral dendrodendritic inhibition of M/T cells and reduces the frequency of the induced gamma oscillations in the OB (Breton-Provencher et al., 2009). Furthermore, the survival of newly generated neurons is regulated by sensory experience (Yamaguchi and Mori, 2005; Lledo et al., 2006; Yokoyama et al., 2011). Together, these findings suggest that neurogenesis has a key role in olfaction-related plastic activities in the OB.

To understand the functional role of neurogenesis in the OB, various behavioral analyses have been applied. To address this question, various methodologies for inhibiting neurogenesis have been used: pharmacological, irradiation, and genetic targeting (Gheusi et al., 2000; Kim et al., 2007; Bath et al., 2008; Imayoshi et al., 2008; Breton-Provencher et al., 2009; Lazarini et al., 2009; Sultan et al., 2010; Sakamoto et al., 2011). Conversely, an apoptotic inhibitor was used to suppress cell death of newly born neurons (Mouret et al., 2009; Sultan et al., 2011).

One of the simplest behavioral tests for olfaction is to check spontaneous odor exploration toward a novel odor without any rewards (**Figure 2A**). In this task, the ability of odor discrimination can be assessed by repeated presentations of the same odor (habituation) followed by the presentation of a novel odor (dishabituation). The sniffing time decreases during habituation sessions, but then increases when the odor is recognized as a new one in a dishabituation session. This can be applied to evaluate the odor detection threshold by comparing sniffing times between different concentrations. This test can also assess the short-term olfactory memory by changing time interval between sessions. Blockade of neurogenesis by infusion of an antimitotic drug impairs the ability of odor detection and short-term memory (60 min), suggesting that new neurons are involved in odor detection and processing odor memory (Breton-Provencher et al., 2009). In addition, gene deficient mice, which result in a decrease of new neurons in the OB, could not discriminate between dissimilar odors (Gheusi et al., 2000; Bath et al., 2008). However, not all findings have supported this result. Mice treated with γ-ray irradiation to block neurogenesis showed normal sensitivity (Lazarini et al., 2009), suggesting that spontaneous odor discrimination is not affected in these mice. Similar results were obtained from other studies (Kim et al., 2007; Imayoshi et al., 2008; Sakamoto et al., 2011). The discrepancies between these findings may be due to different ablation methods. Regarding the target specificity, blocking adult neurogenesis by using conventional knock in/out mice is far from specific.

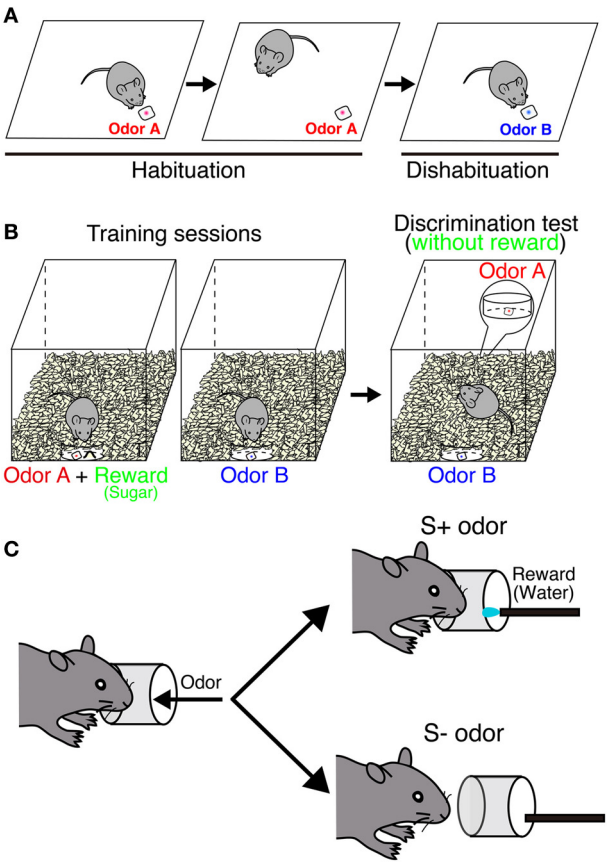

FIGURE 2 | Behavioral paradigm of odor discrimination test. (A) Habituation-dishabituation test. In habituation sessions, odor A (red) is presented repeatedly. A mouse is habituated to this odor, and sniffing time is getting decreased. In a dishabituation session, sniffing time increases when the mouse can recognize a novel odor B (blue). **(B)** Odor-reward association test. During the training, a mouse is associated with one of the odors with reward (sugar). In the test, both odors are placed under the bedding separately without reward. Digging time of each odorant is measured to judge whether the mouse can discriminate between the two odors. In an odor memory retention test, mice are exposed to the same test without further training. **(C)** Go/no-go olfactory conditioning test. A mouse is associated with one of the odors with reward (water). Each trial is counted as correct if the mouse licks continuously upon presentation of a rewarded (S+) odor or does not lick continuously with a non-rewarded (S−) odor.

Odor-reward association learning is another major paradigm that has been used to evaluate the function of neurogenesis in the OB (**Figures 2B,C**). This test can evaluate the capacity to associate an odor with reward (food or water) and the ability of odor discrimination and memory retention. Genetically ablated mice can acquire the odor-associated memory and maintain it for at least 2 months (Imayoshi et al., 2008; Sakamoto et al., 2011). Similar results were obtained from other animal models (Schellinck et al., 2004; Breton-Provencher et al., 2009). These results indicate that continuous neurogenesis is not required for simple discrimination between similar odors or retention of

odor-associated memory. Pre-existing granule neurons born in embryonic and neonatal stages may compensate for these functions in the absence of new neurons. However, conflicting results were also reported following odor-reward association learning. Although mice showed normal odor discrimination ability, their long-term odor-associated memory retention was impaired by irradiation- or drug-induced inhibition of neurogenesis (Lazarini et al., 2009; Sultan et al., 2010). This discrepancy likely results from the difference in experimental paradigms. In addition, parameters to evaluate odor memory are totally different depending on behavior tests [digging time (seconds) or correct decision (%)].

These discrepancies may result from the target specificity for blockade of neurogenesis. Strictly speaking, these past methods did not specifically target new neurons in the OB. Neurons in other cortical regions might be affected to some extent. Therefore, there is a need to generate more sophisticated model that can target only newly generated neurons in the OB. One possibility to increase the target specificity is an intersectional strategy with dual site-specific recombinases (Cre and Flp) (Imayoshi et al., 2011; Huang and Zeng, 2013). Most of new neurons in the OB are GABAergic inhibitory neurons, while new neurons in the DG are glutamatergic excitatory neurons. Based on their different transmitter characteristics, new neurons in the OB and DG can be separately targeted. Recently, our group has developed new transgenic mice in which new neurons in the OB and DG can be separately targeted, and found that continuous neurogenesis is important for flexible olfactory associative learning and memory (Sakamoto et al., 2014). Continuous supply of new neurons in the OB is important to rewrite aquired odor memory and modify the value of odor-associated memory.

Adult neurogenesis in the hippocampus is required for pattern separation (Clelland et al., 2009; Sahay et al., 2011a,b; Nakashiba et al., 2012). It seems that odor enrichment improves a recruitment of newly born neurons and the olfactory discrimination ability. Blockade of neurogenesis with AraC impaired the improvement of odor discrimination, suggesting that neurogenesis is required for perceptual learning (Moreno et al., 2009). This result implies that neurogenesis in the OB also contributes to pattern separation. It would be interesting to examine the relationship between OB neurogenesis and pattern separation (Sahay et al., 2011b).

OB NEUROGENESIS IN INNATE BEHAVIORS

Adult neurogenesis is physiologically linked to reproductive behaviors, suggesting that continuous neurogenesis plays a pivotal role in pheromone-associated behaviors (Shingo et al., 2003; Mak et al., 2007; Mak and Weiss, 2010; Nunez-Parra et al., 2011). Pregnancy and lactation increase the number of both new GCs and PGCs (Shingo et al., 2003). Around gestation day 7, the proliferation reaches a peak in the SVZ/lateral ventricles. After the delivery, the number of new neurons integrated into OB circuits increases, and their dendritic spines exhibit stable features (Shingo et al., 2003; Kopel et al., 2012). These phenomena during early pregnancy and parenting might be important for fine-tuning of olfactory response to mating partners and pups. This induction of neurogenesis is mediated by prolactin (Shingo et al., 2003; Larsen and Grattan, 2010). Reducing the prolactin levels decreases neurogenesis in the SVZ/lateral ventricles and impairs maternal behaviors (Larsen and Grattan, 2010). Neurogenesis in females is also induced by pheromones of dominant males (but not other males) and is important for sexual behaviors (Mak et al., 2007; Oboti et al., 2009, 2011). Relevant increase of new neurons also occurs in male mice when they interact with their postnatal offspring (Mak and Weiss, 2010). This increase of neurogenesis mediated by prolactin appears to depend on the odor of their offspring and is involved in offspring recognition (Mak and Weiss, 2010).

These results indicate that OB neurogenesis is really related to sexual and maternal behaviors, suggesting that neurogenesis plays an important role in such pheromone-associated innately-programmed behaviors. Genetic inhibition of adult neurogenesis revealed that new neurons are essential for mating and maternal behaviors (Sakamoto et al., 2011). Blocking neurogenesis by injecting antimitotic drugs also impairs mating behaviors (Oboti et al., 2011). Pregnancy block (Bruce effect) is a well-known phenomenon; females terminate their pregnancy when they are exposed to the scent of unfamiliar males (Bruce, 1959). Although the detailed mechanism of this pregnancy block remains to be determined, it was shown that the pregnancy failure rate is highly increased by the blockade of continuous neurogenesis (Sakamoto et al., 2011). These results indicate that continuous neurogenesis is essential for pheromone-associated innately-programmed behaviors and activities. However, conflicting results were also reported. Disruption of neurogenesis in the OB by γ-ray irradiation left sexual and maternal behaviors unaffected (Feierstein et al., 2010). The discrepancy between these studies might derive from different models and target specificity as described above. One possibility is that newly born neurons in the DG might be involved in such behaviors. Moreover, because current available methods ablate new neurons in both the main and accessory olfactory bulb, it is difficult to conclude which is important for these pheromone-associated behaviors. More restricted ablating method will be required to address these questions.

Although the majority of newly born neurons are incorporated into the main olfactory bulb (MOB), a small number of new neurons migrate into the accessory olfactory bulb (AOB) (Oboti et al., 2009, 2011; Sakamoto et al., 2011). Genetic ablation of newly born neurons revealed that continuous neurogenesis is required for the maintenance of neuronal circuits in the AOB, as observed in the MOB (Sakamoto et al., 2011). However, unlike the MOB, adult neurogenesis does not lead to substantial replacement of GCs in the AOB. This result highlighted a unique integration mode of new neurons in the AOB, suggesting that intrinsic cellular and molecular properties of GCs may be different between the AOB and MOB. Further studies are necessary to elucidate cellular and molecular mechanisms underlying distinct features of GCs in the AOB.

OPTICAL IMAGING AND MANIPULATION OF NEW NEURONS

Odor information processing is influenced by the activity of OB interneurons, including pre-existing neurons and newly born neurons. As mentioned above, neurogenesis contributes to various olfaction-related behaviors. However, how new neurons

contribute to such behaviors is still unclear. New technologies are required to monitor and manipulate the activity of new neurons during such behaviors. Neuronal imaging technologies can help to tackle this issue. During the past decade, two-photon microscope has become a key tool for monitoring the structure, function, and plasticity of neurons *in vivo*. Calcium imaging is widely used to image the activity of many neurons simultaneously (Grienberger and Konnerth, 2012). In addition, two-photon calcium imaging can monitor the activity of OB GCs in the head-fixed awake state (Kato et al., 2012). However, calcium imaging operates too slowly to track the rapid firing of neurons and is also unable to measure the inhibitory signals. An alternative technique, voltage imaging, has a potential to overcome these problems and may ultimately enable to monitor spatiotemporal activity patterns with millisecond order (Peterka et al., 2011). Various kinds of genetically encoded voltage indicators have been developed (Knopfel, 2012). Recently, Akemann and his colleagues succeeded in voltage imaging at a single cell resolution with two-photon microscope *in vivo* (Akemann et al., 2013). It would be interesting to apply these technologies to examine physiological functions of newly born neurons in the OB. Because most OB newborn neurons are inhibitory interneurons, voltage imaging of M/T cells may help to clarify the contribution of GCs in olfactory circuitry more precisely than calcium imaging.

Optogenetics is also a powerful tool in the field of OB neurogenesis. Over the last decade, a wide variety of different kinds of opsins have been developed and become available, and now optogenetic approach is a standard methodology for investigating the functional properties of neurons at the circuit and behavioral level (Fenno et al., 2011). Recently, it was reported that the activation of newly born neurons by channelrhodopsin can accelerate difficult odor discrimination learning and improved odor-associated memory (Alonso et al., 2012). This strategy may also be useful to examine how newly born neurons contribute to pheromone-associated behaviors. Furthermore, optogenetic tools can control centrifugal input from the olfactory cortex to the OB (Boyd et al., 2012; Markopoulos et al., 2012). It will be interesting to examine how the top-down input affects the survival of new neurons and the effect of odor-associated learning (Yamaguchi et al., 2013). It would be also useful to express optogenetic probes in neurons under activity-dependent control. This approach can allow the reactivation or inactivation of only the subset of neurons that had been activated during a training phase and identify minimal ensemble that are required for behaviors. Light-reactivation of hippocampal neurons that are activated during the training can recall the fear memory of training task (Liu et al., 2012). Because new neurons express immediate-early genes in response to odor stimulation, this approach might be able to identify and manipulate newly born neurons that have been activated by odor stimulation (Magavi et al., 2005).

Although neurogenesis continues throughout life, newly generated neurons dramatically decrease in number with age, and this decline may be involved in memory deficit (Seki and Arai, 1995; Cameron and McKay, 1999; Encinas et al., 2011). In addition, aged mice are impaired at fine olfactory discrimination (Enwere et al., 2004). Furthermore, neurodegenerative diseases are relevant to OB function and adult neurogenesis. For instance, olfactory dysfunction is well known as an early symptom in Parkinson's disease although there is no specific change in the olfactory epithelium (Braak et al., 2003; Haehner et al., 2009). In Parkinson's disease model (α-synuclein overexpressing mice), the ability of odor discrimination is impaired and the survival of adult born neurons is reduced (Neuner et al., 2014). The next key challenge is to increase neurogenesis in aged/neurodegenerative brain and restore brain functions. Light-sensitive promoter system has a strong potential to achieve it (Wang et al., 2012; Imayoshi et al., 2013; Imayoshi and Kageyama, 2014). This system can control gene expression by blue-light illumination with reversibility. By applying this method *in vivo*, it might be possible to promote adult neurogenesis even in aged brains and lead to restore brain functions.

CONCLUSION

Olfaction is indispensable in mammalian life. GCs are the most common GABAergic inhibitory neurons in the OB and modulate the activity of M/T cells to shape odor representations. The OB neural circuits are reorganized by incorporation and elimination of newly generated granule neurons throughout life. Furthermore, blockade of neurogenesis results in various olfaction-related behavior defects. Therefore, continuous neurogenesis is important to acquire plasticity in the olfactory system and thereby adapt neural circuits to environmental changes. However, there are still a lot of problems about adult neurogenesis to be solved. For example, molecular mechanisms integrating new neurons into the OB neural circuits is still unclear. In addition, there are some discrepancies about behavioral analyses. More sophisticated animal model and standardized behavior paradigms should be established. Further studies will contribute to solution of these problems and lead to the development of therapies and drugs for treatment of neurodegenerative diseases.

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Critical periods in adult neurogenesis and possible clinical utilization of new neurons

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CRITICAL PERIODS IN ANIMAL AGE AND IN CELLULAR AGE IN MAMMALS

In mammals, sensory and motor experiences during the neonatal and infant stages of growth induce remarkable plastic changes in the structural organization and functional properties of neuronal circuits in the brain. Following this critical period, however, experience-dependent plasticity declines significantly. The term "critical period" therefore refers to a specific period in early life during which experience-dependent plasticity is greatly potentiated (Levelt and Hübener, 2012), presumably because neuronal circuits and their constituent young neurons transiently express a high capacity for plasticity.

The presence of adult neurogenesis in the olfactory bulb and dentate gyrus of the hippocampus raises the possibility that the critical period might be extended into adulthood in these particular brain regions. Intriguingly, while ocular dominance plasticity of the visual cortex, a non-neurogenic region in the adult brain, occurs only during a few weeks in the neonatal period in rodents, transplantation of new neurons to the adult visual cortex restores the ocular dominance plasticity (Southwell et al., 2010). The occurrence of adult neurogenesis raises the possibility that the critical period of plasticity can be extended into or restored in adulthood by supplying new neurons with high plastic capacity (**Table 1**).

In parallel with the idea of a "critical period in animal age," we previously noted that adult-born neurons have "critical periods in cellular age," during which the newly generated neurons are either incorporated into or eliminated from the preexisting host neuronal circuits (**Table 1**) (Yamaguchi and Mori, 2005). Manifestation of the highly plastic features of adult-born neurons might therefore depend on their cellular age. The idea of a "critical period in cellular age" appears fundamental to the study of adult neurogenesis. The experience-dependent plasticity of neuronal circuits in the olfactory bulb and hippocampus appears to result from interaction between newly generated neurons and preexisting neuronal circuits, the former having higher plasticity and latter having lower plasticity. As a result, the differences observed in the plastic potential of new neurons between that during the critical period and that during the subsequent period are attributable to the different properties of new neurons at different cellular ages. In fact, analysis of adult-born neurons has revealed many aspects of the critical period of experience-dependent plasticity, including the presence of critical periods for the survival or death decision, for synapse formation, for synaptic plasticity, and for incorporation into functional neuronal circuits (Yamaguchi and Mori, 2005; Ge et al., 2007; Kee et al., 2007; Tashiro et al., 2007; Kelsch et al., 2009; Nissant et al., 2009; Belnoue et al., 2011).

A second important issue in adult neurogenesis is the time of day at which the plastic change in neuronal circuits occurs. We previously found that the life and death decision of new neurons in the olfactory bulb occurs within a narrow time window, in association with a specific behavioral state of the animal (Yokoyama et al., 2011). In food-restricted mice, extensive apoptotic elimination of new olfactory bulb neurons occurs during several tens of minutes of rest or sleep after food eating. The extent of neuronal elimination during postprandial rest or sleep is influenced by olfactory sensory experience. Thus, the life and death of new olfactory bulb neurons is likely determined within a particular "behavioral state-correlated time window", which consists of the sequence of olfactory behavior during wakefulness and subsequent rest or sleep behavior (**Table 1**). The behavioral state-correlated time window may be further interpreted as a "critical time window of preexisting neuronal circuits" that determines whether to incorporate or eliminate new neurons.

CONTRIBUTION OF CRITICAL PERIOD ANALYSIS OF ADULT NEUROGENESIS TO BRAIN REPAIR BY CELL TRANSPLANTATION

We consider that the "critical period of adult-born neurons" and "behavioral state-correlated time window for plasticity" ideas have important implications for the possible clinical utilization of new neurons. To supplement new neurons to diseased or injured brains, neural stem/progenitor cells are the plausible candidate for clinical use. Neural stem/precursor cells from various sources can be utilized, including those from embryonic brains, those induced from ES cells or iPS cells, and those endogenously present in the patient's brain (Bellenchi et al., 2013; Sandoe and Eggan, 2013). Whatever the origins of stem/precursor

Table 1 | Time windows for experience-dependent plasticity in adult neurogenesis and cell transplantation therapy.

Types of time windows	Adult neurogenesis	Cell transplantation
Animal age	Extended plasticity of neuronal circuits into adulthood	Functional restoration and improvement of damaged or aged brain
Cellular age	Developmental stage of adult-born neurons with high experience-dependent plasticity	Developmental stage of transplanted neurons with high rehabilitation-dependent plasticity
Behavioral state	Sequence of experience during wakefulness and subsequent rest or sleep	Sequence of rehabilitation and subsequent post-rehabilitation period

cells, it is assumed that transplantation or recruitment of stem/precursor cells into damaged neuronal circuits might restore the function of the neuronal circuits via the process of their development into mature neurons and integration into the preexisting neuronal circuits. Rehabilitation following brain damage is critically aided by physical exercise, intellectual exercise and extensive sensory stimulation. In stem/progenitor cell transplantation, a primary aim of rehabilitation would be to provide proper experience-dependent activity to transplanted cells and recipient neuronal circuits, with the expectation that the transplanted cells develop and survive and are appropriately incorporated into the recipient neuronal circuits for the recovery of circuit function. On this basis, knowledge of the critical period of experience-dependent plasticity of adult-born neurons will provide a scientific basis for cell transplantation-based rehabilitation therapy (Döbrössy et al., 2010).

Application of our understanding of the critical period in the cellular age of adult-born neurons provides insight into how the timing of rehabilitation and cell transplantation is coordinated (**Table 1**). Adult neurogenesis studies suggest that critical periods of experience-dependent survival, synapse formation, synaptic plasticity and circuit incorporation of adult-born neurons correspond to their synaptogenesis or post-synaptogenesis period (Kelsch et al., 2010; Toni and Sultan, 2011; Drew et al., 2013; Lepousez et al., 2013). By analogy, rehabilitation during the period when transplanted neurons make synaptic contacts with preexisting neurons might crucially regulate the appropriate incorporation of transplanted neurons into recipient neuronal circuits.

Knowledge of the "behavioral state-correlated time window for plasticity" also indicates the possibility that the occurrence of specific behavioral states after rehabilitation might be crucial (**Table 1**). In the hippocampus and neocortex, neuronal activity during rest or sleep is considered to reflect neuronal activity during the preceding waking period (Diekelmann and Born, 2010). The life and death decision of adult-born olfactory bulb neurons might be promoted by neuronal activity during rest or sleep which reflects the neuronal activity during the preceding waking period with olfactory experience (Yamaguchi et al., 2013). If we assume that this "olfactory experience" period during wakefulness corresponds to the rehabilitation period in cell transplantation therapy, the fate decision of transplanted neurons about whether they will be incorporated or eliminated does not occur during rehabilitation, but may actually be promoted during the post-rehabilitation period. If this is the case, significant attention should be paid as to how patients spend time after rehabilitation. How long is the effect of rehabilitation maintained until the following period of neuronal selection? Is it better to rest or sleep immediately after rehabilitation? Is the effect of rehabilitation perturbed or erased by other unrelated experiences before rest or sleep? These questions cannot now be answered with any reliability, but consideration to the possible role of post-rehabilitation period will likely optimize the effect of rehabilitation.

Further, applying the idea that the critical period of plasticity can be extended or restored by supplying new neurons suggests that brain repair by cell transplantation may allow not only the recovery of brain function, but also the improvement of function. Use of the highly plastic potential of transplanted neurons might allow an improvement in brain function beyond its original pre-pathological/trauma level, which cannot be achieved solely by preexisting old neurons (**Table 1**).

HOW FAR ARE WE FROM THE CLINICAL UTILIZATION OF BASIC KNOWLEDGE GATHERED FROM CRITICAL PERIOD STUDIES IN ADULT NEUROGENESIS?

As discussed, the basic idea of critical periods in the study of adult neurogenesis is applicable to cell transplantation therapy. However, the actual time window for experience-dependent plasticity is unlikely to be uniform. Critical periods in cellular age would depend not only on the type of transplanted neurons but also on the type and condition of recipient neuronal circuits (recipient brain regions). The coordinated timing of rehabilitation after cell transplantation needs to be identified in individual cases. The difficulties of studying the human brain *in vivo* require extensive study in experimental animals and a way to extrapolate the results back to humans.

At present, little is known about the molecular mechanisms underlying the critical periods of plasticity. Knowledge about the expression and function of neurotransmitter receptors, including NMDA, AMPA, and GABA receptors, during the development of adult-born neurons is accumulating, and signals via these receptors have been found to be crucial to the survival and synapse formation of adult-born neurons (Kelsch et al., 2010; Toni and Sultan, 2011; Drew et al., 2013; Lepousez et al., 2013). However, the developmental relationship between the expression of these receptors and the opening and closing of the critical periods of plasticity is unclear, and the molecular mechanisms underlying the behavioral

state-correlated time window for plasticity are not understood at all. Further detailed understanding of the mechanisms of these critical time windows will enable the development of pharmacological and molecular interventions to better coordinate the rehabilitation to cell transplantation therapy.

A further critical point is that while most studies of adult neurogenesis involve healthy brains, cell transplantation therapy is primarily conducted in aged or damaged brains. Recipient neuronal circuits in cell transplantation therapy are unlikely to provide an ideal environment and experience-dependent activity to new neurons. To best utilize our understanding of adult neurogenesis and optimize outcomes from cell transplantation, the recipient brain must be brought to a healthy state as possible. Cell transplantation therapy needs to be combined with the understanding of the nature of the disease/injury of the recipient brain and the application of promising interventions to it (Gerin et al., 2011; Gandy and DeKosky, 2013). Rehabilitation before cell transplantation (Döbrössy and Dunnett, 2005) might have positive effect on the outcomes. We need to know more about the interaction between new neurons and preexisting neuronal circuits in the appropriate utilization of new neurons, and to understand the effect of rehabilitation and pharmacological/molecular interventions on both new neurons and recipient neuronal circuits.

Rehabilitation depends on the active participation of patients, and requires the cooperation of patients, doctors and paramedical staff. A scientific understanding of rehabilitation in relation to the critical time windows of experience-dependent plasticity would boost motivation for rehabilitation, and enable efforts to be concentrated on particular time windows. Research into the critical time windows of plasticity in adult-born neurons will contribute to the development of effective rehabilitation programs, and support patients in their hopes and motivation to recover from brain damage.

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New insights into the role of histamine in subventricular zone-olfactory bulb neurogenesis

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The subventricular zone (SVZ) contains neural stem cells (NSCs) that generate new neurons throughout life. Many brain diseases stimulate NSCs proliferation, neuronal differentiation and homing of these newborns cells into damaged regions. However, complete cell replacement has never been fully achieved. Hence, the identification of proneurogenic factors crucial for stem cell-based therapies will have an impact in brain repair. Histamine, a neurotransmitter and immune mediator, has been recently described to modulate proliferation and commitment of NSCs. Histamine levels are increased in the brain parenchyma and at the cerebrospinal fluid (CSF) upon inflammation and brain injury, thus being able to modulate neurogenesis. Herein, we add new data showing that *in vivo* administration of histamine in the lateral ventricles has a potent proneurogenic effect, increasing the production of new neuroblasts in the SVZ that ultimately reach the olfactory bulb (OB). This report emphasizes the multidimensional effects of histamine in the modulation of NSCs dynamics and sheds light into the promising therapeutic role of histamine for brain regenerative medicine.

Keywords: subventricular zone, olfactory bulb, neural stem cells, histamine, neurogenesis

INTRODUCTION

Brain diseases represent a very demanding worldwide health challenge. Nevertheless, no effective cure exists for the majority of these disorders. The discovery of NSCs in restricted regions of the adult brain redefined it as a plastic organ. Thus, the search for new drug candidates that may enhance stem cells properties and a full knowledge of NSCs biology are crucial to fulfil the actual healthcare and scientific demands.

NSCs reside in two niches of the adult brain: the SVZ lining the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus. Newly born neurons generated in the SGZ migrate short distances toward the granular cell layer, whereas SVZ-derived neuroblasts migrate long distances through the rostral migratory stream (RMS) toward the OB (Eiriz et al., 2011). Interestingly, upon brain injury, some neuroblasts can leave the SVZ/RMS axis to migrate toward damaged areas and differentiate into the specific neuronal/glial phenotype of the injured region. Therefore, a great effort has been taken on the design of stem cells-based strategies to promote brain repair (Ruan et al., 2014). For that purpose it is crucial to identify new factors that can enhance NSCs capabilities to produce new neurons. Herein, we comment on recent data supporting the role of histamine as a robust proneurogenic factor *in vivo* and we also discuss the profits vs. challenges for its usage in stem cell-based brain repair therapies.

