# GENERATION OF NEURONS AND THEIR INTEGRATION IN PRE-EXISTING CIRCUITS IN THE POSTNATAL BRAIN: SIGNALLING IN PHYSIOLOGICAL AND REGENERATIVE CONTEXTS

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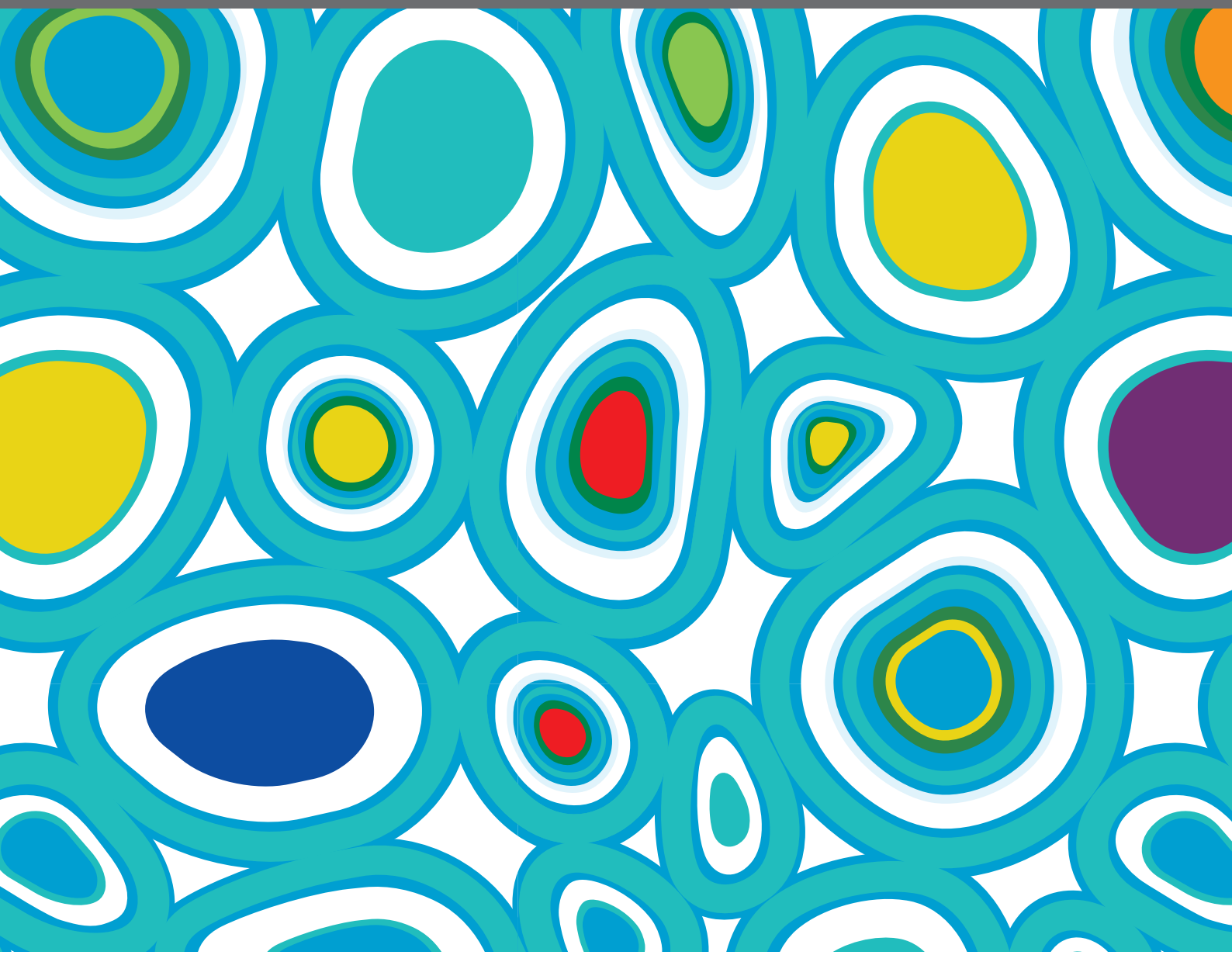

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# GENERATION OF NEURONS AND THEIR INTEGRATION IN PRE-EXISTING CIRCUITS IN THE POSTNATAL BRAIN: SIGNALLING IN PHYSIOLOGICAL AND REGENERATIVE CONTEXTS

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EDITORIAL
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# Editorial: Generation of Neurons and Their Integration in Pre-existing Circuits in the Postnatal Brain: Signalling in Physiological and Regenerative Contexts

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**Editorial on the Research Topic**

**Generation of Neurons and Their Integration in Pre-existing Circuits in the Postnatal Brain: Signalling in Physiological and Regenerative Contexts**

During the development of the nervous system, the production of hundreds of subtypes of neurons and glial cells relies upon the relatively fast generation, amplification, specification, and differentiation of neural progenitors and neural stem cells (NSCs). This strategy, although restricted, is retained in specific niches in the adult nervous system throughout lifetime under physiological conditions. Furthermore, damage to the neural tissue in certain circumstances triggers neurogenesis leading to regeneration.

Many of the signaling cascades governing neurogenesis and circuit formation during embryogenesis are harnessed by the adult brain niches, although their regulation is substantially different in the postembryonic context. The progression from neural progenitors to differentiated new neurons must adapt to a new context with novel states emerging such as the ability of adult progenitors to enter quiescence. In addition, newly born neurons differentiate and establish their connections in an already functional network where the signals that were used to guide circuit formation during development are no longer present.

In this Research Topic, we include both original and review articles covering essential aspects of the generation and maintenance of the most prominent neurogenic niches both in teleost and in mammals such as the optic tectum in zebrafish and the hippocampal dentate gyrus (DG), the ventricular-subventricular zone (V-SVZ), and the hypothalamus in rodents. The most relevant cellular populations in those niches [neural stem cells (NSC) and immature neurons] are discussed, as well as their regulation by intrinsic programs (such as cell cycle components) and extrinsic signals such as extracellular matrix (