GENERAL ROLE OF HISTAMINE IN THE CENTRAL NERVOUS SYSTEM

Histamine is an amine that has been classically associated with peripheral inflammatory reactions (Dale and Laidlaw, 1910). However, new evidences also highlight its function as a neuromodulator and neuroinflammatory agent. Four receptors mediate the effects driven by histamine: two postsynaptic (H1R, H2R), one presynaptic (H3R), and a forth receptor mainly present in the immune system (H4R). All receptors belong to the family of rhodopsin-like class A receptors coupled to guanine nucleotide-binding proteins (Brown et al., 2001). Neurons, microglia and mast cells are the three cellular reservoirs of histamine in the adult brain (Brown et al., 2001; Katoh et al., 2001). Histaminergic neurons, present in the tuberomammillary nucleus, project numerous ramifications throughout the entire adult brain, allowing histamine to be involved in a broad range of physiological functions, such as sleep-wake control, emotions, learning and memory (Panula and Nuutinen, 2013). Histamine is found at nanomolar levels in the healthy brain (Soya et al., 2008; Croyal et al., 2011; Bourgogne et al., 2012). However, several brain pathological conditions may be associated with an increased degranulation of mast cells in the *choroid plexus*, leading to a massive release of histamine in the CSF and the consequent increase of the blood brain barrier (BBB) permeability. Histaminergic neuronal activity (analyzed by positron emission tomography) was also found to be increased in the lesioned brain parenchyma (Vizuete et al., 2000; Motoki et al., 2005; Yanai and Tashiro, 2007; Kallweit et al., 2013). Importantly, histamine has been described

to be involved in several brain pathologies such as seizures (Bhowmik et al., 2012), stroke (Fan et al., 2011), multiple sclerosis (Ballerini et al., 2013; Krementsov et al., 2013), Parkinson and Alzheimer's disease (Shan et al., 2013). Remarkably, histamine may have a dual role and exert either neuroprotective or neurotoxic effects depending on the animal disease model, the receptor/signaling pathway activated and the diversity of histamine and histamine agonists/antagonists administration protocols. A clinically relevant therapeutic platform should take in account all of these distinct criteria, to be successful. Regarding neurogenesis, and although a recent review (Panula et al., 2014) highlights the role of histamine as a stem cell modulator during brain development, very few research studies on the role of histamine as a proneurogenic factor within the postnatal and adult brain were reported.

HISTAMINE EFFECTS ON NEURAL STEM CELL CULTURES

It is currently clear that histamine is involved in several brain functions but just recently its role as a modulator of stem cell biology has been revealed. We and others have shown that histamine transiently increases intracellular free calcium levels ($[Ca^{2+}]_i$) in SVZ stem/progenitor cells, embryonic stem cells and carcinoma cells (Tran et al., 2004; Agasse et al., 2008), suggesting the presence of functional histamine receptors in undifferentiated stem/progenitor cells. Particularly, we found that SVZ cells express the three types of histamine receptors, H1R, H2R, and H3R, being H1R the one responsible for the selective increase of $[Ca^{2+}]_i$ in immature cells (Agasse et al., 2008).

Recently, it was shown that histamine has a strong proneurogenic effect in neonatal SVZ (Bernardino et al., 2012) and in embryonic cortical cell cultures (Molina-Hernández and Velasco, 2008; Rodríguez-Martínez et al., 2012; Molina-Hernández et al., 2013) *via* H1R activation. Histamine may trigger increased transcription of FGFR1 and increased cell proliferation culminating in the differentiation of FOXP2 neuronal cells both *in vitro* and *in vivo* (Rodríguez-Martínez et al., 2012; Molina-Hernández et al., 2013) (**Figure S1A**). We also showed that histamine induces an increase of the expression of Mash1, Dlx2 and Ngn1 proneurogenic genes and ultimately favors the GABAergic neuronal phenotype. Thus, histamine may be used as an efficient inductor of neuronal differentiation *in vitro* prior NSCs transplantation. In fact, SVZ cells pretreated with poly(lactic-*co*-glycolic) acid (PLGA) microparticles that release histamine succeeded to survive, integrate and differentiate into newly born doublecortin (DCX)-neurons when transplanted into organotypic hippocampal slice cultures and into the DG or striatum of adult mice (Bernardino et al., 2012). Altogether, these data showed that histamine may be a key player in the priming of NSCs toward the neuronal phenotype.

ROLE OF HISTAMINE IN THE ADULT SVZ NEUROGENIC NICHE *IN VIVO*

Despite the absence of *in vivo* studies disclosing the role of histamine in the regulation of the SVZ neurogenic niche, *in vitro* studies have already shown that SVZ NSCs express functional H1R receptors that may be involved in neuronal commitment (Agasse et al., 2008; Bernardino et al., 2012). The relevance of investigating the effects of histamine on SVZ neurogenesis *in vivo* relies on the fact that both inflammation or brain injury may elicit *choroid plexus* mast cells degranulation, increasing the levels of histamine in the CSF and brain parenchyma leading to increased BBB permeability (Anichtchik et al., 2000; Yoshitake et al., 2003; Soya et al., 2008; Kanbayashi et al., 2009; Kallweit et al., 2013). The presence of histamine in the CSF that baths the SVZ neurogenic niche may affect SVZ GFAP-positive stem cells (type B cells) and its progeny *in vivo* by the direct contact of their cilia with the lumen of the lateral ventricles or by the interaction of stem/progenitor cells with the monolayer of ependymal cells (paracrine effect). Interestingly, it was observed that histamine is part of the adult mouse *choroid plexus* transcriptome signature (Marques et al., 2011). Taking into account these considerations, herein we disclose the role of chronic histamine administration in the adult SVZ neurogenic niche *in vivo*. For that, sustained intraventricular infusion of histamine was performed by using mini osmotic pumps that delivered histamine at the CSF for 21 days. All experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (86/609/EEC; 2010/63/EU). Weight matched wild-type C57BL/6 8-10 week old male mice were infused at the right lateral ventricle (Anterior-posterior: −0.5 mm, Medial-lateral: 0.7 mm, Dorso-ventral: 3.0 mm) with osmotic mini pumps (Alzet 1004, Charles River, flow rate: 0.10 μl/h) containing histamine (0.8 mg/Kg, Sigma-Aldrich) dissolved in artificial cerebrospinal fluid (aCSF: 150 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.8 mM Na_2HPO_4, and 0.2 mM NaH_2PO_4) or aCSF alone, as vehicle for 21 days. To ensure stable releasing rates, pumps were incubated before implantation in sterile 0.9% saline at 37°C for 48 h. During the first 3 days after surgery, 50 mg/Kg BrdU was administered intraperitoneally twice a day (**Figure 1A**). After brain fixation in 4% PFA and cryopreservation in 30% sucrose, 40 μm coronal slices were then cut and stained against Ki67, BrdU, DCX, and NeuN (1:1000 Rabbit polyclonal anti-Ki67—Abcam; 1:1000 Rat monoclonal anti-BrdU—Serotec; 1:1000 Rabbit polyclonal anti-DCX—BD Pharmingen; 1:4000 Mouse monoclonal anti-NeuN—Millipore). Sections were then rinsed and incubated with the appropriate AlexaFluor-conjugated secondary antibodies, stained for Hoechst-33342 and mounted. Confocal digital images were obtained on a LSM 510 Meta; Carl Zeiss microscope. The Software used was Axiovision, release 4.6 (Carl Zeiss) and Image J. Cell counting was performed in confocal images from five slices at 240 μm intervals, both at the SVZ and OB. Only counts performed in the contralateral hemispheres (left) are shown in order to exclude any possible bias induced by inflammatory reactions and/or lesion due to the cannulation at the ipsilateral hemisphere (right).

We found that the intracerebroventricular (i.c.v.) infusion of histamine in the lateral ventricles for 21 days induced a trend increase in the number of BrdU retaining cells (BrdU+DCX−) at the SVZ (Control: 25.8 ± 2.7; Histamine: 34.4 ± 4.7; statistically not-significant). This increase is statistically significant if we consider the BrdU+DCX+ double positive cells (Control: 4.6 ± 0.4; Histamine: 10.1 ± 1.9; $p < 0.05$; **Figure S1B** and **Figures 1B,C**). Accordingly, the number of DCX+ cells increased from 67.6 ± 3.9 in control to 129.1 ± 7.9 in histamine treated mice ($p < 0.001$, **Figure 1C**). No significant differences were found in counts of

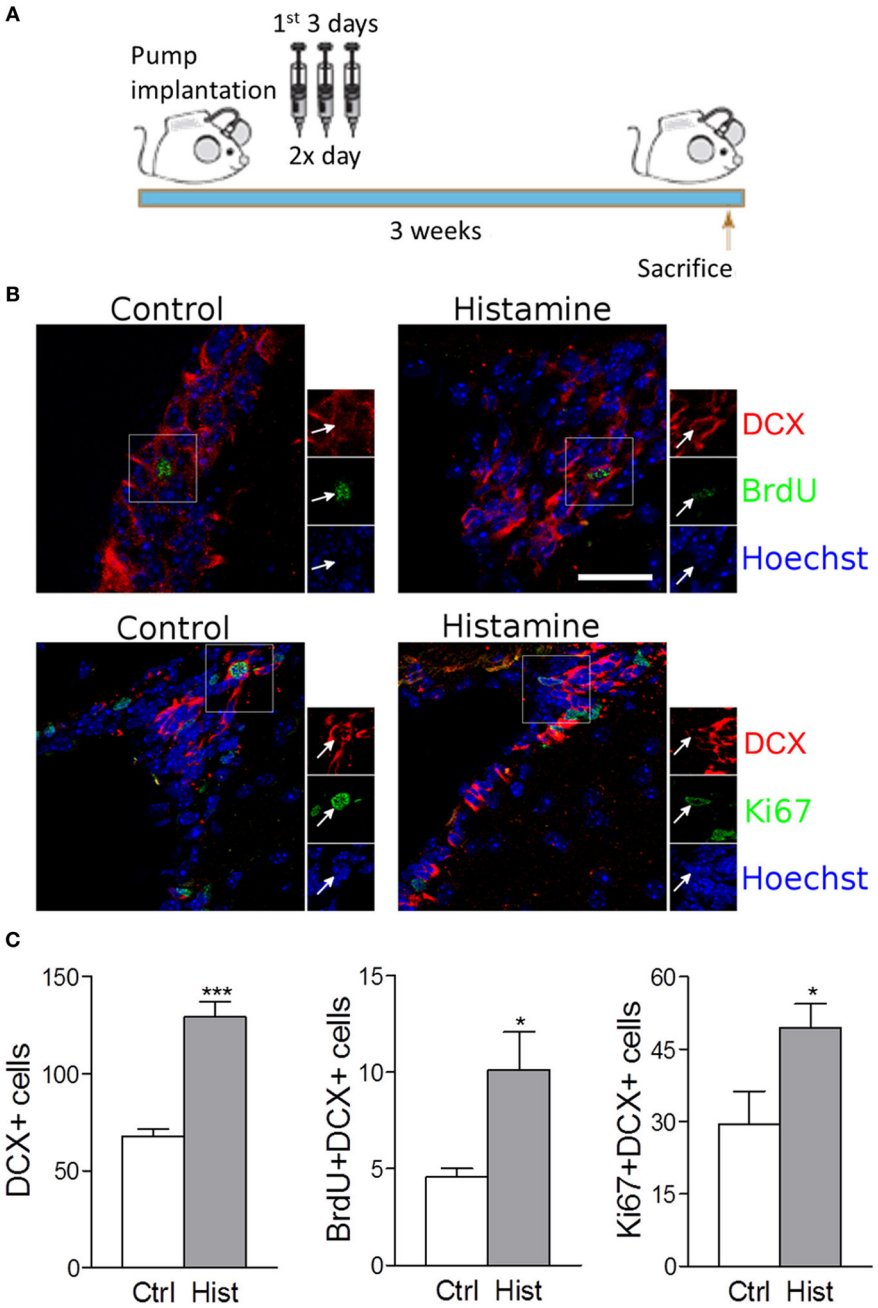

FIGURE 1 | Intracerebroventricular infusion of histamine triggers neuronal commitment in the SVZ. (A) Design of the 3 weeks experiment, consisting in 3-day BrdU treatment (twice daily with 12 h interval) starting at the day after surgery. Animals were sacrificed 3 weeks after surgery. **(B)** Representative fluorescent confocal digital images of BrdU (green; upper panel), Ki67 (green; lower panel) and DCX (red) positive cells observed in the SVZ of control and histamine treated animals *in vivo*, 21 days after pump installation. Scale bar = 20 μm. Arrows highlight double BrdU+DCX+ (upper panel) or Ki67+DCX+ (lower panel) positive cells. Hoechst staining (blue) labels cell nuclei. **(C)** From left to right: bargraphs represent the total DCX+ positive cells, total BrdU+DCX+ double positive cells and total Ki67+DCX+ double positive cells in both control and histamine *in vivo* treated animals, 21 days after pump installation. Ctrl: Control; Hist: Histamine. Data are expressed as mean ± SEM ($n = 5$–7 mice; $^{*}p < 0.05$; $^{***}p < 0.001$). Statistical analysis was performed using Student's unpaired *t*-test.

BrdU+DCX− and BrdU+DCX+ cells between both ipsilateral and contralateral hemispheres (regarding the same experimental condition) and, most importantly, both hemispheres showed the same relative differences between control and histamine treated animals (data not shown), excluding a putative influence of inflammation and/or tissue damage in the ipsilateral hemisphere. These data confirms previous *in vitro* data identifying histamine as a relevant inductor of neuronal commitment.

Curiously, some BrdU+DCX+ cells were retained at the SVZ 21 days upon histamine i.c.v. administration. We may hypothesize that this BrdU+DCX+ cell population at SVZ is derived from BrdU retaining cells, such as quiescent NSCs (B cells) that produce intermediate highly proliferating progenitor cells (C cells). Thus, further studies are also needed to disclose whether this increase of neuroblasts (A cells, BrdU+DCX+ cells) production induced by histamine is due to the activation of B cells which contact with CSF through their apical cilia, or by an increase in the proliferation of C or/and A cells.

Since histamine induced an increase in the number of BrdU+DCX+ cells at the SVZ, we then performed the Ki67 labeling to disclose if histamine had an effect in neuroblast proliferation. Ki67 is a cell marker associated with G1, G2, S and M phases of cell cycle. At the SVZ, Ki67 labelling showed a trend to increase upon histamine treatment (Control: 86.2 ± 24.6; Histamine: 133.0 ± 19.5; statistically not-significant) that was significant when looking to Ki67+DCX+ cells only (Control: 29.4 ± 6.8; Histamine: 49.3 ± 5.0; $p < 0.05$; **Figure S1B,** and **Figures 1B,C**). Interestingly, histamine increased the number of BrdU+DCX+ and Ki67+DCX+ cells but did not significantly affected the population of BrdU+DCX− or Ki67+DCX− cells. Altogether, these data indicate that histamine does not induce an overall increase of cell proliferation in the SVZ, but instead may trigger neuronal commitment (as previously showed by us—Bernardino et al., 2012) and/or induce neuroblast proliferation as previously reported (Rodríguez-Martínez et al., 2012; Molina-Hernández et al., 2013).

We also found that SVZ NSCs labelled with BrdU have differentiated into migrating neuroblasts that reached the OB in control and more densely in histamine treated animals (**Figure S1B,** and **Figure 2**). An increased number of BrdU+DCX+ cells was found in both the granular cell layer (GCL) and glomerular layers (GL) of the OB (Control GCL: 60.0 ± 3.5; Histamine GCL: 117.7 ± 7.4, $p < 0.001$; Control GL: 2.2 ± 0.2; Histamine GL: 5.5 ± 1.0, $p < 0.05$; **Figures 2A,C**). A significant increase of the DCX+ cells was also found in the GCL upon histamine infusion (Control GCL: 583.3 ± 11.5; Histamine GCL: 798.4 ± 33.0, $p < 0.001$; Control GL: 86.0 ± 7.1; Histamine GL: 117.3 ± 12.1; **Figure 2B**). In accordance with the SVZ data, the total number of BrdU+DCX− cells was not significantly different between control and histamine-treated animals in either OB layers (Control GCL: 286.0 ± 29.0; Histamine GCL: 362.6 ± 34.0; Control GL 22.5 ± 5.4; Histamine GL: 33.4 ± 5.3; statistically not-significant). Moreover, Ki67 labeling was almost inexistent at the OB (data not shown). These data may suggest that histamine is not interfering with the overall OB cell proliferation, but, instead, it increases the number of neuroblasts that reach the OB and, therefore, the final population of newly-generated OB neurons (**Figure S1B**). Additionally, some BrdU+ cells found at GL and GCL of control and histamine-treated animals generated NeuN-mature neurons (**Figure 2D**).

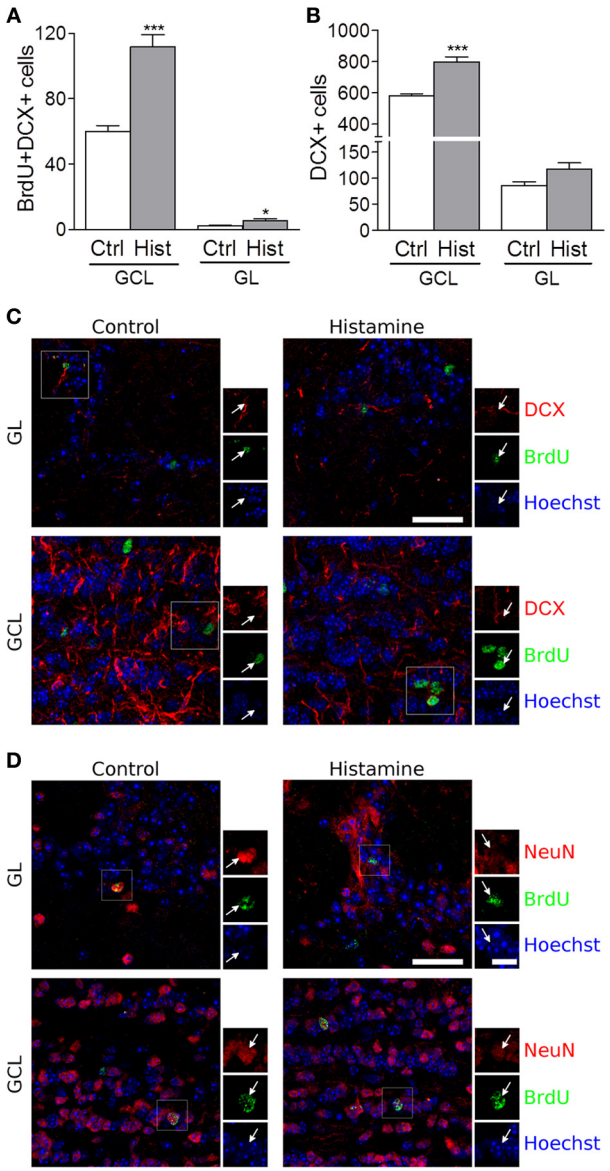

FIGURE 2 | Olfactory bulb integration of newly differentiated neuroblasts upon histamine long-term treatment. Bargraphs represent the total BrdU+DCX+ cells **(A)** and total DCX+ positive cells **(B)** counted in the granular cell layer (GCL) and in the glomerular layer (GL). Ctrl, Control; Hist, Histamine. Data are expressed as mean ± SEM ($n = 5$–7 mice; $^{*}p < 0.05$; $^{***}p < 0.001$). Statistical analysis was performed using Student's unpaired *t*-test. Representative fluorescent confocal digital images of BrdU (green) and DCX **(**red; **C)** or NeuN **(**red; **D)** positive cells observed in the granule cell layer (GCL) and glomerular layer (GL) of control and histamine treated animals *in vivo*, 21 days after pump installation. White arrows point to double positive BrdU+DCX+ **(C)** or BrdU+NeuN+ **(D)** cells. Hoechst staining (blue) labels cell nuclei. Scale bars = 30 μm.

In fact, histamine is responsible for the priming of NSCs at the SVZ toward the neuronal phenotype, which ultimately will reach the OB. The SVZ-derived progenitor cells are committed to the GCL and GL of the OB, where they differentiate mainly into GABAergic (Bédard and Parent, 2004; De Marchis et al., 2004) but also glutamatergic (Brill et al., 2009) and dopaminergic interneurons (Saino-Saito et al., 2004). Although we do not know the neuronal phenotypes generated by DCX+ neuroblasts found in the OB, we do know that, as expected, the majority of them differentiate into SVZ-derived cells located at

the GCL instead of the GL, a structure that mostly contains GABAergic interneurons. Accordingly, we found that histamine induces GABAergic neuronal differentiation in murine SVZ stem cell cultures (Bernardino et al., 2012). Furthermore, the morphology and disposition of DCX+ cells in the GCL and GL of the OB suggest that they are indeed young granule and periglomerular cells (Merkle et al., 2007).

Taken together, our data reveals that histamine is a crucial modulator of neuronal differentiation at the SVZ-OB neurogenic axis. However, we may anticipate some obstacles in using histamine to boost intrinsic regenerative properties of endogenous NSCs. Histamine was also shown to modulate the growth and specification of several cancer types, including gliomas. Increased activity of histidine decarboxylase (HDC), the enzyme involved in histamine synthesis, was found at the surrounding extracellular space of several cancer types, which is suggestive that it may be a crucial factor involved in tumorigenesis. Experiments performed in malignant cell lines and experimental tumors *in vivo* suggest that histamine modulates diverse biological responses related to tumor growth, such as proliferation, survival, and modulation of inflammation and angiogenesis (Eiriz et al., 2014). We could postulate that histamine have the ability to deregulate NSCs dynamics favouring proliferation and boosting the appearance of cancer stem cells especially nearby the neurogenic niches. However, some contradictory reports showed that histamine does not modulate cancer cell proliferation and instead induce their differentiation. Previously, we have shown that histamine does not support proliferation of SVZ stem/progenitor cells *in vitro* (Bernardino et al., 2012). Herein, we showed that the i.c.v. administration of histamine does not induce a significant increase in the total number of BrdU+DCX- or Ki67+DCX- cells at the SVZ, suggesting that, at least after 21 days, histamine does not induce a cancer-like profile of SVZ NSCs cells *in vivo*.

Another limiting factor responsible for the intrinsic difficulties of endogenous brain repair therapies relies on exacerbated inflammatory reactions occurring upon brain lesion that may create a hostile environment for the survival of neural stem/progenitors and neuroblasts. Microglia cells are the main cellular players involved in the innate immune response against brain injury or infection. Microglia phenotypes vary among neurogenic and non-neurogenic regions (Goings et al., 2006) and it may modulate SVZ neurogenesis (Shigemoto-Mogami et al., 2014). In this sense, we recently showed that histamine *per se* stimulates microglia motility and interleukin-1 beta release *via* H4R activation (Ferreira et al., 2012). But, on the other side, in an inflammatory context, histamine inhibited LPS-stimulated microglia activation *via* the same receptor. This dual role of histamine mediating microglial inflammatory responses highlights the need for further studies on the immunomodulatory effects of histamine within neurogenic niches. Increased levels of histamine found upon injury or inflammation can influence the overall cellular micro-environment, including mast cells, ependyma, neurons and microglia, favouring (or not) the survival, proliferation, and differentiation of new cells. This may depend on histamine levels and its distinct actions on different cellular populations present at SVZ-OB neurogenic niches vs. lesioned brain regions.

Recently, Kallweit et al. (2013) have shown that histamine is increased in the CSF of multiple sclerosis patients. In this line, and although we do not show a clear lack of effect of histamine in other neural cell types, such as oligodendrocytes or astrocytes, we have previously shown that histamine did not change NG2 or GFAP expression within the SVZ *in vitro* (Bernardino et al., 2012). Still, more detailed analysis of the effects induced by histamine at the neurogenic niche *in vivo* needs to be accomplished for further therapeutically relevant conclusions.

Upon brain injury, normal cellular dynamics is disturbed and NSCs are de-routed from their quiescent undifferentiated state to an active proliferative state so that new NSCs differentiate into neuroblasts that migrate to the damaged area (Kaneko and Sawamoto, 2009; Grade et al., 2013). Brain repair therapies involving the administration of proneurogenic factors (e.g., histamine) to boost endogenous mobilization of these neuronal precursors is less aggressive than the transplantation of NSCs, but requires a full control of the external booster in order to achieve a fine-tune of the endogenous resources. Alternatively, the transplantation of exogenous stem cells, or stem cell-based progenies at various stages of maturation (e.g., neuroblasts), to replace lost neurons, also raise several limitations, including the possible death of transplanted cells, low number of cells typically available for therapy, inadequate cell differentiation, erroneous cell integration into the host circuitry, immune rejection and variability in the functional outcome of the transplanted cells. Therefore, it is imperative to take in consideration these limitations (endogenous sources vs. exogenous transplantation) to find new effective platforms aiming the repair of damaged brain regions.

Neurogenesis occurring at the SVZ is well documented in rodents, and has also been demonstrated in primates and humans. However, both the cellular organization and the physiologic mechanisms involved on NSCs biology are distinct among these species. The SVZ NSCs found in the human brain younger than 18 months of age actively produce neurons which fate is the OB and the prefrontal cortex (Sanai et al., 2011). However, despite several controversies, a recent report showed that during adulthood human SVZ-derived NSCs lose the ability to migrate toward the OB and, instead, are found in the striatum (Ernst et al., 2014). Importantly, SVZ stem cells extracted from the adult human brain retain the capacities to produce neurons *in vitro*, suggesting that neurogenesis in the SVZ may be boosted under proper stimulation. In fact, several reports showed an increase of striatal neurogenesis in postmortem brains of Huntington disease and stroke patients (Curtis et al., 2003; Macas et al., 2006; Martí-Fàbregas et al., 2010). With our experimental protocol we showed that histamine increased the generation of newly-born neurons at the SVZ that ultimately migrate toward the OB. Thus, differences between human and mouse SVZ niches should be taken in consideration before extrapolating the proneurogenic effect of histamine found in mouse SVZ-OB axis to the potential application in human brain repair strategies. Further studies should disclose whether histamine may also boost neurogenesis under a pathologic condition, such as ischemia, eventually inducing the migration of SVZ-neuroblasts toward the lesioned striatum. Thus, in spite of the potential bottlenecks in triggering an efficient endogenous brain repair, we may asset that histamine efficiently

prime NSCs toward the neuronal phenotype, a phenomenon that may support its application in future brain regenerative medicine therapies.

AUTHOR CONTRIBUTIONS

Maria F. Eiriz: Conception and design; Collection and assembly of data; Data analysis and interpretation; Manuscript writing. Jorge Valero: Conception and design; Collection and assembly of data; Data analysis and interpretation, Manuscript writing. João O. Malva: Conception and design; Provision of study material; Data analysis and interpretation; Administrative support; Critical reading of manuscript. Liliana Bernardino: Conception and design; Provision of study material; Data analysis and interpretation; Financial support; Administrative support; Manuscript writing; Final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnins.2014.00142/abstract

Figure S1 | Integrative scheme of the effects driven by histamine in stem/progenitor cells both *in vitro* and *in vivo*. (A) Histamine has been reported to modulate both neuronal differentiation and cell proliferation in diverse types of stem/progenitor cells cultures. Red cells: neurons; Yellow cells: progenitor cells; Blue cell: stem cell. **(B)** Our *in vivo* results showed that histamine increases the number of neuroblasts at the SVZ that reach the OB.

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Adult neurogenesis in brain repair: cellular plasticity vs. cellular replacement

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INTRODUCTION

The last decade has seen an exponential increase in research directed to the field of regenerative medicine aimed at using stem cells in the repair of damaged organs including the brain. The therapeutic use of stem cells for neurological disorders includes either the modulation of endogenous stem cells resident in the brain or the introduction of exogenous stem cells into the brain. The final goal of these attempts is to replace damaged dysfunctional cells with new functional neurons. Nevertheless, there are multiple concerns regarding the therapeutic efficacy of the cellular replacement approach both from endogenous and exogenous sources. Indeed the extensive heterogeneity of neuronal subtypes in the brain makes it difficult to drive stem cells to differentiate to specific neuronal subtypes (Hawrylycz et al., 2012), which is a major requirement for regaining the lost neurological function. Furthermore, the fact that the brain is a very complex 3D structure with highly complex hierarchically organized connections raises a question on whether new neurons formed outside the brain niche can be functionally integrated into the preexisting circuitry. An alternative approach to cellular replacement can be enhancing plasticity in newborn neurons in the neurogenic niche to take over a function of a remote brain region. This strategy may have a yet unknown potential as it overcomes the limitations of the cellular replacement approach. In this opinion paper, we discuss limitations and potential of cellular replacement and cellular plasticity in the context of brain repair with a special focus on remote plasticity.

CELLULAR REPLACEMENT FOLLOWING NEUROLOGICAL DISORDERS

Cellular replacement upon brain damage involves two main strategies: (i) pharmacological or genetic modulation of endogenous neural stem cells (NSCs) and (ii) transplantation of exogenous stem cells.

NSCs resident in the adult brain are characterized by the ability to self-renew their own pool through cell proliferation and by the potential to differentiate into the three main cell types of Central nervous system (CNS): neurons, astrocytes, and oligodendrocytes (Gage, 2000).

Active neurogenesis occurs throughout adulthood in primates and various mammals including; rodents, rabbits, monkeys, and humans (Ming and Song, 2005; Martino et al., 2011). New functional neurons are produced under physiological conditions in two neurogenic niches: the subventricular zone (SVZ) in the lateral wall of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Gage, 2000). Moreover, various studies have shown the presence of "local" progenitors residing in various brain regions outside the stem cell niches including; neocortex, cerebellum, striatum, amygdala, substantia nigra, and hypothalamus (Ming and Song, 2005; Martino et al., 2011; Crociara et al., 2013).

Endogenous cellular replacement requires either: (i) increase in the number of newborn neurons in the neurogenic niches as compared to physiological conditions, (ii) migration of new neurons from the neurogenic niches to the damaged area, or (iii) production of the new neurons from local progenitor cells in the vicinity of the damaged brain. Indeed, various reports have demonstrated the occurrence of these three phenomenona following brain damage. Specifically, it has been shown that neurogenesis can be upregulated in neurogenic niches in response to different brain insults including ischemia (Jin et al., 2001; Harry, 2008; Osman et al., 2011), seizures (Parent and Lowenstein, 2002; Smith et al., 2005) and traumatic brain injury (Dash et al., 2001; Harry, 2008). Similarly, migration of newly generated neurons to the site of damage has been reported following brain ischemia (Arvidsson et al., 2002; Thored et al., 2007). Furthermore, neurogenesis following brain insults has been also reported in areas outside the neurogenic niches including the cortex, striatum, hippocampus, subcortical white matter, and corticospinal system (Sohur et al., 2006).

Although the reactive increase in neurogenesis that occurs following injury may indicate an attempt of the damaged brain to self-repair, this response fails in promoting functional recovery and in producing adequate amounts of newborn neurons that can survive and integrate.

Therefore increasing the number of functional neural precursor cells by increasing their survival rate, via pharmacological or genetic modulation, could be a promising strategy for brain repair.

The other cellular replacement strategy, following neurological insults, involves the transplantation of stem cells from

exogenous sources into the damaged brain. The most commonly used stem cells are immortalized human neural stem cell lines, mesenchymal stem cells, embryonic stem cells, neuronal progenitors isolated from rodents or humans, and induced pluripotent stem cells (iPSCs) (Liu et al., 2009; Martino et al., 2011). The therapeutic potential of the transplanted stem cells have been validated in various models of diseases and injuries (Shihabuddin et al., 2000; Pluchino et al., 2003; Cummings et al., 2005; Jin et al., 2005). Although varying degrees of functional recovery have been observed, it does not always correlate with the number of newly integrated neurons resulting from the differentiation of the transplanted stem cells. Indeed there is general agreement that transplanted stem cells play various other roles beside cellular replacement in the diseased/damaged brain including neuroprotection and reduction of the inflammatory response via a bystander effect (Martino and Pluchino, 2006; Martino et al., 2011).

LIMITATION OF THE CELLULAR REPLACEMENT APPROACH

Stem cells-based cellular replacement from endogenous or exogenous sources has many limitations including those stemming from the heterogeneity of neuronal subtypes and the highly complex structure of the brain. During the development of the nervous system different types of neurons are produced in highly controlled manner both temporally and spatially. This process is conserved in different species and fate determination of neural progenitor cells result in several postmitotic progenies with distinct phenotypes (Cepko et al., 1996). Importantly, the molecular signature and the transcriptional regulation of different neuronal subtypes vary enormously between different anatomical regions in the brain (Hawrylycz et al., 2012) limiting the differentiation of transplanted stem cells into specific brain regions and neuronal subtypes. One way to overcome this limitation is to develop techniques to direct the differentiation of neural progenitor cells to a specific phenotype. This solution is not easily applicable due to the limited potential of adult neural progenitor cells to differentiate to most neuronal subtypes. Indeed the wide heterogeneity of neuronal subtypes in the central nervous system originates during embryonic development from earlier neural precursors cells.

The functional integration of the newly generated neurons in the existing brain circuits is another major limitation to the cell replacement approach for transplanted cells and for cells produced outside the neurogenic niche. This can be attributed to the fact that the brain is composed of highly entangled set of cells and connections with precise stable spatial organization. The introduction of new neurons in the existing brain structure requires complex processes including: (i) directed migration of the new neurons to the proper site of integration and (ii) directed neurite-growth over long distances, which have not been demonstrated in the adult brain outside the neurogenic niches.

Therefore, the introduction of new neurons directly to the site of damage in the brain either by exogenous or endogenous sources faces major challenges such as differentiation to the correct subtype and integration. This leaves to date the newborn neurons in the neurogenic niches as the only cell type shown to be able to functionally integrate in the adult brain circuitry.

Consequently, one fundamental question is how we can make use of the reactive pool of neural precursor cells residing in the neurogenic niches to take over the function of a remote damaged brain region. In order to address this question it will be important to gain knowledge from the plastic properties of the older brothers of neural stem cells, the postmitotic neurons.

CELLULAR PLASTICITY FOLLOWING NEUROLOGICAL DISORDERS

Postmitotic neurons exhibit a certain degree of plasticity following brain ischemia and traumatic brain injuries. Indeed, despite the permanent structural damage and cellular loss, functional recovery is observed to a certain extent following brain damage (Chollet et al., 1991; Cao et al., 1998).

Neuroplasticity is defined as the brain's ability to reorganize itself by forming new functional synaptic connections throughout life. Continuous remodeling of neuronal connections and cortical maps in response to our experiences occurs to enable neurons to adapt to new situations (Taupin, 2008). Reorganization of brain networks plays also an important role allowing healthy neurons to compensate for damaged neurons (Sbordone et al., 1995; Cramer and Bastings, 2000; Demeurisse, 2000; Weidner et al., 2001). For instance, this functional compensation is evident following brain injury in the hemisphere contralateral to the lesion site. The contralateral hemisphere is reorganized and new connections are formed between intact neurons to take over some of the functions of the injured hemisphere (Takatsuru et al., 2009, 2011). Recent advances in functional imaging, e.g., positron emission tomographic and functional magnetic resonance imaging have indeed confirmed the occurrence of this reorganization (Calautti and Baron, 2003; Butefisch et al., 2006; Crosson et al., 2007; Ward, 2007). There is also clinical evidence that reorganization of the somatosensory cortex contralateral to the lesion site in stroke patients plays important role in the compensation of impaired functions (Chollet et al., 1991; Cao et al., 1998). Furthermore reorganization of brain networks has been reported in patients suffering from aphasia (speechlessness) in which the non-dominant right-hemisphere takes over the function of Wernicke's area (speech center normally present in the dominant left hemisphere) (Weiller et al., 1995).

Despite the consistent reports confirming circuitry reorganization in the brain following injury, the molecular and electrophysiological mechanisms controlling this fascinating phenomenon remain still elusive.

Another unexplored aspect of compensatory plasticity includes the question of whether newborn neurons are involved in the reorganization of brain circuitry that occurs following brain injury. However, because of their peculiar cellular and plastic properties, we believe that newborn neurons in the neurogenic niches are important players in this phenomenon.

Indeed it has been shown that newly generated neurons, as compared to mature granule cells, exhibit a lower threshold for induction of LTP (Schmidt-Hieber et al., 2004). This property, facilitating synaptic plasticity, makes young neurons ideally

suited to adapt to the reorganization of brain networks and to take over a function that is normally played by other brain regions.

Importantly, following brain ischemia, newborn neurons react with a plastic response enhancing not only their proliferation rate but also exhibiting increased spine density and dendritic complexity as compared to resident hippocampal neurons (Liu et al., 1998; Niv et al., 2012).

So far it has not been investigated whether this plastic response includes changes in the pattern of brain connectivity of newborn neurons. However the recent application of retrograde monosynaptic tracing to study the connectome of the newly generated neurons (Deshpande et al., 2013) in neurogenic niches provides us with tools to address this important question.

The next step following the demonstration of the involvement of newborn neurons in brain reorganization would be to increase their plastic potential by increasing their number. This may be achieved, taking advantage of the increase in the proliferation rate of NPCs that normally occurs upon brain damage (Liu et al., 1998), by increasing their integration and survival rate.

Previous work has described a number of intrinsic and extrinsic factors required for newborn neurons survival (see **Table 1**). The modulation of such factors, important to regulate the survival and integration of newborn neurons in physiological condition, may become even more crucial following brain damage. Recently, cytoskeleton regulators such as Rho kinase and Rho-GTPases have been included among the most important intrinsic regulators of the adult neurogenesis (Christie et al., 2013; Vadodaria and Jessberger, 2013; Vadodaria et al., 2013). Interestingly, the modulation of the Rho-Pathway is also critical for growth cone collapse, neurite outgrowth and regeneration after neurotrauma in the CNS (McKerracher et al., 2012), making it an ideal target to enhance both cellular plasticity and survival. In this perspective the identification of molecular mechanisms that can be targeted to increase both the number and the plasticity of newborn neurons can increase the probability of functional reorganization of brain networks following injury.

CONCLUSIONS

The vast amount of information that have been gathered in the recent years about the use of neural stem cells in brain repair indicates that cellular replacement alone cannot lead to effective restoration of function due to the complex anatomical, histological, and functional organization of the brain.

In this perspective, due to their plastic potential and their innate ability to functionally integrate in brain circuits, newborn neurons produced inside the neurogenic niches are the most suitable targets for brain repair. Moreover, the importance of neurogenesis-related plasticity is further supported by the finding that hippocampal neurogenesis occurs in humans throughout adulthood with a modest decline during aging (Spalding et al., 2013). Indeed, the central location of the hippocampus in the medial temporal lobe in the human brain (Haines, 2004) may allow the communication of newborn neurons to various brain circuits.

In this scenario strategies that enhance the survival and the plasticity of newly generated neurons in the dentate gyrus may be the most effective to foster the functional reorganization of brain circuits following injury.

Table 1 | Factors required for newborn neurons survival and integration in physiological conditions.

	Type	Niche	References
EXTRACELLULAR FACTORS			
BDNF	Neurotrophin	DG	Sairanen et al., 2005; Bergami et al., 2008; Waterhouse et al., 2012
GABA	Neurotransmitter	DG	Ge et al., 2006
Glutamate	Neurotransmitter	SVZ	Platel et al., 2010
WNT	Morphogen	DG	Lie et al., 2005; Kuwabara et al., 2009
INTRACELLULAR FACTORS			
NFATc4	Transcription factor	DG	Quadrato et al., 2012
NF-KappaB p50	Transcription factor	DG	Denis-Donini et al., 2008
CREB	Transcription factor	DG	Jagasia et al., 2009
Neuro D1	Transcription factor	DG, SVZ	Gao et al., 2009
PROX1	Transcription factor	DG	Lavado et al., 2010; Karalay et al., 2011
ROCK (inhibition)	Kinase	DG, SVZ	Leong et al., 2011; Christie et al., 2013

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Neurogenic and non-neurogenic functions of endogenous neural stem cells

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Adult neurogenesis is a lifelong process that occurs in two main neurogenic niches of the brain, namely in the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. In the 1960s, studies on adult neurogenesis have been hampered by the lack of established phenotypic markers. The precise tracing of neural stem/progenitor cells (NPCs) was therefore, not properly feasible. After the (partial) identification of those markers, it was the lack of specific tools that hindered a proper experimental elimination and tracing of those cells to demonstrate their terminal fate and commitment. Nowadays, irradiation, cytotoxic drugs as well as genetic tracing/ablation procedures have moved the field forward and increased our understanding of neurogenesis processes in both physiological and pathological conditions. Newly formed NPC progeny from the SVZ can replace granule cells in the olfactory bulbs of rodents, thus contributing to orchestrate sophisticated odor behavior. SGZ-derived new granule cells, instead, integrate within the DG where they play an essential role in memory functions. Furthermore, converging evidence claim that endogenous NPCs not only exert neurogenic functions, but might also have non-neurogenic homeostatic functions by the release of different types of neuroprotective molecules. Remarkably, these non-neurogenic homeostatic functions seem to be necessary, both in healthy and diseased conditions, for example for preventing or limiting tissue damage. In this review, we will discuss the neurogenic and the non-neurogenic functions of adult NPCs both in physiological and pathological conditions.

Keywords: neural stem cells, neurogenesis, inflammation, transplantation, germinal niches, bystander effect

INTRODUCTION

In 1913 Santiago Ramón y Cajal established that neurons of the brain are only generated during the neurodevelopmental phase, thus setting the so called "no new neurons" doctrine (Ramon Y Cajal, 1913). However, he soon reconsidered his conclusions when evaluating the results of an experiment performed a couple of years before by his younger assistant Francisco Tello. This experiment, in fact, showed that regenerating fibers growing from the stump of a transected optic nerve could suture with a "regenerating" peripheral sciatic nerve (Tello, 1907).

Nevertheless, despite this initial hint, the existence of dividing cells of neural origin in the central nervous system (CNS) was still debated (Hamilton, 1901; Allen, 1912) and could not be formally demonstrated until the beginning of the'60 when Smart (1961) and Altman (Altman and Das, 1965) demonstrated the effective presence of proliferating neural cells—i.e., neurogenesis—in the adult rodent brain. However, this finding would have been indisputably confirmed only 20 years later, namely when Fernando Nottebohm showed that neurogenesis in the ventricular zone is a phenomenon that normally occurs in intact adult female canaries (Nottebohm, 1981; Goldman and Nottebohm, 1983). Few years' later, adult neural stem/precursor cells (NPCs) were identified as a source of new neurons also in the brain of non-human primates and humans (Kukekov et al., 1999; Ming and Song, 2005). Later on, *in vitro* stable culturing systems either for rodent and human NPCs were established (Reynolds and Weiss, 1992).

Nowadays, we know that neurogenesis in the adult brain occurs in physiological conditions in specific neurogenic niches that have particular anatomical and functional characteristics. The role of neurogenesis after injury however still needs to be fully clarified. While there is substantial evidence that active, and latent, neurogenic niches might contribute to the formation of new cells upon CNS tissue damage, the precise role of these newly formed cells has not been yet completely understood. Here we review the possibility that endogenous NPCs exert functional roles not directly related to the production of new cells (the so called "non-neurogenic functions").

NEUROGENESIS IN THE ADULT BRAIN: FROM CELLS TO FUNCTIONS

THE ADULT RODENT BRAIN

Neurogenesis in the adult rodent brain occurs during adulthood in two main neurogenic niches, namely in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and in the subventricular zone (SVZ) of the lateral ventricles.

The SGZ is a thin layer of cells located between the two DG layers of granule and hilus cells. The primary role of SGZ is to generate new cells capable to functionally integrate within the

DG granule layer. The DG granule layer is mainly composed by primary excitatory neurons supporting cognitive functions, such as memory and learning (Shors et al., 2002; Zhao et al., 2008). The development of granule cells from NPCs proceeds throughout different intermediate steps (Filippov et al., 2003). NPCs first develop into (i) radial astrocytes (i.e., type I cells) that, in turn, generate (ii) intermediate neural progenitors (i.e., type-D cells or type II progenitors) (Fukuda et al., 2003)—which are immature progenitors (also called neuroblasts) further differentiating into (iii) neuroblasts. Neuroblasts can be further sub divided into D1 (immature) and D2 (more differentiated) cells (i.e., type G or type III cells) (Filippov et al., 2003; Zhao et al., 2008), which progressively acquire electrophysiological characteristics of granule neurons. SGZ neurogenesis occurs in parallel to angiogenesis (Palmer et al., 2000) and endothelial cells act as scaffolding cells for NPCs. Therefore, endothelial cells provide signals and soluble factors that favor angiogenesis but also neurogenesis (Riquelme et al., 2008).

The second main neurogenic niche is the SVZ, a region located in the lateral side of the two lateral ventricles. This region originates from the neuroventricular epithelium of the embryonic ventricular zone, the area where radial glia proliferates during development. Similarly to the SGZ, the SVZ shows a rather heterogeneous population of stem and progenitor cells. Here we can find (i) relatively quiescent stem cells, known as B cells, that give rise to (ii) actively proliferating cells representing intermediate progenitors in transit to the terminal differentiation (i.e., type C cells or transit amplifying cells) (Doetsch et al., 1999). Type C cells differentiate into (iii) neuroblasts (i.e., immature type A cells) that migrate along the rostral migratory stream (RMS) toward the olfactory bulb (OB) to give rise to new OB granule cells (Lois and Alvarez-Buylla, 1994; Belluzzi et al., 2003). The SVZ can be subdivided anatomically into three main structural domains: domain I (wall of the ventricle) contains ependymal cells as well as the primary cilium of type B cells and is in direct contact with the cerebrospinal fluid (CSF); domain II (below the wall of the ventricle) contains the cell bodies of type-B cells, type C cells, type A cells, neuronal terminals, and other supporting cells; domain III contains basal processes of B-cells that terminate in specialized end-feet capable of contacting blood vessels (Fuentealba et al., 2012). Due to their anatomical localization SVZ NPCs are strategically positioned within the brain: on the one hand, they are in direct contact with the CSF through their apical processes, and, on the other hand, they are tightly apposed to blood vessels forming a peculiar "periventricular" blood brain barrier (BBB) that is the barrier circumventing the lateral ventricles and the third and the fourth ventricle. SVZ NPCs are thus in close communication with two different peripheral blood-related microenvironments (Sawamoto et al., 2006; Mirzadeh et al., 2008; Tavazoie et al., 2008). It is still matter of debate whether the periventricular BBB is more permeable thus facilitating type B and C cells to receive blood-borne molecules regulating self-renewal and differentiation. Apart from the blood compartment, the SVZ is also located very close to crucial areas of the forebrain (i.e., basal ganglia, striatum) that contain GABAergic neurons capable of modulating interconnections between several cortical and sub-cortical brain areas (Koos and Tepper, 1999). In fact, NPCs in the SVZ are separated from the caudate nucleus and the striatum only by a layer of myelin and are in intimate contact with surrounding glia and blood vessels (Doetsch et al., 1999; Alvarez-Buylla and Lim, 2004). This peculiar position makes SVZ NPCs susceptible to the action of several neurotransmitters such as GABA (Platel et al., 2008, 2010), glutamate (Platel et al., 2010), ATP (Abbracchio et al., 2009), and acetylcholine (Cooper-Kuhn et al., 2004; Young et al., 2011), all neurotransmitters released from nearby neurons and collaterals. It is highly likely that SVZ NPCs can be directly influenced by the activity of neuronal networks (Tong et al., 2014). The decreased proliferation of NPCs, so far observed in Parkinson's disease, has been attributed to the loss of dopaminergic innervation of the SVZ (Curtis et al., 2007a). *Post-mortem* studies in humans have identified dopaminergic fibers in contact with epidermal growth factor receptor (EGFR)- positive cells in the SVZ (Hoglinger et al., 2004). In addition, the SVZ area is innervated by serotoninergic fibers (Diaz et al., 2009) and serotonin has been documented to increase neurogenesis in the SVZ (Encinas et al., 2006; Kazanis, 2009).

THE ADULT HUMAN BRAIN

Although it has been variably shown that the two main neurogenic regions of the rodent brain, the SGZ and the SVZ, are also present in the adult human brain, human neurogenesis has some peculiarities that need to be highlighted.

In the 1990s, a study by Eriksson and colleagues performed in a group of cancer patients receiving the DNA labeling nucleotide Bromodeoxyuridine (BrdU) showed the BrdU signal in hippocampal neurons (Eriksson et al., 1998). This work formally established the presence of adult neurogenesis in the human hippocampus during adulthood. However, the observed neurogenesis could also have been attributed to the underlying pathology. Some years later Knoth et al. (2010) confirmed the presence of neurogenesis in the adult human hippocampus based on data obtained from 54 human autoptic specimens (age 0–100). In the same study, qualitative and quantitative age-related changes—very similar to those occurring in the rodent hippocampus—further confirmed and expanded these findings (Knoth et al., 2010).

The human SVZ behaves, instead, very differently to the human adult hippocampus. In this region, the extent of this continuous neurogenesis as well as the presence of a RMS is still matter of debate. In 2004, Sanai and co-workers described within the SVZ a ribbon of proliferating astrocytes—lining the lateral ventricles of the adult human brain—that behaved as multipotent progenitor cells *in vitro* (Sanai et al., 2004). However, they did not find any evidence of chains of migrating neuroblasts in the SVZ or in close proximity to the OB (Sanai et al., 2007). After this provocative works, an intense debate occurred about the existence of a human RMS. In 2007, Curtis and colleagues showed histological evidence of a human RMS-like structure organized around a lateral ventricular extension reaching the OB (Curtis et al., 2007a,b; Sanai et al., 2007). Two successive reports challenged the existence of a RMS; only a ventromedial prefrontal cortex stream was observed in infants up to 2 years of age but not in adults (Sanai et al., 2011). More recently, a retrospective ^{14}C birth dating study showed that there is rather minimal adult

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INTRODUCTION

In 1913 Santiago Ramón y Cajal established that neurons of the brain are only generated during the neurodevelopmental phase, thus setting the so called "no new neurons" doctrine (Ramon Y Cajal, 1913). However, he soon reconsidered his conclusions when evaluating the results of an experiment performed a couple of years before by his younger assistant Francisco Tello. This experiment, in fact, showed that regenerating fibers growing from the stump of a transected optic nerve could suture with a "regenerating" peripheral sciatic nerve (Tello, 1907).

Nevertheless, despite this initial hint, the existence of dividing cells of neural origin in the central nervous system (CNS) was still debated (Hamilton, 1901; Allen, 1912) and could not be formally demonstrated until the beginning of the'60 when Smart (1961) and Altman (Altman and Das, 1965) demonstrated the effective presence of proliferating neural cells—i.e., neurogenesis—in the adult rodent brain. However, this finding would have been indisputably confirmed only 20 years later, namely when Fernando Nottebohm showed that neurogenesis in the ventricular zone is a phenomenon that normally occurs in intact adult female canaries (Nottebohm, 1981; Goldman and Nottebohm, 1983). Few years' later, adult neural stem/precursor cells (NPCs) were identified as a source of new neurons also in the brain of non-human primates and humans (Kukekov et al., 1999; Ming and Song, 2005). Later on, *in vitro* stable culturing systems either for rodent and human NPCs were established (Reynolds and Weiss, 1992).

Nowadays, we know that neurogenesis in the adult brain occurs in physiological conditions in specific neurogenic niches that have particular anatomical and functional characteristics. The role of neurogenesis after injury however still needs to be fully clarified. While there is substantial evidence that active, and latent, neurogenic niches might contribute to the formation of new cells upon CNS tissue damage, the precise role of these newly formed cells has not been yet completely understood. Here we review the possibility that endogenous NPCs exert functional roles not directly related to the production of new cells (the so called "non-neurogenic functions").

NEUROGENESIS IN THE ADULT BRAIN: FROM CELLS TO FUNCTIONS

THE ADULT RODENT BRAIN

Neurogenesis in the adult rodent brain occurs during adulthood in two main neurogenic niches, namely in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and in the subventricular zone (SVZ) of the lateral ventricles.

The SGZ is a thin layer of cells located between the two DG layers of granule and hilus cells. The primary role of SGZ is to generate new cells capable to functionally integrate within the

long term potentiation (LTP) (Kitamura et al., 2009). They also showed that decreased neurogenesis is accompanied by a prolonged hippocampus-dependent period of associative fear memory: this mechanism has been proposed to play a role in clearing disused old memories to preserve the learning capacity of the hippocampus (Willshaw and Buckingham, 1990). Animals exposed to an environmental enrichment showed enhanced hippocampal neurogenesis (Kempermann et al., 1997).

The functional role of NPCs residing within the SVZ is certainly more controversial. As said before, newly formed NPCs in the rodent SVZ migrate along the RMS to the OB where they integrate as interneurons within the granule and glomerular cell layers; a process considered important in maintaining and reorganizing the OB system (Imayoshi et al., 2008). The integration of the new neurons in the OB and DG is varied: in the OB, neurogenesis contributes to the maintenance and reorganization of the whole system while in the DG new neurons are added to modulate and refine the existing neuronal circuits (Imayoshi et al., 2008). While SVZ neurogenesis in the adult brain seems not to exert a role in retaining the memory of spontaneous odor discrimination and innate olfactory preference (Imayoshi et al., 2008), it seems to be involved in consolidating long-lasting olfactory traces (Gheusi et al., 2000; Lazarini et al., 2009). Indeed, the increased survival of new-born granule cells observed after the enrichment is necessary for the increased inhibitory activity in the OB and leads to a better discrimination of highly similar odorants (Moreno et al., 2009). Recent studies have confirmed these data and assessed that while easy odor tasks (Mandairon et al., 2006)—e.g., the habituation-dishabituation test—do not need neurogenesis (Kageyama et al., 2012), more difficult odor tasks, instead, do require modulation of the new-born neuron survival (Mandairon et al., 2006).

Besides the role in smell recognition, the instinctive response to pheromones is also processed by the main and accessory olfactory systems; SVZ neurogenesis plays an essential role in this context (Kageyama et al., 2012). For example, olfactory activities are very important for the maintenance of pregnancy (Bruce, 1959; Kaba et al., 1994): pregnancy induces biphasic stimulation of neurogenesis in the SVZ, leading to a biphasic increase in the production of both granule cells and periglomerular cells in the OB (Shingo et al., 2003). Neurogenesis in females is also induced by dominant male pheromones and seems to be important for sexual behaviors (Mak et al., 2007). Also in male, paternal-offspring recognition behaviors seem to rely on postnatal offspring interaction and are coupled to increased neurogenesis in the paternal OB and hippocampus (Mak and Weiss, 2010; Kageyama et al., 2012). Finally, SVZ neurogenesis might be required for predator avoidance and sex-specific responses that are olfaction dependent and innately programmed (Sakamoto et al., 2011).

NON-NEUROGENIC FUNCTIONS

In the last few years, other non-neurogenic functions of NPCs in the brain have been unraveled. NPCs are in fact able to produce and secrete a wide variety of factors that regulate and drive complex functions of the brain. A recent report showed that neuroblasts derived from both neurogenic niches (the SVZ and SGZ) exert a physiological phagocytic activity in clearing apoptotic neuronal precursors, and that this phagocytic activity is critically important in maintaining neurogenesis in the brain. Interestingly, NPC phagocytosis requires the intracellular engulfment protein ELMO1 to promote Rac activation downstream of phagocytic receptors (Lu et al., 2011).

Moreover, recent evidence supports the importance of non-neurogenic functions of NPCs. Sierra et al. demonstrated in fact that apoptotic new-born cells are rapidly cleared out through phagocytosis by unchallenged microglia present in the adult SGZ niche and that microglia is important in maintaining the homeostasis of the baseline neurogenic cascade (Sierra et al., 2010). Mosher et al. expanded this finding by demonstrating that NPCs are able, through the secretion of vascular endothelial growth factor (VEGF), to modulate microglial activation, proliferation and phagocytosis (Mosher et al., 2012). Furthermore, a bilateral cross-talk between NPCs and microglia seems to take place (Mosher et al., 2012).

Another "homeostatic" function coupled to NPCs has been recently described. Despite not having classical features of a neurogenic niche, median eminence tanycytes may also generate new-born neurons (Kokoeva et al., 2005; Lee et al., 2012). After a first study supporting the idea that hypothalamic neurogenesis in adult mice has a role in the control of energy-balance, including the capacity of regulating leptin-induced phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Kokoeva et al., 2005), another recent work showed that median eminence tanycytes have a role in regulating the weight and metabolic activity of adult mice (Lee et al., 2012).

Moreover, newly generated neuroblasts residing within the SGZ seem to be able to dynamically regulate stress reactivity at both the endocrine and behavioral levels by buffering stress responses, through the regulation of the hypothalamic–pituitary–adrenal axis (Snyder et al., 2011). In fact, neurogenesis-deficient mice also showed increased food avoidance after acute stress, increased behavioral despair in the forced swim test, and decreased sucrose preference, a measure of anhedonia (Snyder et al., 2011). It would be interesting to understand whether the observed alterations can be attributed to an alteration of median eminence tancytes (and vice versa), given that the models used for ablation of NPCs in this work did not exclusively target a single NPC subpopulation.

These data altogether support the concept that NPCs might exert, besides pure neurogenic functions, also a broad spectrum of "bystander" non-neurogenic functions aimed at maintaining the homeostasis of the brain (**Figure 1**) (Martino and Pluchino, 2006).

ROLE OF ENDOGENOUS NPCs DURING CNS PATHOLOGY

NEUROGENIC FUNCTIONS

Different types of brain damage—such as stroke, epileptic seizures, trauma—induce the proliferation of NPCs in neurogenic areas, i.e., the SGZ and the SVZ (Riquelme et al., 2008). The majority of neurons formed in SGZ after an insult become dentate granule cells, similar to what occurs in the intact brain, while in the SVZ newly generated cells often migrate, away from the RMS, toward the lesion site (Jin et al., 2001).

Adult brain reacts to an ischemic injury by a long-lasting generation of neuroblasts from the SVZ; SDF-1a/CXCR4 signaling

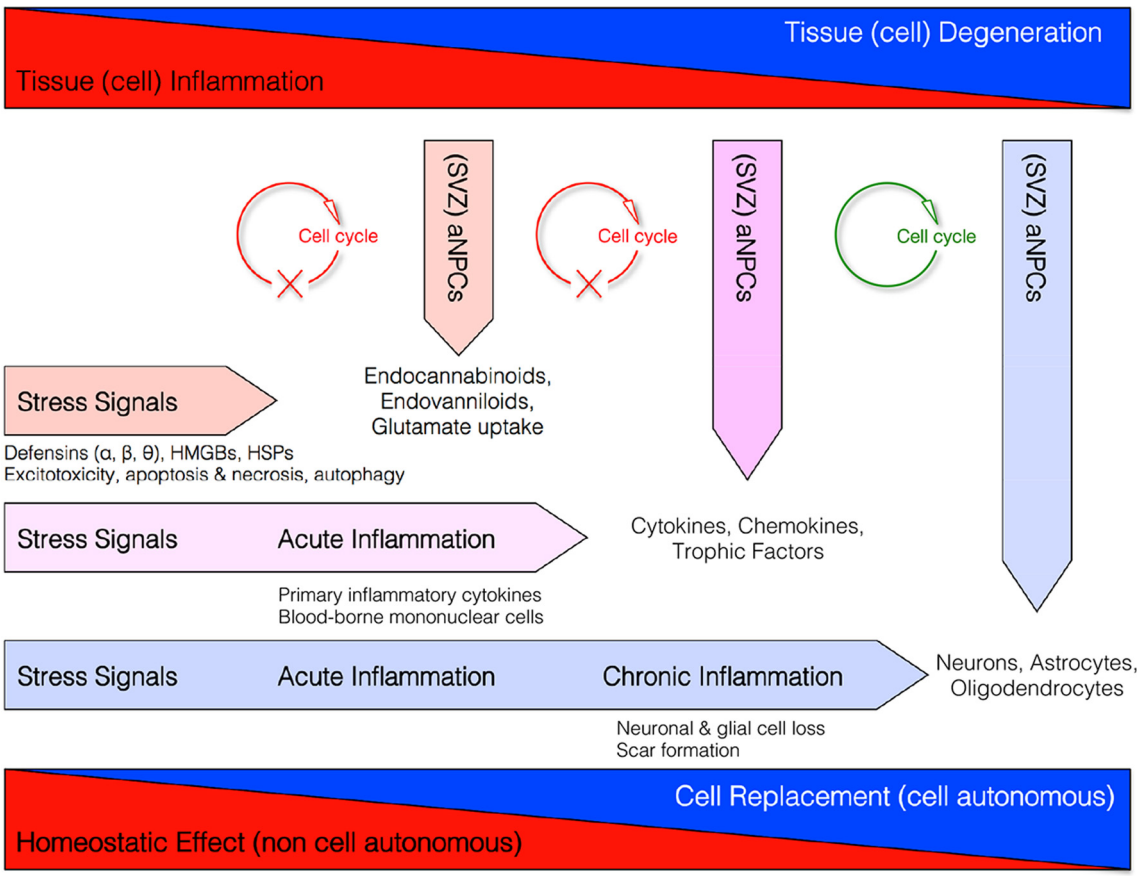

FIGURE 1 | Homeostatic multi-step actions exerted by endogenous NPCs: from maladaptive (stressful) conditions to pathological chronic tissue damage. Endogenous NPCs adapt their homeostatic functions to the needs of the tissue. In order to reduce excitotoxicity so to prevent reactive inflammation, endogenous NPCs release neuroprotective molecules (i.e., endocannabinoids, endovanilloids) and increase glutamate uptake as soon as the occurrence of early stress signals. If this barrier fails and acute inflammation occurs, endogenous NPCs release different neuroprotective and anti-inflammatory molecules (e.g., cytokines, chemokines, and trophic factors) that, in turn, restrain the CNS infiltration of blood-borne inflammatory cells and the acute inflammatory reaction. This latter second-step process is also finalized to reduce the secondary tissue damage. Finally, during chronic inflammatory conditions when tissue architecture is already compromised, NPCs might differentiate into new cellular elements in order to replace endogenous cells lost.

regulates the migration of new striatal neurons generated from endogenous NPCs toward the ischemic damage (Imitola et al., 2004). Within these newly formed peri-infarct neurovascular niches, newly-born immature neurons interact with the remodeling vasculature thanks to their production of stromal-derived factor 1 (SDF1) and angiopoietin 1 (Ang1) (Ohab et al., 2006). Interestingly neurogenesis and angiogenesis, another important reparative process taking place in the peri-ischemic tissue, are tightly coupled after stroke by VEGF that stimulates cell genesis (Teng et al., 2008).

A long-lasting neurogenic response reactive to ischemic injury has been observed not only in animal models but also in stroke patients (Marti-Fabregas et al., 2010). Interestingly, the enhanced neurogenic response is paralleled by increased microglia recruitment, probably due to a stroke-induced up regulation of CXCL10 in the SVZ; this chemokine might act as chemoattractant of CXCR3-expressing microglia (Rappert et al., 2004; Thored et al., 2009). About 80–90% of newly formed striatal neurons that potentially could replace the dead neurons will eventually die (Arvidsson et al., 2002; Thored et al., 2006, 2007). In fact, only a small portion of SVZ-derived cells migrated into striatum does assume features of mature neurons with action potentials in a rodent model of ischemic stroke (Arvidsson et al., 2002).

Similarly to stroke, also epilepsy is associated with an increased level of progenitor proliferation paralleled with an accelerated maturation and integration of only few newly generated neurons (Rotheneichner et al., 2013). Neurons formed in the DG, after an epileptic insult, undergo caspase-mediated apoptotic death, similarly to NPCs isolated from the adult SVZ (Ekdahl et al., 2001). In animal models of epilepsy, the initial rise in neurogenesis is then followed by a long-lasting reduction of neurogenesis (Hattiangady et al., 2004). Reduced cell proliferation has been also observed in the hippocampus of children during the chronic phase of a frequent seizure convulsive disorder (Mathern et al., 2002; Rotheneichner et al., 2013). In experimental epilepsy, SVZ-derived cells migrate toward the hippocampus and differentiate terminally into glial but not neuronal cells (Parent et al., 2006). Newly born neurons might

exacerbate chronically epileptic hippocampus if they aberrantly migrate and incorporate in the dentate hilus (Hattiangady and Shetty, 2008). Interestingly, inflammation might influence the functional integration of adult-born hippocampal neurons as a high degree of synaptic plasticity of the new neurons has been reported in an inflammatory environment. This effect seems to be finalized to counteract inflammation-induced increase of excitatory input (Jakubs et al., 2008). However, the extent to which seizure-induced neurogenesis might contribute to the formation of newly formed neurons destined to integrate into the damaged epileptic hippocampus still need to be clarified.

NON-NEUROGENIC FUNCTIONS

As said above, there is an increased reactive neurogenesis followed by a scarce integration of newly formed neurons into neuronal damaged circuits. This chain of events appears to be paradoxical. Several hypotheses have been proposed. One of these states that NPCs might exert tissue protective functions by deviating from their neuronal default into a glial differentiation pathway or remaining undifferentiated and secrete neuroprotective molecules in a bystander fashion.

Instead of differentiating into the neuronal default pattern, NPCs (from both SVZ and SGZ) may turn into both astroglial and oligodendroglial cells—a gliogenic rather than a neurogenic response—in order to constrain and/or prevent tissue damage. Several recent evidence supports this NPC-mediated phenomenon reacting to a CNS injury.

Localized photothrombotic/ischemic cortical injury triggers the production of BBB stabilizing astrocytes from the postnatal SVZ niche; an event controlled by the Notch modulator thrombospondin 4 (Thbs4). Indeed, knockout mice for Thbs4 had a distorted neuroblast-astrocyte production, an abnormal glial scar formation, and a significant delayed increase of perilesional microvascular hemorrhages (Benner et al., 2013).

In demyelinating diseases, such as multiple sclerosis (MS), NPCs in the rodent SVZ niche become activated, upon demyelination, and provide a potential source of myelinating oligodendrocytes. SVZ-derived cells expand and migrate to the lesions, undergo oligodendrogenesis (Nait-Oumesmar et al., 1999), acquire morphology of myelinating cells, and express myelin proteins (Menn et al., 2006).

Using genetic fate mapping, it has been shown that, after a spinal cord injury (SCI), ependymal cells lining the central canal of the spinal cord have neurogenic potential. Indeed, in mice undergoing SCI, ependymal cell progeny starts migrating from the ependymal layer toward the injury site within 3 days after the injury; once within the lesion site, proliferating cells predominately differentiate into scar-forming astrocytes (Barnabe-Heider et al., 2010). In fact the glial scar that forms after SCI is composed by resident astrocytes and, in its central part, by ependymal cell–derived astrocytes (Barnabe-Heider et al., 2010). Ependymal cell–derived astrocytes might thus contribute to reinforce the injured spinal cord thus avoiding the expansion of the cystic cavity (Barnabe-Heider et al., 2010). Finally, cells recruited by the SCI not only produce scar-forming glial cells, but also, to a lesser degree, oligodendrocytes (Meletis et al., 2008).

The production of new neuronal or glial cells seems not to be the prevailing and sole mechanism of reactive neurogenesis occurring in response to tissue damage.

In stroke, not integrating newly formed SVZ-derived cells seem to protect from tissue injury through the secretion of neurotrophic factors (Jin et al., 2010; Wang et al., 2012; Sun et al., 2013). In a recent work SVZ NPCs were indeed shown to protect striatal neurons from glutamatergic excitotoxicity (as that occurring in the early phase of ischemic stroke and epilepsy) by releasing endogenous endocannabinoids (AEA and 2-AG) capable of binding to their specific receptors (CB1 and CB2) (Butti et al., 2012). Interestingly endovanilloids secreted by SVZ NPCs were found to suppress the growth of high-grade astrocytomas (HGA). NPCs by releasing endovanilloids activate the transient receptor potential vanilloid subfamily member-1 (TRPV1) on HGA cells that, in turn, triggers tumor cell death and prolongs overall survival time of the mice (Stock et al., 2012).

Also in another CNS injury model, SCI, scar stabilizing NPC-derived astrocytes do not only restrict secondary enlargement of the lesion and further axonal loss (Sabelström et al., 2013), but also exert a non-neurogenic action via the secretion of growth factors acting as neuroprotectant to enhance the survival of neurons adjacent to the traumatic lesion.

As previously pointed out, whether a homeostatic function of the endogenous NPCs might occur, the SVZ zone seems to be the more appropriate area. In fact, as stated before, SVZ NPCs are in close communication with two different microenvironments being tightly apposed to blood vessels and in contact with the CSF, and also very close to crucial areas of the midbrain containing GABAergic neurons. A further confirmation of this working hypothesis came from a recent work showing that dendritic cell (DC) traffic within the CNS—from the choroid plexus to the cervical lymph nodes—along the RMS in order to modulate CNS-infiltrating regulatory T cell (Treg) function. This migration of DC seems to dampen experimental CNS auto-inflammatory diseases, thus suggesting that it ultimately prevents pathogenic T-cells from entering the CNS (Mohammad et al., 2014).

NEUROGENIC vs. NON-NEUROGENIC FUNCTIONS

Another important, but so far only partially solved issue, concerns how of NPCs can determine their fate between neurogenic and non-neurogenic functions in pathological conditions. The predominant view, supported by NPC transplantation studies, but confirmed to be valid also for endogenous NPCs as well, is that inflammation is in part responsible for the fate decision of newly formed NPCs.

Inflammation, as process occurring as a consequence of autoimmunity and/or traumatic and ischemic injuries, alter endogenous NPC proliferation and differentiation characteristics in a non-cell autonomous fashion (Pluchino et al., 2005). When inflammation fades away and neurodegeneration prevails, endogenous NPCs tend to differentiate into multiple neuronal lineages, depending on the situation, partially capable of integrating into damaged neuronal circuits (Kokaia et al., 2012).

However, in acute inflammatory conditions, while remaining undifferentiated, transplanted SVZ-derived NPCs might promote CNS tissue healing via the secretion of immunomodulatory

and neuroprotective molecules, capable of reducing detrimental tissue responses. Instead, in chronic inflammatory conditions NPCs seem to be driven toward cell replacement (Martino and Pluchino, 2006).

TRANSPLANTATION OF NPCs IN INFLAMMATORY CNS DISEASES

As underlined before, NPC replacement-based studies allow to investigate the multimodal—neurogenic vs. non-neurogenic functions—mechanism of action of endogenous NPCs (Pluchino et al., 2005).

Whatever the therapeutic action exerted, transplanted NPCs show a certain degree of pathotropism toward inflammatory foci. This is due to the fact that such cells constitutively express an armamentarium of chemokines and chemokine receptors (e.g., CCR1, CCR5, CXCR3 and CXCR4), cell adhesion molecules (e.g., CD44) (Rampon et al., 2008) and integrins (e.g., VLA4) (Campos et al., 2004, 2006; Leone et al., 2008). Transplanted NPCs, very similarly to endogenous NPCs, have the characteristic to be able to follow and reach chemoattractant foci both when intraparenchymally and/or systemically injected (Ji et al., 2004; Pluchino et al., 2005).

When transplanted in acute or chronic inflammatory diseases (e.g., stroke, SCI, or MS), the majority of NPCs survive close to perivascular inflammatory foci (i.e., the atypical ectopic niche) where they interact with many other cell types such as CNS-infiltrating blood-borne inflammatory cells, endothelial cells and CNS-resident astrocytes and microglia. Within these ectopic niches, inflammatory molecules [e.g., interferon (IFN)γ, tumor necrosis factor (TNFα)] inhibit NPCs differentiation by blocking their cell cycle by up regulating the expression of cell cycle dependent kinase inhibitors (Pluchino et al., 2008). As undifferentiated cells, NPCs can produce a wide array of both secreted and transmembrane molecules which, in turn, exert both immunomodulatory and neurotrophic factors (Irvin et al., 2004; Pluchino et al., 2005; Seifert et al., 2005; Martino and Pluchino, 2006; Bacigaluppi et al., 2009; Cusimano et al., 2012).

In relapsing-remitting experimental autoimmune encephalomyelitis (EAE), the experimental model of MS, intravenously (i.v.) transplanted NPCs promote the apoptosis of encephalitogenic T cells either via the expression of death receptor ligands (for example, FasL, Trail and Apo3L) or the production of soluble mediators—i.e., NO synthase (iNOS), IFNγ—involved in mitochondrial-mediated apoptosis (Einstein et al., 2003, 2006; Pluchino et al., 2005). In the post-acute phase of ischemic or haemorrhagic stroke, i.v. transplantation of NPCs reduced activation of macrophage/microglia cells and CNS recruitment of blood-borne inflammatory cells (Lee et al., 2008; Bacigaluppi et al., 2009). Similarly, in the immediate time points following SCI, intrathecally (i.c.) as well as intralesionally transplanted NPCs modulate the local T cell, the microglial response (Ziv et al., 2006) and the recruitment of CNS infiltrating classically activated pro-inflammatory macrophages (Cusimano et al., 2012). Interestingly, it has been recently shown that also embryonic like induced pluripotent stem cell (iPSC)-derived NPCs—once transplanted intrathecally into mice with EAE—protect oligodendrocytes and OPCs from cell death. This transplantation promotes myelin tissue reconstruction via the selective production of leukemia inhibiting factor (LIF), and this production is guided by the inflammatory microenvironment (Laterza et al., 2013).

NPCs are therefore able to prevent inflammation-induced neuronal programmed cell death and glial scar formation—occurring, for example, in EAE, SCI, stroke—mainly via the paracrine secretion of the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial-derived neurotrophic factor (GDNF) (Teng et al., 2002; Lu et al., 2003; Pluchino et al., 2003, 2005; Chu et al., 2004; Ryu et al., 2004; Ziv et al., 2006; Redmond et al., 2007; Bacigaluppi et al., 2009).

Another bystander effect exerted by transplanted NPCs is to directly modulate neuronal circuit plasticity (Zhang and Chopp, 2009). In an experimental model of ischemic stroke human foetal NPCs significantly improved functional outcomes by promoting neuronal dendritic arborization in both hemispheres and axonal projections within the corticostriatal and corticospinal pathways. These effects have been attributed to the capacity of transplanted NPCs to re-express developmental molecules such as guidance molecules (i.e., slit, thrombospondin 1 and 2) but also trophic factor such as VEGF (Andres et al., 2011).

CONCLUSIONS

NPCs in the adult brain exert an important homeostatic role either by producing new cells (neuronogenic or gliogenic function) or by orchestrating important processes (non-neurogenic functions): both actions are pivotal for the maintenance of the proper functioning of the CNS. Those neurogenic and non-neurogenic functions are in part NPC autonomous but are also driven by the microenvironment that might foster, according to the tissue needs, one of these functions. Our understanding of the complex interplay between neuronal, macroglial, and microglial cells in physiological and pathological conditions is continuously evolving, and we have now to consider NPCs as integral part of this interplay. New techniques of molecular biology and genetics will allow us to further understand the neurogenic vs. non-neurogenic functions of endogenous NPCs, and this knowledge would certainly help the scientific community to design efficacious stem cell-based treatment for still incurable neurological disorders.

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Endogenous stem cells for enhancing cognition in the diseased brain

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INTRODUCTION

Adult neural progenitor cells or neural stem cells (NSCs) persist in the adult human brain in two well-established regions, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus. Newborn neurons have been observed in the human SGZ in adults and contribute to specific forms of memory encoding at least in rodents (Braun and Jessberger, 2014). Neurogenesis from the adult SVZ was primarily identified in the olfactory bulb in rodents and was shown to stop early in life in humans despite the continuous presence of NSCs (Sanai et al., 2011). However, a recent study reports neurogenesis in the striatum from the adult human SVZ (Ernst et al., 2014). This finding highlights the difference between rodents and humans and the fact that some brain regions display an unexpected capacity for newborn neuron migration and survival. The SVZ is a prime region to consider for brain repair considering that it spans the entire cerebrum while the SGZ is limited to the hippocampus. Other regions are now known to contain NSCs or progenitor cells such as the hypothalamus, but this will not be discussed here. Several milestones need to be achieved prior to considering functional repair. These include, but may not be limited to: (1) Understanding the mechanisms leading to NSC quiescence and loss with aging. Several mechanisms are involved in the different regulatory steps of NSC self-renewal and loss. We will emphasize some of the mechanisms leading to NSC loss with aging. Once these mechanisms are identified, we should be able to amplify the pool of NSCs and direct their differentiation. (2) Identifying the molecules responsible for fate determination of NSCs and their daughter cells to generate glia or neurons of different types, including interneurons and long projection neurons. (3) Determining the inhibitory molecules that make the adult brain resistant to repair. Some repair has been reported in the cortex of rodents, but it is abortive possibly due to an unfriendly environment. (4) Finally, although we can genetically manipulate NSCs in rodents, it is a different issue in humans. Delivery systems need to be improved. Each of this point is further discussed below.

UNDERSTANDING THE MECHANISMS LEADING TO ADULT NSC QUIESCENCE AND LOSS WITH AGING

Significant amount of work has been focused on understanding the signaling molecules and pathways involved in the regulation of NSC quiescence (Basak and Taylor, 2009). For example, two major players are the Notch and Wnt signaling pathways. Most of the identified pathways are conserved between embryonic and adult NSCs although some molecular players may differ. They are also conserved across NSC niches including in the periphery (Fuchs et al., 2004). Although a large repertoire of molecules have been identified, some confusion remains regarding the true molecular identity of the dormant, quiescent and activated (i.e., proliferative) NSCs. The question why a dormant NSC becomes activated remains unanswered. In others terms, what are the molecules necessary and sufficient to wake up NSCs? Whether these molecules are the same for all NSCs remain unclear.

In addition, an injury to the brain may dramatically affect the activation state of molecular pathways in NSCs as well as their microenvironment. Very little is known on how NSCs respond to injury in terms of their molecular signature driving them out of dormancy. To make matter more complicated, every injury may not alter NSCs in similar fashion. These questions need to be fully examined.

Aging is a natural phenomenon affecting NSCs and their microenvironment (van and Franklin, 2013). One clear outcome of aging is a loss of NSCs and thus reduction in neurogenesis. The extent and mechanisms of NSC loss are likely different in the SGZ and NSC (Shruster et al., 2010). In the SGZ, NSCs terminally differentiate into astrocytes (Encinas et al., 2011). In the SVZ, there is a progressive loss of transit amplifying cells with aging (Paliouras et al., 2012). At the molecular level, one key player in aging is the mammalian target of rapamycin complex 1 (mTORC1) pathway, which controls cap-dependent protein translation (Johnson et al., 2013). There are no data for mTORC1 contribution to aging in the SGZ. In the SVZ, mTORC1 has been involved in the loss of NSC with aging (Paliouras et al., 2012). In addition, activation of this pathway during aging has been proposed to be involved in the terminal differentiation of NSCs into daughter cells, thus contributing to NSC loss (Hartman et al., 2013). Thus, whether small amount of the mTORC1 blocker, rapamycin, could prevent progressive NSC loss is to be examined. mTORC1 activity increases with aging in other systems and rapamycin has been shown to prolong life of animals.

IDENTIFYING THE MOLECULAR MECHANISMS CONTROLLING FATE DETERMINATION TO GENERATE EVERY NEURON TYPE

Adult NSCs essentially generate neurons, most specifically interneurons. In the SVZ, they were shown to be genetically predetermined to generate specific subclasses of olfactory interneurons (Merkle et al., 2007). The genetic network involved in this specificity remains to be identified. In addition, identifying the molecular program determining this fate restricted genetic network needs to be examined earlier during development. In other terms, we will learn from studies focused on the identification of the programs at play in embryonic NSCs that determine their restricted fate once adult NSCs.

With brain repair in mind, there is a need to generate long range projection neurons and not just interneurons. Progress needs to be made in our abilities to reprogram adult NSCs to resemble embryonic NSCs with broader fate determination. Novel reprogramming technologies are developed exponentially in line with the discovery of induced pluripotent stem cells. This technologies need to be applied to adult NSCs *in vitro* and *in vivo*.

DETERMINING THE INHIBITORY MOLECULES MAKING THE ADULT BRAIN RESISTANT TO REPAIR

Perhaps as a protective mechanism for maintaining long-term memory, the adult brain displays very limited repair despite the presence of NSCs in the human SVZ and the reported existence of cells with neural progenitor property in the parenchyma. The adult brain contains repulsive cues (e.g., NOGO and similar factors) and lack nurturing cues for NSC and newborn neurons to survive and integrate, thus providing a non-permissive environment for neurogenesis. There is a definite need for studies aimed at identifying the repulsive cues present in the adult parenchyma preventing NSC and immature neuron survival and integration in normal and injury conditions. In addition, it will be important to compare the molecular signature of different brain regions that display different permissiveness to repair such as the striatum versus the cortex (Ernst et al., 2014). Large molecular screens as well as hypothesis-driven approaches could be taken to address this issue.

One remaining major limitation is access to human brain tissue. The human brain is likely more resistant to repair than the mouse brain. Efforts to develop approaches to perform screen in human tissue and identify factors preventing endogenous neurogenesis need to be developed.

IN VIVO THERAPEUTIC MANIPULATIONS

Even if we address all of the issues listed above, we are left with the task of developing strategies to manipulate cells in the human brains in the least invasive way possible. So far, the best strategy has been to use pharmacological treatments in humans. However, reprogramming NSCs to generate new types of neurons will require transferring DNA or genetic material into cells. In the rodent brain, such strategy is achieved in specific cell types using transgenic animals, viral delivery, electroporation, nanoparticle or exosomal delivery systems, and to some extent cell-penetrating peptides. Nanoparticle and exosomal strategies are promising for humans. In particular, non-invasive intra-nasal delivery is attractive and should be further explored. For success in developing *in vivo* delivery systems in humans, biologists and bioengineers will need to exchange concepts and work together.

CONCLUSION

Despite the hurdles outlined above and the length of time that will be required for achieving brain repair and cognitive enhancement, we cannot fail to pursue our investigations of the four fields outlined above. In the past decade, there has been an exponential increase in studies related to the field of "adult neurogenesis." This is outlined in **Figure 1** following a search in PubMed with this keyword compared to a search with "long term potentiation," which outlines the growth of the neuroscience field. Although there is an apparent stabilization of the growth of the adult neurogenesis field since 2011, this likely reflects the expansion of the field and thus the need to use additional keywords in our search such as adult NSC, repair in the adult brain, adult SVZ or SGZ. This is my hope and belief that despite economic recession affecting scientific growth the number of labs studying adult neurogenesis and NSCs is still increasing.

Overall, the present energetic study of stem cell biology and brain delivery systems will provide a better understanding of brain development, endogenous responses to injuries, and additional therapeutic approaches for brain repair, and hold great promise for broadening the

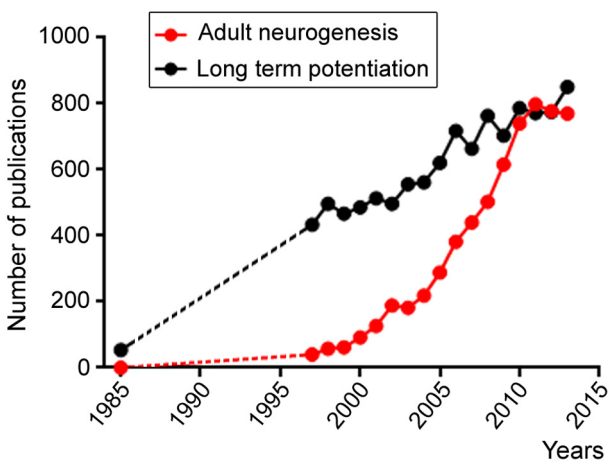

FIGURE 1 | Scattered plots illustrating the number of publications per year obtained in Pubmed using the following two key words: adult neurogenesis (red) and long term potentiation. Both fields display increases over time, but the adult neurogenesis field displays an exponential increase starting in the late 1990's while the long term potential field displays a linear increase likely reflecting the increase in the number of scientists during this period.

therapeutic options available for maintaining and restoring cognition following brain injury and during neurodegenerative diseases. Finally, validating that findings obtained in animal models apply to humans is a must. While some molecules may be the same, the pathways may be different or act differently and this needs to be validated in human adult NSCs prior to trying to repair the human brain.

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NG2 cells (polydendrocytes) in brain physiology and repair

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NG2 cells, also referred to as oligodendrocyte precursor cells (OPCs) or polydendrocytes, represent a major resident glial cell population that is distinct from mature astrocytes, oligodendrocytes, microglia, and neural stem cells and exist throughout the gray and white matter of the developing and mature central nervous system (CNS). While their most established fate is the oligodendrocyte, they retain lineage plasticity in an age- and region-specific manner. During development, they contribute to 36% of protoplasmic astrocytes in the ventral forebrain. Despite intense investigation on the neuronal fate of NG2 cells, there is no definitive evidence that they contribute substantially to the neuronal population. NG2 cells have attributes that suggest that they have functions other than to generate oligodendrocytes, but their exact role in the neural network remains unknown. Under pathological states, NG2 cells not only contribute to myelin repair, but they become activated in response to a wide variety of insults and could play a primary role in pathogenesis.

Keywords: NG2, polydendrocyte, oligodendrocyte, myelin, demyelination, cell fate, subventricular zone

INTRODUCTION

NG2 cells represent a resident glial progenitor cell population that exists throughout the gray and white matter of the developing and mature mammalian central nervous system (CNS) and are distinct from astrocytes, mature oligodendrocytes, microglia, and neural stem cells (reviewed in Nishiyama et al., 2009; Hill and Nishiyama, 2014). Their widespread existence in the CNS began to be recognized in the 1990s by immunohistochemical labeling for NG2 and the alpha receptor for platelet-derived growth factor (Pdgfra). Currently, NG2 cells are considered as the fourth major glial cell type in the CNS, comprising 2–8% of all the cells in the adult CNS (Dawson et al., 2003; Peters, 2004). These cells are often equated with oligodendrocyte precursor cells (OPCs) because of their ability to generate myelinating and non-myelinating oligodendrocytes. However, not all NG2 cells differentiate into oligodendrocytes, and oligodendrocytes are not their only fate, as discussed below. Different names have been used to refer to these cells. The term OPCs is used when discussing their role in oligodendrocyte production, while the terms "NG2 cells" and "NG2 glia" are used when discussing their property that is not directly related to their OPC role, even though NG2, which is also expressed on vascular pericytes, is not an absolute marker for these cells. To avoid using different names to refer to the same cells in different biological contexts, the word "polydendrocytes" has been suggested as a unified name for these cells, in keeping with the names of other types of glia that are loosely associated with their morphology. This perspective article will discuss recent findings and unsolved questions related to the astrocyte and neuronal fate of NG2 cells and their role in brain pathophysiology, primarily in the rodent CNS.

THE FATE OF NG2 CELLS

NG2 cells expand their population by extensive self-renewal. After their peak proliferation during the perinatal period, they retain their proliferative ability throughout life (**Figure 1**).

OLIGODENDROCYTE FATE

The general consensus from a series of Cre-loxP-mediated genetic fate mapping studies is that under normal physiological conditions, NG2 cells in the adult CNS generate oligodendrocytes as they continue to self renew (**Figure 1**) (Dimou et al., 2008; Rivers et al., 2008; Kang et al., 2010; Zhu et al., 2011; Young et al., 2013). It is unlikely, however, that every NG2 cell will differentiate into an oligodendrocyte at some point during the life of the animal, as their uniform distribution does not parallel the distribution of oligodendrocytes (Dawson et al., 2000; Tomassy et al., 2014). It remains to be explored whether all NG2 cells are equivalent in their ability to generate oligodendrocytes or whether there is a subpopulation that is fated to permanently remain as NG2 cells.

ASTROCYTE FATE AND LINEAGE PLASTICITY OF NG2 CELLS

During development, the fate of NG2 cells is not restricted to oligodendrocytes. NG2 cells contribute to 40% of the protoplasmic astrocytes in the gray matter of the ventral forebrain (Zhu et al., 2008, 2011; Huang et al., 2014). The magnitude and the temporal and spatial distribution of protoplasmic astrocytes observed in these studies are quite distinct from the other observations where a small number (1∼5%) of sporadically distributed reporter+ astrocytes were seen from Olig2-creER and Pdgfra-CreER fate mapping in adult (Dimou et al., 2008; Tripathi et al., 2010). Two independently generated tamoxifen-inducible NG2-creER transgenic mouse lines (BAC transgenic

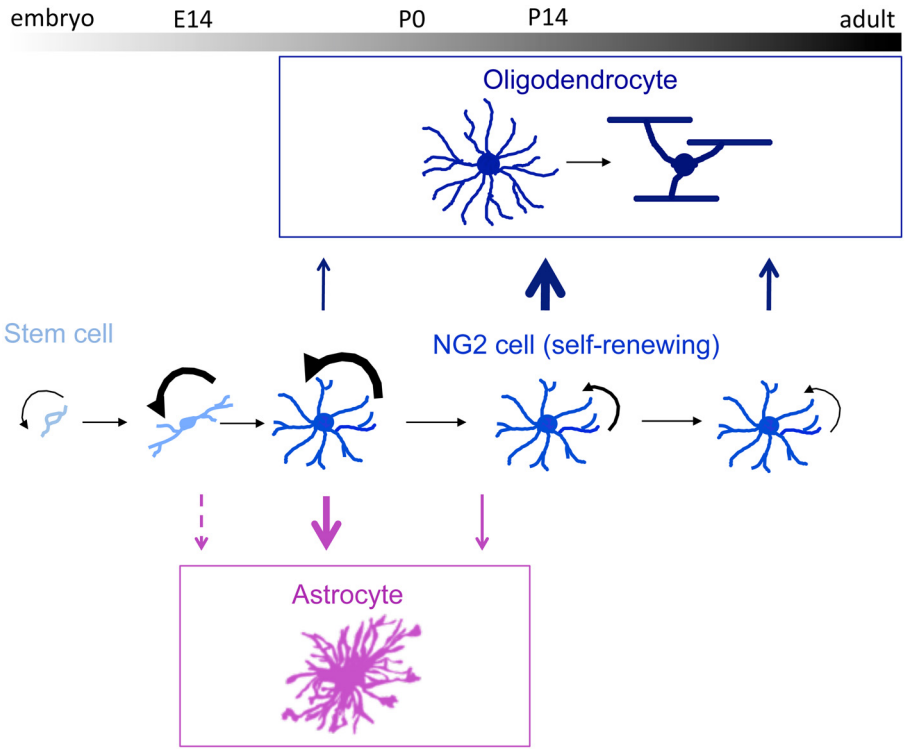

FIGURE 1 | A schematic showing the fate of NG2 cells throughout development. The middle diagram depicts self-renewing NG2 cells, with a chronological scale across the top. Black arrows indicate the rate of proliferation, which is greatest perinatally. Blue upward arrows indicate oligodendrocyte differentiation, peaking during the third postnatal week. Purple downward arrows indicate astrocyte differentiation, which occurs predominantly before birth and gradually ceases shortly after birth. The thickness of the arrows denotes the extent of differentiation. Modified from Nishiyama (2007).

and knock-in) indicate that astrocyte generation from NG2 cells is most robust prenatally and tapers off during the first postnatal week (**Figure 1**) (Zhu et al., 2011; Huang et al., 2014), consistent with the chronology of astrocyte development. This suggests that the astrocyte fate of NG2 cells is a physiological developmental function and not due to radial glial expression of NG2 as suggested in Richardson et al. (2011). None of the other fate mapping studies had attempted to induce Cre prenatally. The reason why NG2 cells lose their astrogliogenic ability shortly after birth could be due to a density-dependent mechanism that regulates astrocyte production (Nakatsuji and Miller, 2001; Zhu et al., 2012).

Are NG2 cells that do not generate astrocytes during early development permanently committed to generating oligodendrocytes? The following observation suggests that under normal conditions, they are restricted to the oligodendrocyte lineage, but that they retain the ability to become astrocytes under certain conditions. When the oligodendrocyte transcription factor Olig2 that is required for NG2 cell specification (Rowitch, 2004; Richardson et al., 2006), was deleted in all NG2 cells, there was a complete fate switch from oligodendrocytes to astrocytes in the neocortex but not in the ventral forebrain, resulting in severe hypomyelination (Zhu et al., 2012). When Olig2 was deleted in early postnatal NG2 cells, only 50% of the Olig2-deleted neocortical NG2 cells switched their fate to astrocytes (Zhu et al., 2012). In the adult, deletion of Olig2 did not convert them into astrocytes, even in response to a stab wound (Komitova et al., 2011), nor was there increased astrocyte generation from NG2 cells in Olig2-creER heterozygous mice (Dimou et al., 2008), although the former study had used an inefficient Cre reporter. Thus, lineage restriction of NG2 cells appears to occur gradually during the first few postnatal weeks. Even after oligodendrocyte specification has occurred during embryogenesis, NG2 cells in certain regions retain some degree of context-dependent lineage plasticity, which is gradually lost in later postnatal life.

NEURONAL FATE OF NG2 CELLS

The neuronal fate of NG2 cells has been one of the most highly debated topics, and Cre-loxP-mediated genetic fate mapping studies have produced inconsistent findings. For example, two studies using Pdgfra-CreER or PLP (proteolipid protein)-CreER transgenic mouse lines observed reporter+ neurons in the piriform cortex (Rivers et al., 2008; Guo et al., 2010), while a subsequent study using an independent line of Pdgfra-CreER mice did not find any evidence for a neuronal fate (Kang et al., 2010). Earlier studies using NG2- and Olig2-Cre driver mice showed no evidence for neurogenesis (Dimou et al., 2008; Zhu et al., 2008; Komitova et al., 2009; Zhu et al., 2011), while a recent study using the same NG2-creER mice showed a few reporter+ neurons in the hypothalamus (Robins et al., 2013). What is the significance

of detecting reporter+ neurons in these fate mapping studies? Is the extent of neurogenesis from NG2 cells sufficiently large to bring about a physiological effect in the neural network? The findings must be interpreted in proper context without overemphasizing observations where sporadic reporter+ neurons are found.

The following example illustrates one of the caveats of the Cre-loxP technology that spurious transient activation of Cre in an unrelated cell could lead to reporter expression in that cell in the absence of lineage progression. In NG2-cre:zeg mice generated by crossing constitutively active NG2-cre mice (Zhu et al., 2008) to the zeg reporter mice (Novak et al., 2000), a significant number of reporter+ neurons appeared in the neocortex after P45 but not at P14 (**Figures 2A,B**). To determine whether reporter+ neurons arose as a result of lineage progression from NG2 cells or due to direct Cre expression in neurons, the zeg reporter plasmid was in utero electroporated directly into neuronal precursors of NG2-cre single transgenic mice at E13.5, and the appearance of reporter+ neurons was examined (**Figure 2C**). The plasmid would be retained in neurons that are undergoing their last cell division and lost from glial cells as they undergo multiple divisions (Bai et al., 2003). The expression of the reporter in neurons would suggest direct Cre activation in neurons. A DsRed plasmid was co-electroporated to mark the transfected cells. When the electroporated mice were sacrificed at P70, all the DsRed+ neurons also expressed EGFP (**Figures 2D–F**), and no NG2 cells expressed EGFP or DsRed. This suggests that there was transient Cre (and possibly NG2) expression in neurons, and that the duration of Cre expression was sufficient for Cre-mediated recombination to allow EGFP expression from the zeg plasmid but not sufficiently long-lasting to be detected by Cre immunohistochemistry or in situ hybridization. It is possible that certain physiological conditions cause a spike in NG2 transcription, which is too transient to be detected in NG2-DsRed transgenic mice (Zhu et al., 2008). Furthermore, no transitional forms between NG2 cells and neurons could be observed, unlike the case for NG2 cells transitioning into astrocytes (Zhu et al., 2012) or oligodendrocytes (**Figure 2G**).

In the new NG2 cell fate mapping study using the NG2creER knockin mice crossed to ROSA-tdTomato reporter, Huang et al. (2014) observed reporter+ neurons with electrophysiological and morphological properties of neurons in the non-neuronogenic regions of the forebrain. By contrast, very few reporter+ neurons were found when the same mice were crossed to the less efficient RSOA-YFP reporter. Since these neurons appeared without evidence of proliferation, it is unlikely that they arose from NG2 cells, as previously reported (Clarke et al., 2012), but rather by some form of Cre-dependent DNA recombination that had occurred in neuronal cells. The Huang study also highlights the different outcomes of studies using Cre reporter lines with different efficiencies. Development of a novel, Cre-independent method is needed to resolve the question of the neuronal fate of NG2 cells.

RELATIONSHIP OF NG2 CELLS TO NEURAL STEM CELLS

The adult SVZ consists of a heterogeneous population including GFAP+ neural stem cells (type B cells), transit-amplifying cells (type C cells), and neuroblasts that migrate to the olfactory bulb via the rostral migratory stream (type A cells) (Gonzalez-Perez and Alvarez-Buylla, 2011). Many early studies were focused on testing the then attractive hypothesis that NG2 cells corresponded to multipotential neural stem cells in the SVZ (Nunes et al., 2003; Aguirre and Gallo, 2004), based on the observation that they could be induced to differentiate into astrocytes and neurons under certain culture conditions (Roy et al., 1999; Kondo and Raff, 2000). However, further examination of NG2 cells and the SVZ revealed that NG2 cells comprise a minority of cells, located mostly at the periphery of the SVZ, and are distinct from the Dlx2-expressing type C cells or neuroblasts that express Doublecortin (Komitova et al., 2009; Platel et al., 2009; Richardson et al., 2011). These studies also showed that NG2 cells are distinct from GFAP+ neural stem cells (type B cells) (Rivers et al., 2008; Komitova et al., 2009; Chojnacki et al., 2011), in contrast to an earlier study that showed expression of Pdgfra on type B cells (Jackson et al., 2006). Neural stem cells do generate NG2 cells, but this fate of neural stem cells seems to be a minor fate compared with their neurogenic fate and is highly region-specific. Interestingly, a recent real-time imaging study of the fate of single cells unequivocally demonstrated that neural stem cell clones that generate NG2 cells do not generate neurons and are primarily found in the dorsal SVZ, while those that generate neurons are more enriched in the lateral SVZ and do not generate NG2 cells (Ortega et al., 2013). Thus, there appears to be an early segregation of neuronal and oligodendrocyte lineages in the SVZ. Under normal conditions, only SVZ type C cells, but not NG2 cells, proliferate in response to epidermal growth factor (EGF) (Doetsch et al., 2002; Hill et al., 2013). However, under pathological conditions such as EGF overexpression or demyelination, EGF can redirect SVZ type C cells to become NG2 cells (Aguirre et al., 2007; Ivkovic et al., 2008; Jablonska et al., 2010; Galvez-Contreras et al., 2013). These observations can be explained if EGF receptor becomes upregulated on a small population of cells that are in transit from SVZ type C cells to becoming NG2 cells.

THE ROLE OF NG2 CELLS IN THE NORMAL CNS

Why has the mammalian brain evolved to maintain such a uniformly distributed glial cell type? Recent studies have revealed that new oligodendrocytes and myelin continue to be produced in the mature CNS (Zhu et al., 2011; Young et al., 2013) and a significant amount of activity-dependent myelin plasticity occurs in the adult (Zatorre et al., 2012; Hill and Nishiyama, 2014). NG2 cells also generate non-myelinating perineuronal oligodendrocytes whose somata lie apposed to neuronal somata (Penfield, 1924). Although the role of the perineuronal oligodendrocytes is not clear, they can produce myelin in response to demyelination (Ludwin, 1979) and could be providing neurotrophic and metabolic support for neurons (Taniike et al., 2002; Fünfschilling et al., 2012; Lee et al., 2012).

NG2 cells are evenly distributed to cover the entire mature CNS parenchyma (Dawson et al., 2000). *In vivo* imaging in 2–3-month-old neocortex revealed non-overlapping territories occupied by adjacent NG2 cells, and their processes appeared to be contact-inhibited (Hughes et al., 2013). Another study using fixed hippocampi from 3–4-week-old rats showed that NG2 cells

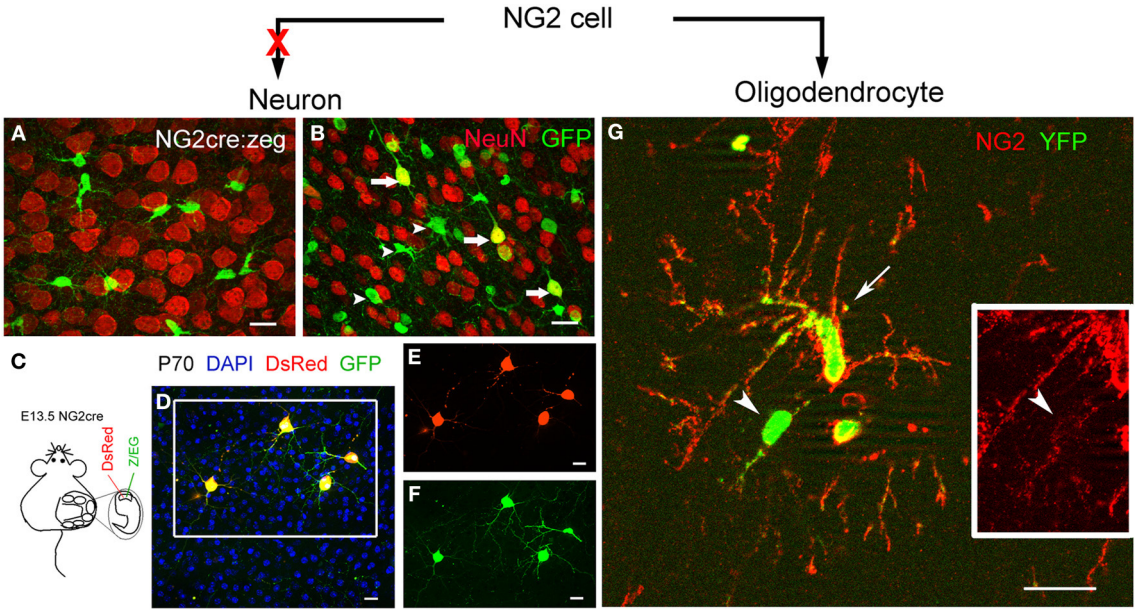

FIGURE 2 | Lineage-dependent and independent reporter expression in Cre-loxP fate mapping. (A,B) Double labeling for GFP and the neuronal marker NeuN in the neocortex of NG2cre:zeg mice at P14 **(A)** and P70 **(B)**. NeuN+ GFP+ cells with mature neuronal morphology (arrows) are seen as well as NeuN- GFP+ NG2 cells (arrowheads) in P70 but not in P14 cortex. **(C–F)** Transfection of zeg reporter plasmid into neuronal precursor cells in NG2cre single transgenic mice. **(C)** Scheme showing co-transfection of DsRed and zeg plasmid DNA into the lateral ventricles of E13.5 NG2cre single transgenic mice by in utero electroporation and positioning the electrodes to target the dorsal pallium. **(D–F)** GFP expression in electroporated neurons at P70. All the GFP+ cells were neurons that had been electroporated (DsRed+), and no GFP+ glial cells were detected. This experiment demonstrates that Cre is activated directly in neocortical neurons of NG2cre mice at some point between P14 and P70, leading to GFP expression in neurons. Unlike the case for oligodendrocytes shown in G, there were no GFP+ cells that appeared to be in transition from NG2 cells to neurons. **(G)** Corpus callosum from P70 NG2creER:YFP mice 14 days after Cre induction with 4-hydroxytamoxifen. A cell that appears to be transitioning from an NG2 cell (NG2+ YFP+) into an NG2- oligodendrocyte (NG2- YFP+) with weak NG2 immunoreactivity in the processes (arrowheads) is seen next to a typical NG2 cell that robustly expresses NG2 (arrow). Inset shows single labeling of the cell marked by arrowhead for NG2.

were tiled but shared approximately 5% of the volume with adjacent NG2 cells (Xu et al., 2013). It is not clear whether the extent of overlap between processes of neighboring NG2 cells changes as the brain matures. Regardless, the uniform distribution of NG2 cells would suggest a yet uncovered homeostatic role in the CNS.

NG2 cells interact uniquely with neurons in that they depolarize in response to receiving direct synaptic input from neuronal axons (Bergles et al., 2000). However, the extent of depolarizations is not sufficient to elicit repetitive action potentials, and thus NG2 cells are still considered as non-excitable glial cells. While the physiological consequences and significance of neuron-NG2 cell synapses remain unknown, and the nature of neuron-NG2 cell communication changes with age and differentiation (Maldonado and Angulo, 2014), it is likely that local increases in intracellular calcium play an important role in mediating downstream cellular effects (Bergles et al., 2000; Ge et al., 2006; Hamilton et al., 2010; Haberlandt et al., 2011).

THE ROLE OF NG2 CELLS IN PATHOLOGY

REPAIR OF DEMYELINATING LESIONS

It is well established that NG2 cells proliferate and differentiate into myelinating oligodendrocytes and repair demyelinated lesions (Di Bello et al., 1999; Watanabe et al., 2002; Tripathi et al., 2010). It still remains to be shown whether replenishment of the NG2 cell population can be a cause for remyelination failure under certain conditions. While repeated acutely demyelinated lesions undergo successful remyelination (Penderis et al., 2003), other studies suggest that NG2 cells can become depleted after acute demyelination (Keirstead et al., 1998) and their repopulation may not occur fast enough to meet the demands of chronic ongoing demyelination (Mason et al., 2004). Recruitment of new NG2 cells could occur by proliferation of local NG2 cells and/or migration and differentiation of cells from the SVZ (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Etxeberria et al., 2010; Tepavcevic et al., 2011). However, evidence is not yet strong that these SVZ-derived cells are capable of fully differentiating into remyelinating cells to the extent that local NG2 cells are.

ACTIVATION OF NG2 CELLS IN OTHER TYPES OF LESIONS

NG2 cells undergo increased proliferation and dramatic morphological changes in response to a wide variety of acute CNS insults besides demyelination, including spinal cord injury (McTigue et al., 2001; Jones et al., 2002), ischemia (Zhang et al., 2013), excitotoxic injury (Bu et al., 2001; Wennström et al., 2004), and viral infection (Levine et al., 1998). The time course of their "activation" and their "reactive morphology" or the extent of proliferation varies depending on the nature of the insult, but the functional significance for these diverse morphological and

proliferative changes is not known. For example, it is not known whether the shorter, thicker processes reflect increased uptake of extracellular fluid/ions or increased phagocytic activity. Nor is it known whether the increased number of thin, elongated process after viral infection reflect a search for something or deregulated cytoskeleton. *In vivo* imaging has revealed that NG2 cell processes are highly dynamic (Hughes et al., 2013; Hill et al., under revision), but it is not known what they are seeking besides axons to myelinate.

In most cases of acute injury, NG2 cell responses occur early, within 24 h (Watanabe et al., 2002; Horky et al., 2006; Simon et al., 2011), which is similar to or slightly lags behind the time course of microglial response and a few days before reactive astrogliosis becomes apparent. Some forms of insult such as excitotoxic injury seem to elicit a greater microglial response than NG2 cell response. NG2 cells exhibit a close spatial relation to astrocyte processes and microglial somata (Nishiyama et al., 2002; Hamilton et al., 2010; Xu et al., 2013), and the latter becomes more pronounced in response to injury (Nishiyama et al., 1997; Bu et al., 2001; Wu et al., 2010). Future studies can be directed to studying how the three types of reactive glia signal to each other to achieve a concerted response specifically tailored to each type of injury.

NG2 CELLS IN PATHOGENESIS

The inherent ability of NG2 cells to remain in cell cycle through life also makes them susceptible to neoplastic transformation. Although the cell of origin of glioblastoma multiforme continues to be debated, cell fate mapping of neural stem cells or NG2 cells with deletions in p53 and NF1 genes revealed that neoplastic changes begin to occur in NG2 cells and not in neural stem cells (Liu et al., 2011). Intriguingly, early proliferative foci arise in perineuronal locations in gray matter rather than in white matter tracts where glioma cells are known to expand and disseminate, suggesting a proliferative paracrine signal imparted by neurons.

Several recent studies have shown that metabolic defects in oligodendrocytes can precede neurodegeneration in amyotrophic lateral sclerosis (ALS) (Lee et al., 2012; Kang et al., 2013; Philips et al., 2013), strongly suggesting a pathogenic role for oligodendrocytes. In addition, a direct pathogenic role for NG2 cells has been shown at the neurovascular interface. In a cerebral hypoperfusion model, metalloproteinase-9 (MMP-9) is secreted from NG2 cells in the vicinity of vascular endothelial cells, leading to degradation of the endothelial tight junction protein ZO-1 and breakdown of the blood-brain barrier (Seo et al., 2013). These findings highlight the importance of the oligovascular niche in normal and pathological conditions that could be an important topic of future investigations (Maki et al., 2013; Miyamoto et al., 2014).

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Beyond cell replacement: unresolved roles of NG2-expressing progenitors

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NG2-expressing parenchymal precursors (NG2+p) serve as primary source of myelinating oligodendrocytes in both the developing and adult Central Nervous System (CNS). However, their abundance, limited differentiation potential at adult stages along with stereotypic reaction to injury independent of the extent of myelin loss suggest that NG2+p exert functions additional to myelin production. In support of this view, NG2+p express a complex battery of molecules known to exert neuromodulatory and neuroprotective functions. Further, they establish intimate physical associations with the other CNS cell types, receive functional synaptic contacts and possess ion channels apt to constantly sense the electrical activity of surrounding neurons. These latter features could endow NG2+p with the capability to affect neuronal functions with potential homeostatic outcomes. Here we summarize and discuss current evidence favoring the view that NG2+p can participate in circuit formation, modulate neuronal activity and survival in the healthy and injured CNS, and propose perspectives for studies that may complete our understanding of NG2+p roles in physiology and pathology.

Keywords: buffering, depolarization, myelin, neuroprotection, neuromodulation

INTRODUCTION

During Central Nervous System (CNS) ontogenesis, myelinating oligodendrocytes originate from highly ramified neural precursors expressing the platelet-derived growth factor alpha receptor (PDGFRa) and the NG2 chondroitin sulfate proteoglycan (Zhu et al., 2008a,b). These precursors persist in the adult CNS parenchyma, where constitute the main proliferative cell type and make up about 5% of all CNS cells (Dawson et al., 2003). At adult stages, they can engage into maturation to sustain a certain degree of basal myelin turnover and plasticity (Wang and Young, 2013; Young et al., 2013) and are rapidly mobilized to replace oligodendrocytes in demyelination (Redwine and Armstrong, 1998; Zawadzka et al., 2010). Herein we will refer to these cells as NG2-expressing precursors (NG2+p), although NG2 is also expressed by pericytes of the vasculature (Stallcup, 2002) and reported in astrocyte subsets (Matthias et al., 2003).

Currently, the only unequivocally established functions of NG2+p are to regenerate themselves and produce oligodendrocytes in the healthy, diseased and aged CNS (Zhu et al., 2008a,b; Kang et al., 2010; Tripathi et al., 2010; Young et al., 2013). However, the persistence of a large pool of quiescent (i.e., neither engaged in proliferation nor maturation) NG2+p with limited differentiation potential at adult ages has suggested that these cells do not only represent a transitional stage along the oligodendroglial lineage, but rather a novel type of glia endowed with specific properties and functions (Nishiyama et al., 2002). Consistently, NG2+p appear uniformly distributed in the gray and white matter and provide a stereotypic reaction to injury independently of the extent of myelin loss, suggesting that they may play roles additional to myelin production. Along this line, it has been proposed that NG2+p may be multipotent progenitors endowed with the ability to generate astrocytes and neurons in defined conditions. However, to date consensus is established only for the generation of astrocyte subsets at perinatal stages (Rivers et al., 2008; Zhu et al., 2008a,b; Huang et al., 2014). Here we will overview and discuss features potentially related to surveillance, neuromodulation and neuroprotection that still render NG2+p very enigmatic and prompt further investigations on this type of glia.

NG2+p ANATOMICAL RELATIONSHIP WITH CNS CELLS AND PARACRINE INTERACTIONS

In both human and rodent CNS, NG2+p appear distributed rather homogeneously in gray and white matter areas, with no correlation between their density and that of myelin (Butt et al., 2005; Staugaitis and Trapp, 2009). NG2+p processes extend tridimensionally to cover non-overlapping fields that are likely maintained by homotypic repulsive mechanisms (Hughes et al., 2013). Accordingly, in the intact tissue contacts among NG2+p processes are rarely observed (Hughes et al., 2013) and, despite some NG2+p express connexin 32 (Melanson-Drapeau et al., 2003), cells are never coupled via gap-junctions (Wallraff et al., 2004; Butt et al., 2005). Thus, at variance with astrocytes, NG2+p do not function as a syncythium, but are rather individual functional units. However, they partly couple to mature oligodendrocytes, indicating some privileged communication with other elements in the lineage (Maglione et al., 2010).

Confocal and electron microscopy analyses showed that NG2+p establish intimate anatomical and functional contacts with other CNS cells. NG2+p processes form multiple contacts with dendrites and axons, and NG2+p arborizations intertwine amongst and can encapsulate neuronal somata (Wigley and Butt,

2009; see also **Figures 1A,A′**) in ways suggestive of their participation in perineuronal nets (Butt et al., 2005). Electron microscopy further revealed that NG2+p processes make contacts with the axonal membrane at the paranodes and nodes of Ranvier (Butt et al., 1999), and interdigitate between pre- and post-synaptic neuronal elements (Ong and Levine, 1999). Contacts with axons include also functional neuron-to-NG2+p synapses (see below). Of note, tight NG2+p-neuron associations exist also in the CNS of adult non-mammalian vertebrates (i.e., zebrafish; März et al., 2010), in line with a fundamental mechanism of communication conserved through species.

Intimate physical interactions also occur with astrocytes. Both cell types often contact the same neurons/axonal terminals (Wigley and Butt, 2009) but NG2+p never ensheat neuronal synapses as astrocytes do. Interestingly, areas of immediate apposition in NG2+p and astrocyte processes are sites of communication, where astrocyte-derived signals induce Ca^{2+} transients in NG2+p (Hamilton et al., 2010). At those sites synaptophysin-positive clusters indicative of secretory vesicle accumulation were observed in NG2+p, pointing to potential secretory spots (Wigley and Butt, 2009). NG2+p also make direct contacts with microglia (Nishiyama et al., 1997), pericytes and myelin (Butt et al., 2005). Hence, NG2+p connect to distinct cell types and functionally-relevant cellular domains, suggesting that they may actively sense and integrate information from diverse sources. An additional level of integration occurs through paracrine signals produced by neighboring cells including neurons, astrocytes and microglia, and influences NG2+p during developmental myelination and in pathology (see Clemente et al., 2013 for review). Is the output of this integration (see below) limited to the regulation of NG2+p differentiation or survival? It is surprising that NG2+p are generally considered only as a sink and not as a source of signals. Hence, if and how NG2+p affect surrounding cells remains essentially unknown.

PRIVILEGED CONTACTS WITH NEURONS

What makes NG2+p unique amongst other glial cells is their connection with neurons through synapses that sense neuronal activity at the quantal level with high temporal and spatial resolution. These contacts emerge in parallel with neuronal synaptogenesis and appear ubiquitary, being present in all regions examined so far (cerebellar and cerebral cortex, Lin et al., 2005; Kukley et al., 2008; Ge et al., 2009; Tanaka et al., 2009; Vélez-Fort et al., 2010; hippocampus, Lin and Bergles, 2004; Mangin et al., 2008; brain stem, Müller et al., 2009; white matter tracts, Kukley et al., 2007; Ziskin et al., 2007; Káradóttir et al., 2008; De Biase et al., 2010; Etxeberria et al., 2010). Synapses include glutamatergic and Gamma-Aminobutyric Acid (GABA)-ergic inputs, and both produce depolarizations. Full functionality of these contacts *in vivo* is attested by recordings of evoked, spontaneous, and miniature currents both in physiology and during remyelination (Etxeberria et al., 2010; Vélez-Fort et al., 2010). Glutamatergic contacts are lost as NG2+p progress in differentiation (De Biase et al., 2010; Kukley et al., 2010), in line with a role in the regulation of the cell cycle or of functions specific of the progenitor stage. Notably, glutamatergic inputs increase in frequency and amplitude in NG2+p cells during CNS maturation (Mangin et al., 2008), whereas in the cerebral cortex GABAergic signaling shifts from activation of synaptic receptors to indirect activation of extrasynaptic channels through spillover (Vélez-Fort et al., 2010; Balia et al., 2013; Passlick et al., 2013).

Glutamate- and GABA-induced depolarizations in NG2+p are overall modest in amplitude with the notable exception of cerebellar climbing fiber inputs that induce relevant potential variations (Lin et al., 2005). Thus, to affect the cell physiology, a number of convergent inputs from diverse synapses likely require be integrated. Transduction of synaptic signal may also rely on calcium-mediated mechanisms such as calcium entry though permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

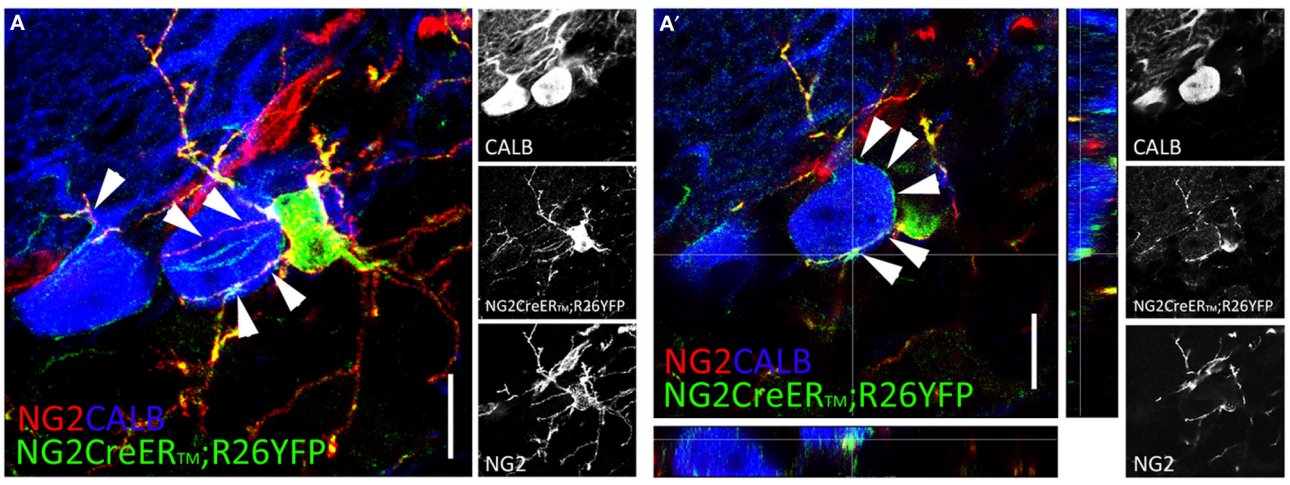

FIGURE 1 | NG2+p distribution in the adult cerebellum. (A,A′) Two weeks after tamoxifen induction, multiprocessed cells identified by NG2 (red), and YFP (green) expression appear closely associated to calbindin+ (blue) Purkinje cells in adult NG2CreER™; R26YFP mouse cerebellum. NG2+p arborizations envelop Purkinje cell somata and dendrites [arrowheads in **(A,A′)**]. **(A)** Confocal stack comprising 20 optical section 1 mm thick. **(A′)** Single confocal plane. Scale bars: 10 μm.

(AMPA) or N-methyl-D-aspartate (NMDA) receptors, and activation of voltage dependent conductances that provide signal amplification and can trigger calcium transients from intracellular stores. Notably, in the hippocampus neuron-to-NG2+p synapses undergo activity-dependent modifications analogous to long term potentiation (LTP) at excitatory synapses (Ge et al., 2006), showing that these contacts possess the machinery to sustain plastic changes. Moreover, glutamate or GABA evoked signals can be integrated intracellularly with responses to other mediators such as adenosine triphosphate (ATP), which, upon release by both axons and astrocytes, triggers calcium currents through P2Y and P2X receptors (Hamilton et al., 2010). Yet, depolarizations and calcium transients are mostly described as very local events that take place at the cell processes, where synapses are mostly found, and could therefore influence spatially restricted functions such as local protein synthesis, motility, or secretion (see also above, Kirby et al., 2006; Tanaka et al., 2009; Haberlandt et al., 2011; Wake et al., 2011; Hughes et al., 2013).

What is the functional significance of neuronal inputs? Since NG2+p do not appear able to transmit electrical signals to other cells, information derived from neuronal activity is likely to instruct functions specific to these progenitors. Several reports showed that neurotransmitters can affect proliferation and migration of NG2+p *in vitro* (Luyt et al., 2007; Gallo et al., 2008; Tong et al., 2009). Other studies related alterations in circuit activities at adult ages (including motor activity, sleep-wake cycles, experimental spreading depression, or enriched environment) to modulation of NG2+p proliferation and maturation (Ehninger et al., 2011; Simon et al., 2011; Tamura et al., 2012; Bellesi et al., 2013). However, these findings, which appeared somewhat contradictory, only established a rather aspecific link between neuronal activity and NG2+p behaviors. In a recent report Mangin et al. (2012) addressed this issue more directly and found that sensory stimuli from the whisker pad regulate NG2+p number and distribution in the neonatal barrel cortex by negatively affecting cell proliferation. These data are in keeping with an inhibitory role of glutamatergic inputs on NG2 cell amplification and suggest that different inputs levels would result in proliferation-mediated accumulation of NG2+p at sites of relatively low electric activity. Such accumulations could then specifically predispose cells to start myelination (Mangin et al., 2012), as the achievement of a critical density is one of the key factors for myelin formation (Rosenberg et al., 2008). Electrical activity itself could likely further support the progression of postmitotic progenitors along the lineage, as it has long been known to be a myelination promoter (Demerens et al., 1996; Stevens et al., 2002; Lundgaard et al., 2013). Thus, the response of NG2+p to neuronal activity appears crucial to regulate their number and engagement in myelination during development, thereby contributing to structure the CNS architecture. Similarly, it could underlie myelin refinements related to learning and memory during adulthood. Yet, how these findings apply to the adult CNS and whether the large abundance of NG2+p present at adult stages is exclusively required to sustain the low grade of myelin turnover so far detected and/or experience-related circuit modulations remain to be assessed. Indeed, the occurrence of myelin remodeling appears rather low in the adult CNS (Zatorre et al., 2012; Young et al., 2013) and at least in the gray matter the overall rate of NG2+p proliferation does not differ in myelin rich vs. myelin low territories (our unpublished observations) thus showing to be unrelated to myelin turnover. Further, exploiting the high metabolic charge of electric activity solely to tonically limit NG2+p proliferation appears poorly efficient, particularly in light of data showing that homotypic density cues play a major role in the regulation of NG2+p proliferation at adult stages (Hughes et al., 2013). Moreover, the observation that an important part of NG2+ clones in the gray matter do not generate oligodendrocytes in the adult brain (Levison et al., 1999; Zhu et al., 2011) raises the question as to whether NG2+p responses to neuronal inputs may include functions other than proliferation control or lineage progression.

NG2+p FUNCTIONS BEYOND MYELINATION?

Recently discovered features of NG2+p point to additional neuromodulatory and neuroprotective actions of this population. Some of these traits appear specifically related to the particular capability of NG2+p to sense and respond to electrical activity. Others, including the expression of growth factors, morphogens, cytokines, chemokines, and extracellular matrix (ECM) components, can instead be viewed as properties inherent in the progenitor nature of these cells, and may well correspond to the reparative bystander actions that germinal neural progenitors exert either *in situ* or upon grafting in the lesioned CNS (Martino and Pluchino, 2006; Butti et al., 2012).

Of note, evidence that NG2+p express synaptophysin suggests they may be capable of regulated secretion and bidirectional communication with astrocytes and neurons (Wigley and Butt, 2009). Of relevance in this context, Maldonado et al. (2013) have shown that during postnatal maturation NG2+p become progressively more sensitive to extracellular potassium increases generated by action potentials thanks to upregulation of Kir4.1 inward rectifying potassium channels. These channels mediate inward currents in conditions of high potassium and constitute one of the mechanisms through which astrocytes perform potassium buffering in the extracellular space, which is a requisite for correct neuronal transmission and excitability. In adult NG2+p, besides contributing to set the resting membrane potential, these channels may help with removing high extracellular potassium and regulating neuronal functions at sites of close appositions to neurons (see above) (Maldonado et al., 2013). This could be a NG2+p specific mechanisms occurring upon single neuron firing, which may remain undetected by astrocytes (Maldonado et al., 2013).

NG2+p are also known to express several ECM components such as tenascins, versicans, neurocans, phosphacan, hyaluronan and even hyaluronan, and proteoglycan link protein 1 (HPLN1), which act as synaptic stabilizers by anchoring the neurotransmitter receptors to the cytoskeleton and contribute to perineuronal nets formation (Butt et al., 2005; Sim et al., 2009; see also Sim and Goldman, 2013). Given the intimate association of NG2 processes with neuronal pre- and post-synaptic elements and that neighboring pairs of NG2+p and neurons receive synaptic contacts from the same neurons (Bergles et al., 2000; Lin et al., 2005; Mangin et al., 2008), it can be hypothesized that NG2+p monitor neuron-neuron synapses and, depending on synchronization levels, act on synaptic stabilization and network organization

by modifying the perisynaptic microenvironment. Interestingly, human NG2+p also show enriched levels of thrombospondin 2 (Sim et al., 2006), which is known as a synaptogenic cue released by immature astrocytes (Christopherson et al., 2005). Seminal studies demonstrating the role of astroglia in synaptic strengthening and formation also attributed a relevant effect to cells of the oligodendroglial lineage (Pfrieger and Barres, 1997). However, these data should be re-considered in light of the need to clarify the purity of cells and actual maturation stages. Yet, in line with an early function in circuit formation, acute deletion of cycling oligodendroglial cells have been reported to induce rapid changes in the expression of molecules involved in synaptic plasticity, axon growth and guidance in the cerebellum at birth, indicative of fast activation of remodeling mechanisms (Doretto et al., 2011). Previous experiments at later developmental stages have shown that myelin formation shapes cerebellar connections by removing exuberant collateral branches of Purkinje neurons (Gianola et al., 2003). These results suggest that cycling NG2+p participate in shaping cerebellar circuits well before myelination starts.

Neuroprotective mechanisms have been also proposed to be activated in NG2+ cells upon lesion. NG2+p provide a stereotyped response to injury, unrelated to the extent of myelin loss and of their own damage, which includes a precocious activation of proliferation and hypertrophy and is mediated by inflammatory and danger related signals (Levine et al., 2001; Nielsen et al., 2006). The sensitivity of these cells to changes in nerve conduction and neurotransmission could also influence NG2+p reactivity. Tanaka et al. (2009) reported that GABA-receptor mediated excitation in NG2+p after ischemic stroke increases brain-derived neurotrophic factor (BDNF) production, which was instead blocked by inhibition of GABA-mediated depolarization. The authors further hypothesize that NG2+p-derived BDNF participate in post-stroke reparative mechanisms. Given the well-known actions of BDNF in promoting synaptic transmission, plasticity, and growth (Lu et al., 2013), these speculations could be extended to include modulatory actions of neuronal functions in physiological conditions. Along this line, reactive NG2+p responding to depolarizing waves induced by experimental cortical spreading depression were reported to upregulate the peptide galanin and proposed to release it to receptor positive cortical neurons with the purpose of limiting excitotoxic damage (Shen et al., 2003). In a recent *in vitro* study Sypecka and Sarnowska (2013) provided first evidence in support of a pro-survival action of NG2+p on the injured nervous tissue. In co-cultures of primary NG2+p with organotypic hippocampal slices subjected to oxygen-glucose deprivation, the authors observed a significant rescue of neuronal viability and identify BDNF, interleukin-10, stem cell factor (SCF) as agents through which NG2+p perform immunomodulatory and protective functions in their experimental setting. Accordingly, NG2+p derived from human embryonic stem cells express transforming growth factor (TGF) β2, a potent inhibitor of inflammation, midkine, and activine A, two neurosupportive factors that are highly upregulated early after injury (Munz et al., 2001; Zhang et al., 2006; Yoshida et al., 2014). NG2+p cells could therefore share the capability of mature oligodendrocytes to produce neurotrophic factors, influence adjacent cells (Wilkins et al., 2001; Dai et al., 2003) and especially neurons in physiology and conditions of cell stress (Lee et al., 2012; Frühbeis et al., 2013). Further, in amyloidosis models it has been reported that NG2+p internalize and degrade β-amyloid1-42 by autophagy (Li et al., 2013). Thus, together with astrocytes and microglia, NG2+ glia could also participate in the clearance of amyloid as part of their reactive response (Boda et al., 2011; Behrendt et al., 2013).

The potential reparative and supportive actions may be specifically triggered by NG2+p reactivity or be already present in resting conditions and become amplified as a consequence of the NG2+p widespread cytogenic response to damage. However, these considerations remain speculative, because a detailed and comprehensive examination of NG2+p changes upon lesion is not available. What is instead better assessed is the contribution of NG2+p to scar formation and the inhibitory role of chondroitin sulfate proteoglycans—including NG2—in axon remodeling and regrowth of transected axons (Galtrey and Fawcett, 2007), recently accompanied by evidence for conduction blockade exerted by NG2 at nodes of Ranvier after spinal cord transection (Petrosyan et al., 2013). While these inhibitory effects are mostly seen as detrimental for circuit rewiring, they could be part of a mechanism necessary to contain excitoxicity and damage extension, as formerly shown for astrocytes (Buffo et al., 2010). Further, actions restricting plasticity may be compensated by an increment in the availability of prosurvival factors determined by both NG2+p reactivity and proliferation.

CONCLUDING REMARKS

Intense research over the last decades has revealed key insights into NG2+p physiopathology related to myelinogenesis. Nevertheless, fundamental aspects of NG2+p biology remain undetermined. For example, while NG2+p are known to respond to signals produced by neurons, astrocytes, and microglia, whether this communication is reciprocal and how it occurs is substantially neglected. NG2+p-derived signaling may include morphogenic, neuromodulatory, and neuroprotective factors whose elucidation may also have therapeutic implications for the implementation of the endogenous reparative potential of injured CNS. *In vivo* approaches aimed at selectively and timely ablating NG2+p in the CNS, together with the identification of active paracrine/juxtacrine factors produced by NG2+p, will be highly instrumental to address these issues, especially to understand how much it is crucial to maintain such a high number of cells to sustain myelin turnover and plasticity in the adult CNS and whether any relevant alteration in neuronal functions occurs in the absence of NG2+p in the intact or injured CNS.

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Modeling physiological and pathological human neurogenesis in the dish

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New advances in directing the neuronal differentiation of human embryonic and induced pluripotent stem cells (hPSCs, abbreviation intended to convey both categories of pluripotent stem cells) have promoted the development of culture systems capable of modeling early neurogenesis and neural specification at some of their critical milestones. The hPSC-derived neural rosette can be considered the *in vitro* counterpart of the developing neural tube, since both structures share a virtually equivalent architecture and related functional properties. Epigenetic stimulation methods can modulate the identity of the rosette neural progenitors in order to generate authentic neuronal subtypes, as well as a full spectrum of neural crest derivatives. The intrinsic capacity of induced pluripotent cell-derived neural tissue to self-organize has become fully apparent with the emergence of innovative *in vitro* systems that are able to shape the neuronal differentiation of hPSCs into organized tissues that develop in three dimensions. However, significant hurdles remain that must be completely solved in order to facilitate the use of hPSCs in modeling (e.g., late-onset disorders) or in building therapeutic strategies for cell replacement. In this direction, new procedures have been established to promote the maturation and functionality of hPSC-derived neurons. Meanwhile, new methods to accelerate the aging of *in vitro* differentiating cells are still in development. hPSC-based technology has matured enough to offer a significant and reliable model system for early and late neurogenesis that could be extremely informative for the study of the physiological and pathological events that occur during this process. Thus, full exploitation of this cellular system can provide a better understanding of the physiological events that shape human brain structures, as well as a solid platform to investigate the pathological mechanisms at the root of human diseases.

Keywords: ESCs, iPSCs, neural tube, human neurogenesis, rosettes, neural crest, *in vitro* disease modeling, self-aggregation

USING hPSC-DERIVED ROSETTE FORMATION TO MODEL EARLY NEURULATION PROCESSES

The unique developmental potential and replicative capacity of hPSCs offers a nearly unlimited source of specific somatic cell types that can be exploited for *in vitro* mechanistic studies or cell transplantation therapies. Remarkably, neuronal cells were among the first lineages to be differentiated using hPSCs (Reubinoff et al., 2001; Zhang et al., 2001). Neuronal induction was first obtained by promoting the differentiation of hPSCs in aggregate-like embryoid bodies. Subsequently, aggregates were placed in stringent serum-free culture conditions, which selectively facilitate the survival and growth of neural cells. This transition toward the neural lineage is readily manifested in hPSCs (but not in their murine counterparts) because of the appearance of rosette-like structures within the differentiating hPSC colonies (Reubinoff et al., 2001; Zhang et al., 2001). These structures develop from progenitor cells, which line up close together to form a round, columnar epithelium that is reminiscent of blooming rosettes when viewed under bright light.

Further examination of these structures revealed that the rosettes are formed from neural progenitors. These neural progenitors are endowed with highly polarized morphology, as indicated by the presence of tight and adherence junctions at the side facing the internal lumen, while the external side is strongly enriched in laminin-rich extracellular matrix (Lazzari et al., 2006; Elkabetz et al., 2008; Colleoni et al., 2010). This architecture recapitulates the cellular organization of the neural tube, the embryonic primordium of the entire central nervous system (CNS), in both shape and function. In fact, within rosettes, the nuclei of neural progenitor cells undergo a stereotyped movement known as interkinetic nuclear migration, which is harmonized to the cell cycle stage and specific to authentic neural tube cells (Taverna and Huttner, 2010). Nuclei undergoing DNA synthesis localize at the outer edge of the neural tube, while mitotic divisions are confined to its innermost core, a pattern replicated in the same sequence by nuclei within the rosettes (Lazzari et al., 2006; Elkabetz et al., 2008; Colleoni et al., 2010). Therefore, rosettes share the same elementary organization of the developing neural tube; hence,

they are equivalent to the developing neural tube with respect to structure and function (**Figure 1**). Rosette neural progenitors intertwine to create overlapping cellular layers; however, they remain constrained to the surface where they anchor. How rosettes can be adapted to the three-dimensional (3D) space, and which morphology and growth pattern they will follow in these culture conditions, have not yet been ascertained. This setting might enable the *in vitro* reproduction not only of neural tube organization, but also (and even more challenging) its organogenesis.

During development, the neural tube is highly patterned along the anterior-posterior and ventral-dorsal axes (Muñoz-Sanjuán and Brivanlou, 2002). In particular, its anterior portion is invariably committed to give rise to the different brain regions. It is worth noting that neural rosettes, when cultured in the mentioned culture conditions, acquire a specific regional code that coincides with the anterior-dorsal section of the developing neural tube, as demonstrated by the acquisition of a specific set of regional molecular markers (Elkabetz et al., 2008; Colleoni et al., 2010; Hu et al., 2010) (**Figure 1**). Considering that rosettes are induced in a medium that lacks any molecular inducer, these findings suggest that the commitment of the anterior-dorsal region represents the default state during neuronal induction, and only subsequent inductive clues, either cell or non-cell autonomous, might override this basal state, imposing other regional identities.

FROM NEURAL TUBE-LIKE ROSETTES TO NEURAL CREST CELL PROGENITORS

In serum-free neurobasal culture conditions, each differentiating hPSC rosette is scattered in an array of disorganized and unpatterned cells. Remarkably, the expression of two specific markers (p75 and HNK1) identified many of these cells as neural crest cells (Lazzari et al., 2006; Gossrau et al., 2007; Lee et al., 2007, 2010). Molecular analyses corroborated the acquisition of the neural crest identity, as shown by expression of the associated molecular determinants. Temporal analysis illustrated how neural crest cells originated by delaminating from rosette structures in a process similar to the epithelial-mesenchymal transition (EMT), which normally occurs during development when nascent neural crest cells arise from the neural tube (Curchoe et al., 2010). Spontaneous emergence of the neural crest component *in vitro* provides additional evidence that PSC-derived rosette structures acquire dorsal identity, since this is the side of the neural tube from which the neural crest is selectively induced during development (Le Douarin et al., 2004).

In agreement with this view, treatment of rosettes with Sonic hedgehog (Shh), a ventralizing morphogen expressed by the floor plate of the ventral neural tube, inhibits the expression of neural crest-specific genes while activating ventral neural tube markers (Colleoni et al., 2010). More importantly, subsequent culturing of neuralized hPSC colonies that had been exposed to epigenetic stimulation with the growth factors bFGF and EGF enabled the

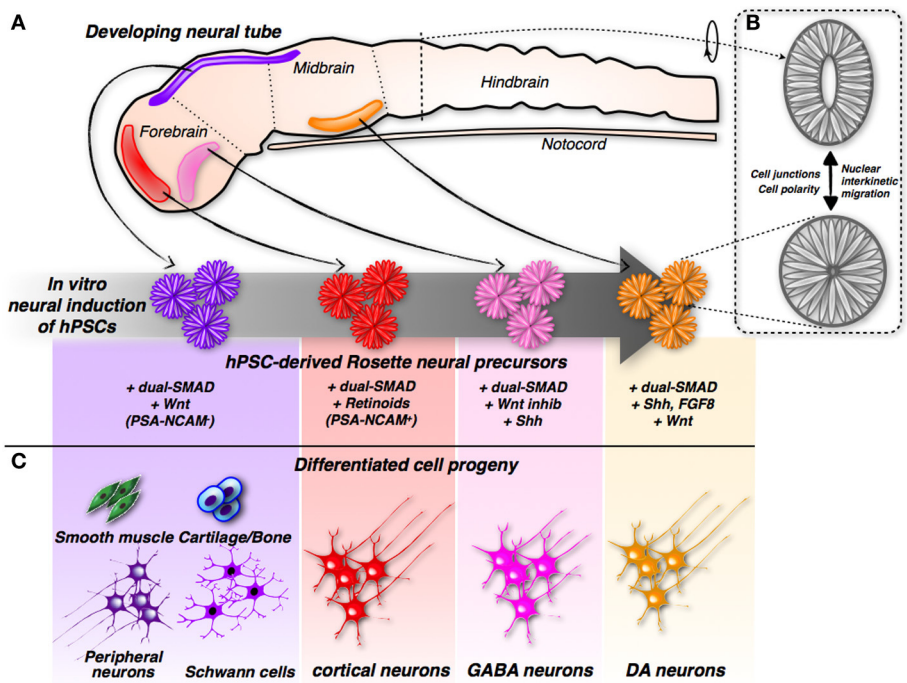

FIGURE 1 | Induction and differentiation of iPSC-derived rosette neural progenitors. (A) Modulation of neurodevelopmental molecular pathways by different combinations of small molecules can direct hPSCs into rosette neural progenitors with different positional identities along the developing neural tube. **(B)** The hPSCs-derived rosette and the neural tube share the same spatial organization showing cells with a remarkable polarization and cell-junction compartmentalization. In addition, relative nuclei position is depending by the cell-cycle stage in both systems. **(C)** According to their specific early commitment, hPSCs-derived rosette neural progenitors generate distinct neuronal sub-types and, in the case of hNCPCs, even non-neuronal somatic cells.

generation of stable cell lines of human neural crest progenitor cells (hNCPCs) (Lazzari et al., 2006; Lee et al., 2007, 2010; Colleoni et al., 2010). hPSC-derived hNCPCs exhibited a stable phenotype, even after sustained proliferation *in vitro* and multiple single cell disaggregation passages.

Notably, although hPSC colonies initially included CNS-like rosette progenitors, the rosette progenitors stopped multiplying and died out over few passages, giving rise to a population of pure hNCPCs (Colleoni et al., 2010). This probably occurred because rosette neural progenitors are extensively polarized epithelial cells, and therefore are susceptible to cell death upon single cell dissociation. Conversely, hNCPCs retained an intrinsic resilience to cell death that might have been gained with the EMT process and the acquirement of mesenchymal-like cell properties (Vega et al., 2004; Robson et al., 2006). Interestingly, the withdrawal of mitogens induced cell cycle exit and the differentiation of neural crest progenitors into an array of somatic cells, including sympathetic and sensory neurons, glial cells, melanocytes, myofibroblasts, cartilage, and bone cells (Lee et al., 2007; Colleoni et al., 2010). Although neural lineages are well represented, their *in vitro* maturation is a weeks-long process. In addition, glial cells progressed to a Schwann cell progenitor stage; these progenitors were positive for S100β and GFAP, although no sign of occurring myelination was ever detected (Lee et al., 2007; Colleoni et al., 2010). Therefore, it remains unknown whether hPSC-derived Schwann cells can be induced to differentiate, or whether they are able to generate functional myelin sheaths around axonal tracts. Clarification of this point will enable the establishment of a valuable model of human myelination.

Furthermore, taking into consideration other neural crest derivatives, an interesting study has recently determined the experimental conditions necessary for the maturation and enrichment of melanocytes from differentiating hNCPCs (Mica et al., 2013). Finally, mesodermal derivatives, including cartilage and bone, have been equally generated from hNCPCs (Lee et al., 2007; Colleoni et al., 2010). This finding may be of great interest for regenerative therapies, exploiting bone and cartilage replacement to treat, for example, trauma or osteoporosis. However, to continue along this direction, it is necessary to address the quality and type of cartilage that hNCPCs are able to generate, as well as (even more importantly) its stability, robustness, and longevity after *in vivo* transplantation.

The induction of both neural and mesenchymal cell lineages indicates that hPSC- derived NCPCs acquire a cranial identity in culture, because only head neural crest contributes to mesoderm-derived structures in the embryo (Le Douarin et al., 2004). Remarkably, clonal analysis identified hNCPCs with mesodermal and/or ectodermal potential similar to what has been described regarding hPSC-derived neural crest cells (Baroffio et al., 1991; Lee et al., 2007). However, different culture conditions or durations in culture can strongly drive hNCPCs from a neural to a mesenchymal phenotype (Lee et al., 2007; our unpublished results). Indeed, the exposure of hNCPCs to a serum-containing medium upregulated mesenchymal markers (e.g., CD73) and stimulated their differentiation into mesodermal derivatives (e.g., smooth muscle, cartilage, and bone cells) (Lee et al., 2007). Therefore, hNCPCs represent an ideal cellular system in which to investigate the molecular mechanisms that regulate this dual cell fate potential, as well as their subsequent final differentiation into a variety of distinct cell types.

LONG-LASTING NEURONAL PROGENITORS CELLS (hNPCs)

While isolating and expanding hNCPCs from hPSC-derived neural rosettes is a straightforward process, the generation of hNPCs is unfortunately hampered by their low survival rate upon rosette dissociation (Elkabetz et al., 2008; Koch et al., 2009). However, this hurdle has been significantly attenuated by the introduction of the Rho-associated protein kinase (ROCK) inhibitor Y-27632, which inhibits single-cell-induced death (Watanabe et al., 2007). hNPCs, which are capable of extensive self-renewal, clonogenicity, and multipotency, have been derived from rosettes by generating neurosphere-like floating aggregates or monolayer cultures (Zhang et al., 2001; Conti et al., 2005). The purity of hNPC cultures has yet to be convincingly assessed, and might be jeopardized by the presence of contaminating hNCPCs. This is an important caveat because many protocols rely on the manual isolation of rosettes, and therefore likely permit the incorporation of closely abutting hNCPCs, which can easily outnumber the hNPCs owing to their better survival and growth rate in the same culture conditions. Taking all of this into consideration, it is always worthwhile to test the presence of p75+ cells in hNPC cultures in order to verify neural crest cell contaminants. Kim et al. (2012) have conceived a valuable approach to prevent such contamination; they demonstrated that polysialylated-neural cell adhesion molecule is strongly enriched in rosette hNPCs, and thus cell sorting for this epitope enables the isolation of a pure population, devoid of any cellular contaminants.

The polysialylated-neural cell adhesion molecule-negative cell fraction is mainly comprised of hNCPCs, thus providing an approach to purify both cells types in a single-step procedure (Kim et al., 2012). Moreover, long-lasting proliferative hNPCs represent a renewable source of neurons and glia that, once established, is independent of hPSC culture. However, growth factor-dependent hNPC expansion might also lead to undesired effects. Indeed, Bilican et al. (2014) noted that chronic exposure to EGF might cause NPCs to lose their anterior identity while assuming a hindbrain regional code. Interestingly, the expansion of hNPCs in the presence of bFGF and physiological levels (3%) of O_2, is sufficient to enable long-term proliferation while maintaining the anterior character that is fundamental for stable differentiation of committed cortical neurons. How hNPCs are able to modify their competent state with respect to regional specification and give rise to different neuronal subtypes is still unclear. Some reports suggest that early hNPCs might still respond to inductive signals in order to acquire a different regional identity (Koch et al., 2009); however, they are unlikely to be able to maintain this capability without change over time and after extensive cell passaging. Given these last observations, several procedures have been established with the aim of generating a single neuronal subtype using direct differentiation of hPSC-derived neural rosettes, some of which are described in the next Section.

CORTICAL NEURONS

Although neural rosettes exhibit a default anterior regional code, their dissociation and subsequent neuronal differentiation in standard culture conditions (e.g., absence of morphogen supplementation) leads to a population of mature neurons that are representative of various brain regions. However, prolonged exposure to the BMP inhibitor Noggin is sufficient to efficiently induce differentiation mainly of cortical neurons (Espuny-Camacho et al., 2013) (**Figure 1**). In addition, the presence of retinoids (retinol-acetate and all-trans retinol) during neural induction and differentiation maximizes the conversion of cortical neurons (Shi et al., 2012). Both procedures converge on the progression of cortical neuronal differentiation with the initial generation of deep layer neurons; superficial layer neurons are generated later, during the development of the cerebral cortex. Therefore, the temporal sequence of neuronal subtype specification in this system mirrors the *in vivo* occurrence of the same process (Greig et al., 2013). Based on these data, one can envisage that this pattern of *in vitro* neurogenesis is mainly mediated by cell autonomous mechanisms, which might be intrinsically encoded within the neuronal progenitors. The generation of both deep and upper cortical neurons from hPSCs covers a very extensive timeframe, which might last for up to 3 months of culture, which dovetails with the delay in cortical neurogenesis that occurs in human embryos compared with mouse embryos (Shi et al., 2012; Espuny-Camacho et al., 2013). More importantly, hPSC-derived cortical neurons grafted into the frontal cortex of the neonatal brain were able to survive, mature, and extend their axons to the proper neuronal targets according to their layer specification (Espuny-Camacho et al., 2013). These data indicate that the use of this *in vitro* system enables the full acquisition of subtype identity, which is then fully elaborated after transplantation. Finally, the grafted human neurons developed functional properties and synaptic connectivity within 3 months after transplantation. It would be of great interest to challenge the transplantation of these neurons in healthy and injured adult brain, in order to verify whether they are able to mature and functionally integrate even in more prohibitive conditions.

GABAergic NEURONS

In contrast to excitatory (glutamatergic) cortical pyramidal neurons that project long distance, inhibitory (GABAergic) cortical interneurons make local synapses and are essential for the maintenance of balanced activity within neural circuits (Marín, 2013; Kepecs and Fishell, 2014). Interneuronal dysfunctions have been described in several disorders, including epilepsy, autism, schizophrenia, and Tourette's syndrome (Valiente and Marín, 2010; Alvarez Dolado and Broccoli, 2011; Marín, 2012). The direct differentiation of hPSCs into telencephalic GABAergic neurons has been achieved recently (Maroof et al., 2013; Nicholas et al., 2013). In both studies, early telencephalic GABAergic commitment of hPSCs was reported by the expression of green fluorescent protein (GFP) under the control of the Nkx2.1 promoter, which is fundamental for setting up the appropriate conditions for the specification of this neuronal subtype. In fact, optimal conditions were attained by combining exposure to BMP and Wnt inhibitors, which prime telencephalic identity, with Shh signaling stimulation, which induces ventral neuronal character, thus reaching up to 70% of Nkx2.1-GFP-positive, hPSC-derived, GABAergic neuronal precursors (**Figure 1**). Cortical interneurons include a different set of neuron types that have distinct roles in cortical development and function (Marín, 2013; Kepecs and Fishell, 2014). In these conditions, the prevalence of somatostatin-positive cells was observed, while many fewer interneurons expressing parvalbumin or VIP were detected. Thus, more work is needed to identify permissive conditions for the effective derivation of the later subtypes. Co-cultures with astrocytes or hippocampal neurons promoted hPSC-derived GABAergic neuronal maturation and functionality, including repetitive action potential firing, ionic channel currents, and synaptic currents. To directly prove their inhibitory synaptic activity, GABAergic neurons were excited by an optogenetic protocol that evoked robust postsynaptic currents in neighboring neurons, which were sensitive to the GABA antagonist bicuculline (Nicholas et al., 2013). Thus, hPSCs can be induced to differentiate into functional inhibitory GABAergic interneurons. It is interesting to point out that upon transplantation into neonatal brains, a fraction of the grafted neurons matured in the host neural tissue, gaining functional properties and synaptic connectivity, although several months of engraftment were required (Nicholas et al., 2013). These last results are particularly compelling in light of the fact that the transplantation of mouse native GABAergic precursors has proven therapeutic in treating animal models of epilepsy, schizophrenia, and Parkinson's disease (PD) (Southwell et al., 2014; Tyson and Anderson, 2014). All of these different pathological conditions have benefited from GABAergic neuronal transplantation, likely because it favors the re-organization of the neuronal circuit connections and the re-establishment of the correct balance between excitation and inhibition in cortical circuits (Southwell et al., 2014; Tyson and Anderson, 2014). In particular, this approach might have a straightforward application regarding the many forms of epilepsy that eventually reach an irreversible, drug-resistant stage.

DOPAMINERGIC NEURONS

Substantia nigra (A9) midbrain dopaminergic neurons are specifically lost during the initial phase of PD. The consequent loss of dopamine availability in striatal tissue is responsible for the troublesome motor impairments that severely affect PD patients. In fact, most of the drugs used to treat PD aim simply to restore sufficient levels of dopamine in the striatum, but cannot reverse or block the loss of dopaminergic neurons (Meissner et al., 2011). In this context, dopamine neuron replacement has been envisioned as a potential therapeutic strategy during the last decades (Dunnett et al., 2001; Lindvall and Björklund, 2011).

Transplantation of midbrain human fetal tissue provided a solid basis for a cellular approach to PD, confirming the ability of transplanted neurons to integrate into the host striatal tissues and sustain functional integration and a therapeutic effect (Barker et al., 2013). However, this approach had little clinical benefit, mainly because of the broad cell heterogeneity of the transplanted tissue, its minimal availability, and scarce immuno-compatibility (Lindvall and Björklund, 2004; Barker et al., 2013). Even worse, these issues were responsible for the development of the troubling

side effect of graft-induced dyskinesia, which is experienced by some of the patients (Lane et al., 2010).

Given this context, hPSCs have long been considered a better renewable source of dopamine neurons that could potentially overcome these hurdles. To this purpose, many protocols have been developed for the *in vitro* differentiation of hPSCs into this particular class of neurons. Combined exposure to the morphogens Shh and FGF8, known to contribute to ventral midbrain neural commitment in the developing neural tube, was also found to instruct mouse and human PSCs to differentiate into tyrosine hydroxylase (TH)-positive dopamine neurons (Kim et al., 2002; Perrier et al., 2004; Lee et al., 2009) (**Figure 1**). However, the brain contains distinct dopamine neuronal clusters that differ with regard to function, connectivity, and molecular marker expression. This is a crucial issue because only transplantation of A9 [and not, for example, VTA (A10)] resident dopamine neurons can achieve maximal behavioral recovery in a mouse model of PD (Grealish et al., 2010). Reasonable graft-induced recovery depends on the correct innervation (and thereby the functional activation) of the striatum, which represents the physiological target structure of A9, but not A10, dopamine neurons (Grealish et al., 2010).

These results have far-reaching implications, indicating that the therapeutic benefits of cell transplantation approaches depend on the replacement of native neurons with cells that are close, if not identical, in nature and identity. Improvements in the differentiation of hPSCs into fully committed midbrain dopamine neurons have been attained by two recent studies (Kriks et al., 2011; Kirkeby et al., 2012). Both employed sustained stimulation of Wnt signaling (by inhibiting the enzyme glycogen synthase kinase 3β, GSK-3β) to robustly induce the differentiation of hPSCs into dopamine neurons (**Figure 1**). Neurons derived using this protocol exhibited midbrain character, as confirmed by the expression of the regional markers FoxA2 and Lmx1a (Kriks et al., 2011; Kirkeby et al., 2012). Remarkably, transplantation of these midbrain-specific dopamine neurons in the striatum resulted in significant behavioral recovery in both rodent and primate models of PD (Kriks et al., 2011; Kirkeby et al., 2012).

This transplantation strategy is designed to allocate transplanted dopamine neurons close to their physiological target (striatal neurons) in order to facilitate their functional connectivity, although grafted neurons would be ectopically located regarding their natural midbrain position. It is unknown whether this new neural circuit, its components necessarily re-organized, can fully restore physiological function without any concomitant behavioral alterations. To overcome this limitation, it would be interesting to test whether hPSC-derived neurons transplanted in the substantia nigra are able to re-establish nigro-striatal connections. Although the adult brain parenchyma is minimally permissive to axonal regrowth, grafted fetal dopaminergic neural precursors are surprisingly capable of generating a nigro-striatal pathway with an outgrowth pattern that matches the anatomy of the intrinsic system (Thompson et al., 2009). These results reveal some opportunities to reconstruct native neuronal circuits using a cell transplantation approach in the adult brain. Whether a similar approach can be envisioned using hPSC-derived dopamine neurons remains to be demonstrated.

IN VITRO NEURONAL MATURATION AND AGING

In vitro differentiation of hPSCs into neurons is a long and multistep process that matches all of the stages of embryonic neuronal commitment and differentiation. Therefore, the neurons that result from hPSC induction appear to be very immature soon after their specification; they acquire functional properties only after several weeks in culture. Mature functional and synaptic properties are observed in the majority of the hPSC-derived neurons after 8–12 weeks of culture (Ricciardi et al., 2012; Verpelli et al., 2013). Thus, this delay in functional maturation of the hPSC-derived neurons *in vitro* certainly hampers the conduct of functional studies using this system.

Supplementation of the culture medium with neurotrophins and cAMP can enhance neuronal differentiation and restrain cell death events (Soldner et al., 2009). However, a major differentiating stimulus is provided when hPSC-derived neurons are co-cultured on a primary astrocyte feeder layer. In fact, neuronal differentiation, dendrite development, excitability, and synaptic activity are all accelerated from 8- to 20-fold when hPSC-derived neurons are cultured on astrocytes rather than on laminin (Tang et al., 2013). Zhang et al. (2013) recently devised an interesting alternative. They have shown that hPSC differentiation can yield a high level of functional glutamatergic neuronal cells in less than 2 weeks through the forced expression of only one transcription factor, Neurogenin 2. This is an attractive procedure because neurons are generated directly from naïve hPSCs in a single manipulation step, thereby skipping all of the intermediate steps and saving time and labor. A similar approach has been employed recently to accelerate the generation of functional dopamine neurons by expressing the Ascl1, Nurr1, and Lmx1a genes in naïve hPSCs (Theka et al., 2013).

As mentioned above, neurons derived from hPSCs require time to mature fully and are comparable to cells at the immature fetal stage (Marchetto and Gage, 2012). This is particularly troublesome when one aims to study the mechanisms of neuronal aging and associated neurodegenerative disorders. In fact, neuronal aging is a major contributor to late-onset neurodegenerative disorders and must be taken into account when modeling such disorders. In order to model late-onset diseases, recent studies have employed hPSC-derived neuronal *in vitro* cultures that have been grown for up to 200 days (Dimos et al., 2008; Ebert et al., 2009). hPSCs represent an interesting system with which to follow changes in the morphological and functional properties of neurons over a long period of time *in vitro*, possibly mimicking some of the processes that occur during aging. However, new solutions are needed to accelerate the aging process of these neurons *in vitro*. Notably, a new approach has been recently proposed that involves the expression of progerin, a truncated form of lamin-A that is associated with premature aging (Miller et al., 2013). In fact, the expression of progerin in hPSC-derived fibroblasts and neurons induced multiple aging-related markers and characteristics, including the appearance of neuromelanin in dopamine neurons (Miller et al., 2013). It remains to be established which features of late-onset disease can be authentically recapitulated by the progerin-induced model *in vitro*. Similar to this study, other known age-specific molecular pathways might be explored to promote neuronal aging *in vitro* (Newgard and Sharpless, 2013).

PATHOLOGICAL NEUROGENESIS: MODELING HUMAN DISEASES USING NEURONS GENERATED *IN VITRO*

The advent of reprogramming adult human cells to become human induced pluripotent stem cells (hiPSCs) has revealed new opportunities to model disorders linked to a genetic cause. hiPSCs are able to self-renew and give rise to the affected somatic cells, providing an unlimited source of informative material to investigate diseases of interest. To date, a number of studies have validated this approach, modeling both neurological and neuropsychiatric disorders (Tiscornia et al., 2011; Zhu et al., 2011; Sandoe and Eggan, 2013). Monogenic diseases in which the mutated gene is known are particularly suitable for this approach. In this scenario, the function of the gene of interest can be restored, thus proving that the original mutation was indeed the causative factor of the disease-specific phenotype (Soldner et al., 2011; Liu et al., 2012). The approach is particularly relevant because the individual genetic variability of human sample often hampers any comparison between patient and healthy donor cells, disabling the detection of phenotype differences (Saha and Jaenisch, 2009). In fact, it would be necessary to analyze a large cohort of control and patient cells in parallel in order to address this matter, which would require a huge effort to generate and analyze a large range of hiPSC lines. Some compelling studies have been reported recently that illustrate the power of this approach in helping to identify new cellular and molecular mechanisms of disease; we report a few notable examples here.

Lee et al. (2010) developed an hiPSC model of familial dysautonomia, a disorder that causes depletion of the autonomic and sensory neurons, induced by mutations in the IKBKAP gene. In the hiPSC system, they were able to correlate the loss of IKBKAP gene expression with defects in neurogenesis and diminished cell motility in hiPSC-derived neural crest cells. Similarly, Marchetto et al. (2010) used neurons derived from the hiPSCs of individuals affected by Rett syndrome (RTT), an autism-like disorder caused by mutations in the methyl CpG binding protein MECP2. Lack of MeCP2 protein in hiPSC-derived neurons was associated with decreases in dendritic spines, synapses, and calcium oscillation frequency, as well as a concurrent reduction in nuclear size compared with control cells. A more recent study used an isogenic human embryonic stem cell model of RTT to identify a significant reduction in nascent protein synthesis (Li et al., 2013). Moreover, cells manifested a severe defect in AKT/mTOR pathway activity that resulted from the lack of MECP2 expression (Ricciardi et al., 2011; Li et al., 2013). More interestingly, the activation of AKT/mTOR signaling was sufficient to ameliorate the disease phenotypes in mutant hiPSC-derived neurons, by boosting protein synthesis. These data clearly show how the hiPSC modeling system is a convenient platform for the development of pharmacologic and genetic approaches that aim to revert a mutant phenotype using either a hypothesis-driven procedure or a more systematic solution.

A relevant question is whether hiPSC-based modeling can be fruitful for studying late-onset neurodegenerative diseases. In a recent study, hiPSC-derived neurons from patients with genetic or sporadic forms of Alzheimer disease (AD) did not exhibit enhanced susceptibility to cell death or changes in activity compared with controls (Israel et al., 2012). However, they did exhibit significantly higher levels of the pathological markers amyloid-β, phospho-Tau, and active GSK-3β than control cells. These hiPSC-derived neurons from AD patients also accumulated large RAB5-positive early endosomes compared with controls. Importantly, treatment of purified neurons with β-secretase inhibitors, but not γ-secretase inhibitors, caused significant reductions in phospho-Tau and GSK-3β levels (Israel et al., 2012). Therefore, a direct relationship can be drawn between the proteolytic processing of amyloid precursor protein, but not that of amyloid-β, in GSK-3β activation and Tau phosphorylation in hiPSC-derived neurons. These data suggest that hiPSC technology can be used to observe early pathological changes, possibly associated with the prodromal phase of AD, even if it does not faithfully recapitulate the phenotypes that characterize the late-onset phase.

NEURAL INDUCTION FROM A SINGLE LAYER TO THREE DIMENSIONS

The employment of cutting-edge culture procedures has recently elevated the potential of neural rosettes to recapitulate the events and mechanisms that underlie neural tube development to another dimension (Eiraku et al., 2008; Lancaster et al., 2013). Studies have recently reported the generation of *in vitro* 3D cell aggregates (organoids) that not only reproduce an organ's structure, but also recapitulate its function and development (Sato et al., 2009; Suga et al., 2011; Kadoshima et al., 2013). The applications of this technology to the investigation of still-unapproached aspects of organogenesis promise to bear relevant knowledge that is instrumental to a full understanding of tissue formation and pathogenesis.

The possibility of modeling human brain disease in a Petri dish (Eiraku et al., 2008) is certainly the most attractive, but innumerable implications can be imagined. Two different approaches have attempted to reproduce corticogenesis (and, even more surprisingly, brain morphogenesis) using hPSCs: quick re-aggregation of cells was determinant in the first case (Eiraku et al., 2008; Kadoshima et al., 2013), while larger and more complex structures (cerebral organoids) were achieved in the second case using a spinning bioreactor (Lancaster et al., 2013). Each approach generated a complex, multilayered structure that was able to self-organize and possessed morphogenetic features highly reminiscent of brain development. Among the most impressive: formation of an apical and basal membrane and specification of the anterior-posterior axis. Nonetheless, the specification of a larger number of brain areas (e.g., sub-pallial structures) was only detectable within cerebral organoids. This finding is particularly relevant because it has permitted the recapitulation of a key process of brain development that is highly correlated with numerous cortical deficits, such as the invasion of the neocortex by migrating GABAergic neurons originating in the sub-palladium.

Corticogenesis itself was also partially recapitulated in both cellular systems. Cortical development is a highly dynamic and complex process in which an internal layer of neuroepithelial progenitors (the ventricular zone) generates post-mitotic neurons that migrate and stratify in an inside-first outside-last fashion depending on their birth date (Molyneaux et al., 2007). As expected, the cardinal features of the neuroepithelial progenitors, already identified in neural rosettes, were also observed in the

apical or luminal side of 3D structures, where progenitors reside. Further characterization of these cells revealed features typical of radial glial cells (RGCs, late progenitors of the neocortex), such as apical and basal processes. Moreover, a population of transient Tbr2-positive amplifying cells (which normally occupy the sub-ventricular zone of the developing cortex) was also identified adjacent to RGC-like cells. It is worth noting the description of a third type of progenitors predominantly derived from human cultures. The appearance of these cells (mainly characterized by the absence of the apical process) is highly reminiscent of outer-radial glial (oRG), an important hallmark of human neurogenesis in contrast to murine neurogenesis. The abundance of these cells in the developing human cortex has been associated with more complex structural features, such as cortical gyrification; thus, the presence of oRG-like cells indicates the faithful reproduction of human corticogenesis in 3D culture.

The identification of a progeny of post-mitotic neurons that form layers that are positioned basally over proliferative areas according to their time of birth has further confirmed the identity of the progenitor niche. The distribution and specification of post-mitotic neurons in quickly re-aggregated cells and in cerebral organoids has managed to recapitulate the early events of corticogenesis, although the realization of a fully layered cortex still remains to be established. Nonetheless, a simpler but equally laminated neuroepithelial structure, the retina (Eiraku et al., 2011; Nakano et al., 2012), was reproduced *in vitro* in similar culture conditions, once again confirming the ability of neuroepithelial cells to self-organize. The importance of this cardinal feature might lie in the ability of these cells to polarize (and consequently, to form rosettes). It was recently suggested that the same processes, polarization and rosette formation, are fundamentally involved in epiblast specification and self-organization, which emphasizes the intriguing hypothesis of a possible common mechanism in tissue morphogenesis.

The advent of 3D cultures can significantly change the way we see not only regenerative medicine, but also cell research en bloc. Although this system can provide unique information, great effort will have to be deployed to shepherd these expectations into solid reality.

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Therapeutic potential of neural stem cells: greater in people's perception than in their brains?

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The discovery that the mammalian brain contains neural stem cells, and that such cells produce new neurons during adulthood through a process known as "adult neurogenesis," led to the hypothesis that their underlying biology could be exploited for central nervous system (CNS) repair purposes. Yet, in spite of the large amount of knowledge gathered on adult neurogenesis during the last two decades, no substantial translational advances have been reached for most neurological diseases (Rossi and Cattaneo, 2002; Arenas, 2010; Lindvall and Kokaia, 2010). This may be linked to the intrinsic complexity of the nervous tissue at the structural, developmental, and evolutionary level, that preclude cell regeneration and repair (Weil et al., 2008; Bonfanti, 2011). As a result, neural cell replacement is at present not possible neither by implementation of endogenous neurogenic potential nor through transplantation of highly neurogenic stem cell sources.

However, in the gold rush-like phenomenon that has followed the discovery of neural stem cells the scientific community appeared often unaware of the limits associated with endogenous cell replacement and underestimated some problems and hurdles, which remain unknown to the general public and are sometimes undetected by the scientists themselves. In addition, the misleading communication of a series of basic scientific results that are often biased toward their possible translation, along with an amplification of interpretation from the media to the public have led to the perception that new therapeutic "possibilities" were at reach. What has been missed is a real vision of the state of the art: the present inability of medical science and fundamental research to overcome the gap between *in vitro* stem cell behavior and their adaptation to brain environment, as well as the need of more fundamental research prior to proposing therapeutic perspectives. As a consequence, the complex intermix emerging from stem cell discoveries, scientists' press releases, media, and society has produced substantial failure in the communication of scientific results, ultimately putting pressure toward premature translation of still insufficient pre-clinical data. In this Opinion article we suggest that a better communication from scientists to the public, without distortion by media and scientists themselves, is needed to avoid misunderstanding and to increase trust and constructive dialogue between science and society.

STEM CELL DISCOVERIES, ADULT NEUROGENESIS, AND THE TRANSLATIONAL GAP

Since the beginning of the nineties, the fact that adult neurogenesis can occur in mammals, at least within certain brain regions, led many scientists to exploit this endogenous capacity of the CNS for reparative/regenerative strategies. In parallel, several attempts have been put in place to bring the neural stem/progenitor cells in a dish or to obtain them from pluripotent stem cells. Yet, very small advances in the translation of this knowledge toward brain repair have been achieved. Many breakthroughs have been obtained in culture systems, thus increasing our ability to grow and manipulate stem cells (from the old neurosphere assay to the most striking example of the induced pluripotent stem cells—iPS—from the Yamanaka lab or of the direct reprogramming strategy). Yet, what hampers the possible therapeutic exploitation of stem/progenitor cells is a gap of knowledge between their activity/product and the host tissue environment. Paradoxically, the discovery of adult neurogenesis as a process which destroys the dogma of a static brain also shows that the exceptions are restricted to small neurogenic sites. Moreover, spontaneous neurogenesis in adult mammals is primarily linked to homeostatic roles and hardly directed to repair (Bonfanti, 2011). In other words, endogenous stem cells work well in their niches as do isolated stem cells *in vitro*, both sources of progenitors failing to properly act in the mature nervous tissue (normal and pathological). Hence, particularly in the CNS, unlike tissues that undergo continuous cell renewal (e.g., skin, blood, etc.), it is not granted that the goal of brain repair could be solved by gaining additional knowledge and/or by improving the availability of highly neurogenic stem cell sources.

Today, excellent protocols have been developed that recapitulate human brain development *in vitro* starting from human embryonic stem cells or human iPS cells. In the most extraordinary achievement, authentic dopaminergic neurons have, for the first time, been obtained which are capable of persistent functional recovery when transplanted into a mouse and rat model of Parkinson's disease (Kriks et al., 2011). In spite of such progress, it is still

unclear how these cells which are highly plastic in culture systems may function and persist long-term *in vivo* and whether a permanent reconstruction of damaged brain circuits in adulthood may be realistic (Rossi and Cattaneo, 2002). Furthermore, quite surprisingly, in the large literature on adult neurogenesis and neural stem cells there are a few reports analyzing the factors hampering brain repair following the use of exogenously delivered and/or endogenous stem/progenitor cells; most studies are focused on the other side of the coin, e.g., the factors promoting neurogenesis.

While pursuing the cell replacement strategy, a further therapeutic approach might be that of exploiting the neuroprotective and immune modulatory capacities of both transplanted (Martino et al., 2011) and endogenous (Kokaia et al., 2012) stem/progenitor cells. Yet, the recent discovery of such "bystander effect" foreseeing transplanted cells as biological minipumps able to release beneficial factors, although promising under the profile of research, can be a further source of confusion between scientists, physicians, and patients. At the present time, more than 300 clinical trials have been started in the world to test the effect(s) of different stem cell sources in neurodegenerative diseases (Donegà et al., 2013). In most cases, little is known about the mechanisms by which different stem cell lines are expected to function *in vivo*, what is their survival and distribution, what are the beneficial (rather than detrimental) factors they release, whether their release is sustained over time or, rather, represent an acute reaction after transplantation which may vanish at prolonged survival times, and which aspect of the neuropathology is expected to be targeted.

In conclusion, it is our perception that the translation of basic knowledge discoveries on both endogenous and exogenous stem cells into robust, biologically, and therapeutically relevant cell-based strategies for neurological diseases still remains a far-reaching goal which requires further years of research, possibly with the notable exception of Parkinson's disease. In parallel, current adult neurogenesis research, although largely not tailored toward restoration and regeneration, might yield new perspectives through insight into fundamental principles of plasticity and particular roles of new neurons in the brain, most notably in the hippocampus (Kheirbek et al., 2012; Snyder and Cameron, 2012; Freund et al., 2013). Yet, when communicating science, a clear distinction should be made between translational perspectives having indirect implications for our understanding of aging and brain disease, and those directed at obtaining cell replacement goals.

STEM CELL DISCOVERIES: SCIENTISTS, MEDIA, AND SOCIETY

As for many other fields, the stem cell arena has been characterized by repeated breakthroughs resulting in the public perception that "stem cells" act like magic bullets that can cure many diseases. Because of this high level of expectation, it has become increasingly difficult to maintain a balance between crude reality, realistic perspectives, and unjustified hopes. This failure in the dissemination of complex scientific concepts depends on the actions carried out by people bringing different kinds of responsibilities at various levels of the communication process. In particular, during the communication process, a number of significant steps may be overlooked as follows: (i) the original communication of the results obtained within the scientific community (scientific papers) is often too unbalanced toward the possible translation of basic science discoveries; (ii) there is an overemphasized release of information concerning the publication of a paper to the media; (iii) the above is followed by an amplification of interpretation/communication from the media to the public (which always stresses the "possible"—but at present non-existing—therapeutic use of a new discovery without paying any attention to the discovery process and to the value of the knowledge-acquisition process); (iv) patients and/or their families are often exposed to simplified interpretations of new therapeutic "possibilities"; (v) there is frequent misunderstanding about the real role of clinical trials in ascertaining the effectiveness and security of a given cell-based strategy; (vi) the recent description of the so called "bystander effect" has become a further source of overestimation of stem cell potential by incompetent physicians. What is not clearly perceived is the inability of medical science and fundamental research to overcome the gap between stem cell potential to regenerate new neurons and the non-permissive CNS environment preventing cell integration. Ultimately, a general thinking has prevailed supporting the view that stem cell treatments can produce new neurons in the diseased brain or that the bystander effects of given stem cells are already "therapies."

A consequence of such misunderstanding has been particularly deleterious in Italy, where last year political decisions have led to the authorization by the Parliament of the first governmental sponsored clinical trial intended to evaluate an unverified cell-based "treatment." This treatment was without a scientific basis, ineffective, and dangerous. Such a decision has been taken under the pressure of a small group of protesting lay people and patients without involving scientists and experts in the field. The State-sponsored trial has been stopped by the current Ministry of Health on the basis of a negative evaluation by an *ad hoc* appointed scientific committee. However, as we write, a regional court declared that committee unlawfully biased, and a new committee has now been nominated. Protesters and patients, however, are still in tribunals, several of them having authorized the injection of the same, unproven, unknown, and ill-prepared "stem cell potion" into people with diseases as severe as Parkinson, Amyotrophic Lateral Sclerosis, Spinal Muscular Atrophy, or even coma on the basis of a "constitutional right for a cure."

CONCLUSIONS AND POSSIBLE SOLUTIONS

There are several gaps and distortions in the process of science communication on regenerative medicine in general—and for neurological diseases in particular—that can lead to serious abuses, misunderstanding, and heavy consequences for the patient's life. It is necessary to address such issues in the future by identifying the single steps and responsibilities in order to prevent/overcome the problem. One possible solution might be the introduction of an additional regulation of stem cell therapies, as suggested in a recent paper (Bianco et al., 2013): "The scientific community must consider the context—social, financial, medical, legal—in which stem cell

science is currently situated and the need for stringent regulation. Additional concerns are emerging. These emanate from the novel climate, created within science itself, and stem cell science in particular, by the currently prevailing model of "translational medicine." Only rigorous science and rigorous regulation can ensure translation of science into effective therapies rather than into ineffective market products, and mark, at the same time, the sharp distinction between the striving for new therapies and the deceit of patients."

Yet, such a remedy might be ineffective in the absence of a more direct involvement of scientists into the communication process. In this context, the biological issues involved in the process of adult neurogenesis viewed as an *in vivo* product of neural stem cell activity, are even more tight to be grasped. The different ways by which spontaneous and/or lesion-induced neurogenic plasticity might be exploited for therapeutic strategies must be clearly explained to the public, as well as it should be made clear that long-term efforts are required to test how effective such strategies might be. Recently, the neuroscientist David M. Eagleman focused on the dilemma: "Communicating science to the public takes time away from busy research careers. So why would you do it?" (Eagleman, 2013). His answer consisted of six reasons which embrace the whole meaning of science and its beauty, from the need to acknowledge the funders, to stopping the flow of bad information, to the need of clarifying what science is and is not. These tasks should remain within the responsibilities of scientists themselves because that is exactly what they are "well set up for."

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Major unsolved points in adult neurogenesis: doors open on a translational future?

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In spite of many data gathered during the last two decades on adult neurogenesis (AN) it is evident that such knowledge is not sufficient for granting translational outcomes in brain repair, especially if the ultimate goal is to promote cell replacement. Alternative strategies aimed at fostering AN physiological functions (restorative approaches) are still undefined. By asking the question whether AN research field has to be considered as a dead end in the context of brain repair, here we review some unresolved issues: multifaceted evolutionary constraints in mammals, stem/progenitor cell type/availability and tissue permissivity, impact on other brain functions, interplay with other forms of plasticity, and relevance in humans. We suggest that full understanding of AN biology is an essential step for its possible exploitation in brain repair, and that further fundamental, multidisciplinary research is required to reach translational outcomes. Scientist's attitude and their communication skills are also important. To avoid overestimation of AN reparative potential in a translational perspective, more distant goals of cell replacement should be kept clearly distinct from restorative approaches involving AN functional plasticity.

Keywords: brain repair, neurodegenerative diseases, regenerative medicine, therapeutic approaches, neural stem cells, parenchymal progenitors, cell therapy, brain evolution

Two decades of investigation on adult neurogenesis (AN) yielded an utterly new vision of brain plasticity and opened new perspectives for brain repair/regeneration strategies. Nevertheless, the ultimate goal of exploiting neurogenic processes for obtaining cell replacement is still far from being achieved. Starting from this antinomy, the big question is: should be the AN research field considered as a dead end in the perspective of brain repair, or, alternatively, is it worthwhile to put in place further efforts in order to solve the problem? By reading the scientific literature, it is clear that all neurobiologists, even believing in an AN translational future, do not share the same answer. Non-univocal visions are normal in a field that has developed by progressively ramifying in many directions accordingly to the different goals pursued by each research group. Some scientists primarily deal with AN physiological roles/mechanisms, apparently being less interested in direct translation of results. Others are mainly focused on aspects that implement AN, or directly address the issue of injury-induced, reactive neurogenesis, paying less attention to the peculiar limits of the mammalian CNS in repairing damage. New translational perspectives in "restorative" rather than "structural reparative" neurology have been recently raised, what could be useful in slowing down the impact of various neurologic impairments (e.g., those occurring in neurodegenerative, vascular, traumatic diseases, age-related cognitive decline), even in the absence of cell replacement. Nevertheless, it is evident that knowledge gathered during the last two decades is not yet sufficient for granting translation of basic neurobiological research. Such inability is linked to several unresolved issues in both physiological and lesion-induced neurogenesis, and to scarcely integrated views between different approaches used to address AN studies. In other words, even in the absence of current, effective therapeutic outcomes, we may not be at a dead end, rather we are in the middle of a route with many new "perfectly reasonable deviations from the beaten track" (Feynman, 2005).

THE PRESENT KNOWLEDGE IN MAMMALS: SOME LIGHTS IN THE DARK

Our knowledge of AN in mammals might be grouped in two domains: first, some facts and concepts which are definitively accepted and substantially understood by the scientific community ("acquired knowledge"), and second, a number of issues which remain largely obscure and/or underestimated ("gaps of knowledge"). The main blocks of acquired knowledge can be summarized as follows: (i) two canonical neurogenic zones (subventricular zone, SVZ, and subgranular zone, SGZ) harboring stem cell niches provide neural cell addition into the olfactory bulb and hippocampus (Ming and Song, 2011); we know a lot about their anatomical organization and functional regulation as well as the integration of the newly born neurons (Fuentealba et al., 2012; Tong and Alvarez-Buylla, 2014; Vadodaria and Gage, 2014); (ii) wide areas of the central nervous system (CNS) out of the canonical neurogenic sites host cycling and/or quiescent progenitors which give rise to different processes of non-canonical cell genesis: parenchymal gliogenesis (Boda and Buffo, 2010; Trotter et al., 2010), parenchymal neurogenesis (Bonfanti and Peretto, 2011) and periventricular neurogenesis (Migaud et al.,

2010); little is known about non-canonical cell genesis, which seems to lack integration within the parenchyma; (iii) progenitors in both canonical and non-canonical neurogenic sites are activated in different pathological conditions (Arvidsson et al., 2002; Luzzati et al., 2011); in spite of such activation, the response to injury is substantially non-coordinated and/or abortive, not leading to effective brain repair (Kernie and Parent, 2010; Bonfanti, 2011; Bonfanti and Peretto, 2011).

Behind these blocks of acquired knowledge large amounts of unknown facts and concepts are still hidden. First of all, AN remarkable plasticity has introduced a new kind of complexity: that of dynamic, developmental-like processes related to neuronal addition occurring within a substantially static tissue. Moreover, in mammals, the CNS is structurally, functionally, and evolutionarily refractory to repair, healing, and regeneration. These facts make it extremely challenging to exploit AN as a therapeutic tool, also because the variables involved are dependent among each other and linked by different hierarchies (**Figure 1**). Here, we will analyze the key points still remaining open in the AN field, considering them as potential hurdles to a full understanding of the biological process itself, and, in turn, to its possible exploitation for brain repair.

AN PHYSIOLOGICAL FUNCTION(S) vs. BRAIN REPAIR: EVOLUTIONARY ASPECTS

Unlike most vertebrates, in adult mammals spontaneous neurogenesis is primarily linked to homeostatic/physiological roles and hardly directed to repair (Bonfanti, 2011). This view is supported by many studies which addressed the issue of reactive (lesion-induced) neurogenesis, indicating "activation" of neural progenitors which substantially do not provide cell replacement, most of the newly born/mobilized cells being fated to die (Kernie and Parent, 2010; Luzzati et al., 2011).

The fact that many non-mammalian vertebrates can perform brain repair/regeneration (Endo et al., 2007; Grandel and Brand, 2013) underlines the involvement of evolutionary aspects at the developmental, anatomical, stem cell types/availability and tissue-specific environment levels (Bonfanti, 2011). The occurrence of AN in the CNS of all vertebrates suggests the naive (and wrong) view of a direct relationship between neurogenic activity and regenerative capability (Ferretti, 2011): AN is not sufficient for regeneration to occur, and other aspects should be considered. Beside lower intrinsic regenerative properties (and lower availability of stem/progenitor cells), the mammalian CNS is characterized by more detrimental tissue reactions, in fact hampering regeneration. Immune cell activation leading to inflammation is an early response after injury that is common to most animal groups. Yet, whereas in many non-mammalian vertebrates initial acute inflammation stimulates regeneration without subsequent detrimental tissue responses, in mammals neuroinflammation leads to the formation of the glial scar with consequent impairment of regeneration (Mescher and Neff, 2006; Sofroniew, 2009; Kyritsis et al., 2014). In other words, stem/progenitor cell availability alone cannot grant regenerative capacity if glial cell activation and inflammatory reactions also occur. A theory explains the failure in mammalian brain repair as a result of evolutionary constraints in which the injured CNS would not favor a strategy of regeneration, but rather one of minimizing further damage (Weil et al., 2008). Hence, important gaps of knowledge still exist, both in mammals and other vertebrates, concerning homeostastic/metabolic functions and tissue reaction aspects linked to AN, and the role of the immune system, which still remain largely unexplored (Schwartz et al., 2013). All these variables are involved in determining the differences between neurogenic and non-neurogenic tissue local environments, and, in turn, their permissivity to reparative processes.

PROGENITOR CELLS, TISSUE ENVIRONMENT, AND AN OUTCOME(S)

Although cell proliferation exists throughout the intact CNS and is enhanced by several physiological/pathological conditions, it does not produce substantial neurogenic outcomes in the parenchyma out of the canonical sites (olfactory bulb, dentate gyrus). The main aspects that seem essential in granting CNS neurogenic/reparative capacity are: occurrence of specialized progenitor types and tissue permissivity. The SVZ and SGZ neurogenic niches harbor stem cells that appear very specified in their commitment (Obernier et al., 2014) and thus hard to divert toward other fates. As to parenchymal neural progenitors in non-canonical sites it is not yet clear what is their origin, nature, fate, and function(s). Yet, these cells do represent promising substrates for future research for several reasons (abundance, widespread distribution, region-specific differentiative commitment). Wherever stem/progenitor cells are located, both in canonical and non-canonical sites, unraveling the mechanisms underlying their quiescence or activation is also essential for their possible manipulation (Basak et al., 2012). Nevertheless, the functional availability of proper stem/progenitor cells is not sufficient to grant AN and repair in the absence of a receptive tissue environment: olfactory bulb and hippocampus circuits are permissive to neuronal integration, whereas the mature parenchyma allows less or no integration (Bonfanti and Peretto, 2011).

Taken together, these facts add hurdles to the ultimate goal of making mammalian AN processes useful for cell replacement. In spite of a large amount of data concerning the regulation of canonical AN (in terms of modulation; see Kempermann, 2011), very little is known about the cellular/molecular factors which allow the interaction between progenitors and the mature CNS tissue (permissivity) both in neurogenic and non-neurogenic sites. Such tissue permissivity is strictly linked to intrinsic features (adhesion molecules, extracellular matrix, availability of growth factors, etc.) which are maintained and/or delayed from development, thus allowing the AN process to persist during adulthood. In this context, a few studies have thoroughly investigated the steps that drive and regulate the shift between embryonic and AN.

ANIMAL MODELS vs. HUMANS

Most knowledge on AN has been gathered on laboratory rodents, what is a clear limit for translational approaches. Indeed, neurogenic processes differ quite among mammals as to their location, rate, niche organization, and the postnatal temporal windows in which they occur (Bonfanti and Peretto, 2011). For instance,

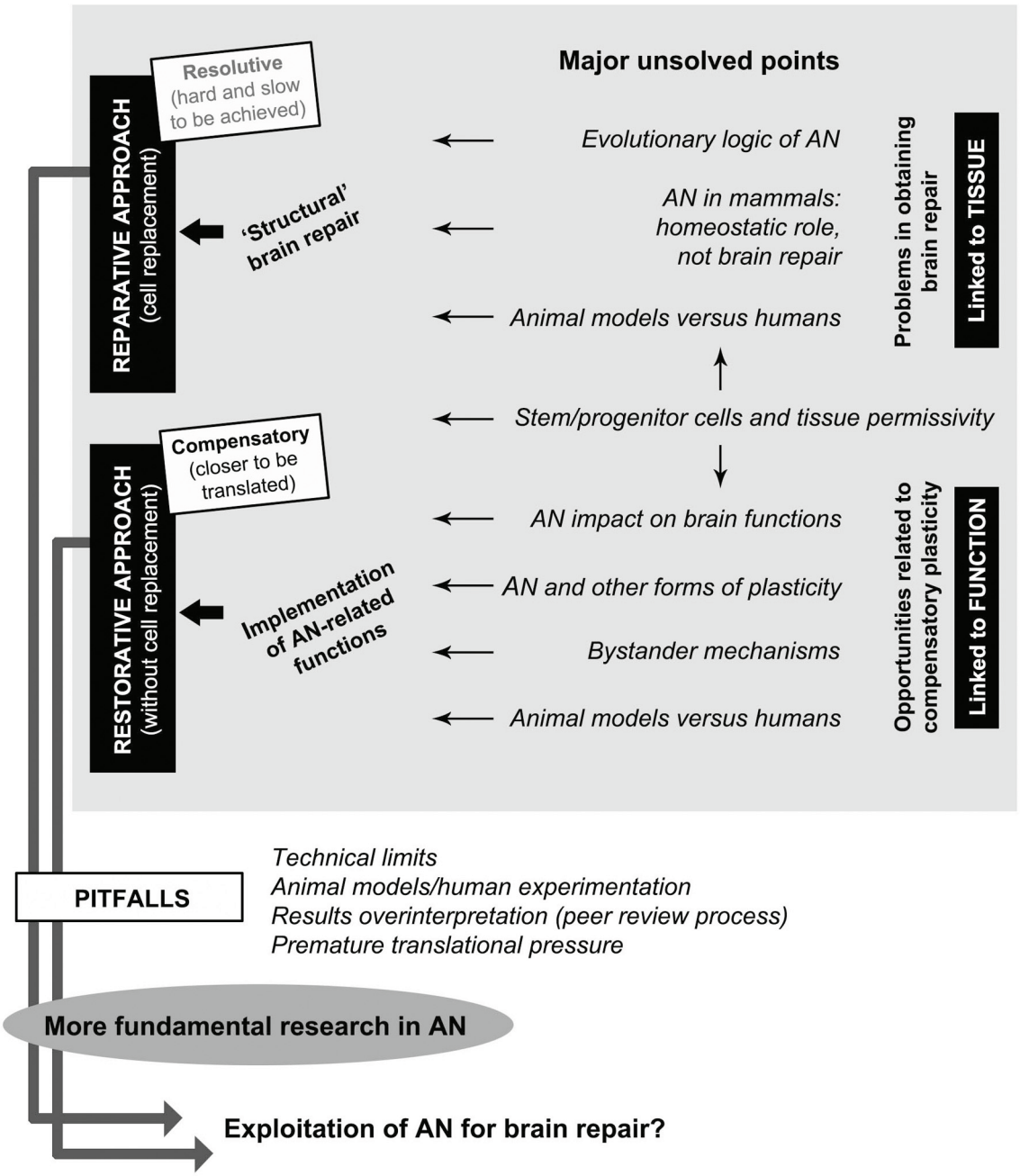

FIGURE 1 | Interplay between adult neurogenesis (AN), some of its major unsolved points, and possible perspectives for brain repair. Gray box: among many open issues still existing in the potential role of AN in neural plasticity (gaps of knowledge), a general distinction should be made between: (i) tissue-related problems depending on evolutionary issues and hampering brain repair/regeneration (top), and (ii) function-related opportunities depending on possible homeostatic roles of AN which could be exploited/implemented for restorative approaches (bottom). Stem/progenitor cell availability and tissue permissivity (middle) are essential aspects for allowing translational perspectives to be figured out in both directions. Reparative approaches, which imply cell replacement as the ultimate goal of regenerative medicine, are not available at present. Restorative approaches include different therapeutic perspectives linked to the implementation of physiological functions of AN aimed at obtaining compensatory plasticity in the absence of cell replacement, both in the damaged and undamaged (age-related decline) brain. Successful achievement of these goals is linked to further investments in fundamental research by overcoming of current pitfalls in the AN field.

while cell migration from SVZ to olfactory bulb persists throughout life in mice, it is exhausted very early in human infants (Sanai et al., 2011). In the human hippocampus, measures of ^{14}C concentration in genomic DNA show a substantially constant rate of AN through ages, in contrast with an evident decrease in young rodents (Spalding et al., 2013). Also parenchymal neurogenesis remarkably varies among mammals, showing species-specific regional localizations (Luzzati et al., 2006; Ponti et al., 2008).

Other inter-mammalian differences concern specific functions related to the ecological needs and behavioral activity of the animals (Barker et al., 2011).

Besides AN heterogeneity, mammals also differ in their brain anatomy and physiology (Carlson, 2012), and this can affect the impact AN might exert on the whole brain function (see below). Also the average lifespan varies among mammals, thus implying that differences in postnatal development of CNS areas, brain maturation, puberty, make it difficult to compare AN in different species (Lindsey and Tropepe, 2006; Kuhn and Blomgren, 2011). Hence, restraining AN research to laboratory rodents may introduce several bias in the search for translational outcomes. If comparative/evolutionary studies through phylogeny are essential to unravel the common logic of AN, the study of differences among mammals are also important for correctly interpreting/modeling the possible contribution of AN to homeostasis and brain repair in humans.

TO WHICH EXTENT AN IMPACTS THE BRAIN FUNCTION?

Addition of newborn neurons in the olfactory bulb and hippocampus optimizes neurological functions/behaviors such as social interaction/reproduction, memory, learning, and pattern separation (Sahay et al., 2011; Feierstein, 2012). These two brain regions are essential for behavioral outputs critical for survival of the individuals and species (Mucignat-Caretta et al., 2012; Snyder and Cameron, 2012). Accordingly, AN is assumed as a mechanism which promotes life-long adaptability of individuals to environmental complexity and novelty (Freund et al., 2013). Regulation of AN is achieved through integration of multiple external and internal stimuli, which implies activity of multiple brain regions/circuits and complex feedback loops (Kempermann, 2011). Thus, though restricted to the olfactory bulb and hippocampus, AN potentially impacts diverse brain functions (Snyder and Cameron, 2012; Lepousez et al., 2013), which might explain that in mammals it occurs only in two regions. Although little is known on this hypothesis, since the anatomical, functional, molecular bases underlying the above mentioned interactions are far from being clarified, the possible impact of AN on other brain functions/circuits can have important translational implications (Leuner and Gould, 2010; Kheirbek et al., 2012; Snyder and Cameron, 2012; Quadrato et al., 2014). Several data are already available on the link between hippocampus, pattern separation/overgeneralization of sensory stimuli and anxiety disorders (Leuner and Gould, 2010; Kheirbek et al., 2012). The recent finding that human hippocampal AN appears substantially maintained during adulthood (Spalding et al., 2013) adds new interest to this issue, also in the perspective of implementing cognitive functions during aging (Bordey, 2014). Yet, proper translational outcomes imply definitive clarification of the real rate/impact of AN in humans during postnatal development and adulthood, in physiological, and pathological condition.

Finally, if AN does extensively affect the brain function(s), it should be emphasized that it is only one among other forms of CNS plasticity and that very little is known about the mechanisms which underlie their mutual relationships.

AN AND OTHER FORMS OF PLASTICITY AND/OR REPAIR STRATEGIES

The CNS of mammals, in spite of having lost most of its regenerative/repair capacity with respect to other phyla, is endowed with different forms of structural plasticity involving pre-existing cellular elements (Bonfanti and Nacher, 2012). Among them, the most known and widespread is the experience-dependent synaptic plasticity that can occur in response to environmental enrichment and after a lesion in the form of compensatory events, i.e., synaptic formation/elimination and axonal sprouting/pruning (Brown et al., 2009; Chen and Nedivi, 2010; Fu and Zuo, 2011). Further levels of structural plasticity are found in a population of "immature," non-newly generated neurons of the cerebral cortex (Gomez-Climent et al., 2008). These cells, in spite of their differentiated neuronal morphology (Luzzati et al., 2009), express immature neuronal markers and show very few synapses on their membrane, thus not being integrated in the adult cortical circuits, like "stand by" elements (Bonfanti and Nacher, 2012). All these forms of structural plasticity could be useful in rehabilitation approaches that mostly exploit compensatory plasticity of undamaged, preexisting structures (Dobkin, 2004). If and how all these forms of plasticity are integrated with AN is a fully open question, also taking into account that mammalian AN itself consists of heterogeneous processes involving the canonical neurogenic niches and progenitors located throughout the CNS tissue (non-canonical cell genesis; Boda and Buffo, 2010; Bonfanti and Peretto, 2011).

A better knowledge of the mutual relationships existing within the vast landscape of neural plasticity is fundamental to correctly figure out restorative therapeutic approaches in neurology (**Figure 1**). In recent years, several studies have started to unravel new modes of communication between stem/progenitor cells (endogenous or transplanted) and resident cells of the CNS, also involving a cross-talk with the immune system. This communication is at the basis of the so called "bystander effects," namely a series of paracrine mechanisms which can exert beneficial effects even in the absence of cell replacement (Martino et al., 2011). An hypothesis supported by several works is that transplanted stem/progenitor cells can exert a bystander immune modulation by modifying the inhospitable microenvironment at the injury site through the release of soluble molecules such as chemokines and cytokines (Pluchino and Cossetti, 2013). More recently, it has been proposed that the same effects might be also exerted by cell mobilization/activation of endogenous stem/progenitor cells toward adjacent injured sites (Kokaia et al., 2012). In perspective, these studies have the added value of considering neural plasticity, AN, and brain repair in the context of a cross-talk between the CNS and the immune system, the latter being far more important than previously thought. Hence, the study of cell-cell interaction/paracrine communication does represent a fully open, promising field of research, aimed at developing "restorative" rather than "cell replacement" strategies.

SOME PITFALLS IN AN FIELD: INTERPRETATION OF RESULTS AND PEER REVIEW PROCESS

Since the beginning, following the emphasis of a new form of CNS plasticity, the reparative potential of AN has been overestimated

by the scientific community, at least under its possible regenerative outcome. This fact is reflected by statements contained in many papers dealing with both spontaneous and lesion-induced AN in which the results obtained are more or less directly linked with potential therapeutic outcomes, in the absence of direct evidence for such a link. These statements, although originally intended as "possibilities" by the authors, are frequently amplified by the media, thus generating unjustified hopes in patients affected by neurological diseases (this aspect is analyzed in Cattaneo and Bonfanti, 2014). The source of this problem is well addressed by Kerner (2006): "Many individual research reports, while suggesting exciting new innovations that may lie ahead in the future, have little or no immediate application in public health and/or clinical practice. Thus, it may be difficult for the practice community to distinguish the signal about what is currently important to practice from the noise of what may or may not become important in the future."

In the neurological context, restorative approaches in the absence of cell replacement, including modulation of physiological/paracrine functions (Martino et al., 2011; Bordey, 2014; Quadrato et al., 2014) should always be kept clearly distinct from the true reparative/regenerative processes involving cell replacement. In the history of AN scientific publications, from the initial "naïve" belief that AN could easily represent the biological substrate for cell replacement in the CNS, to a more recent overestimation of the bystander effect therapeutic potential, it appears that too many unjustified claims actually bypass the filter of peer review. We feel that this habit is not rewarding for the public representation of science and even not for the future of AN field.

FUTURE PERSPECTIVES: AN AND FUNDAMENTAL RESEARCH

It appears evident that having introduced excessive and premature translational issues toward brain repair did not solve the problem of neurological diseases. Moreover, a simplistic view of AN as a ready-made therapeutic tool could even be counterproductive, since it might put down the interest in AN studies. In spite of some oversights along the route, the increasing knowledge gathered during the last 20 years is enormous, considering the changes that AN research has produced in our vision of brain plasticity. Non-invasive technologies are essential to study AN in humans (e.g., Spalding et al., 2013 for ^{14}C detection), although further technical advancements are needed (e.g., cell imaging enhanced resolution; Sierra et al., 2011). Yet, because the AN field has become widely diversified both in its goals and lines of research (**Figure 1**), obtaining of future breakthroughs will require a multidisciplinary integration of high specific expertise in order to gain a whole transversal view of the variables involved (for the several meanings of translation/implementation concepts see Kerner, 2006). The current gaps of knowledge should be filled with new basic research prior to put them into a translational view. We can be confident that applied (beneficial and profitable) products of fundamental research will be eventually achieved in the future, although not visible/predictable at the time of the experimental phases (Press, 2013). Confidence in the eventual usefulness of basic research should be sufficient to bring back AN in the normal context of science.

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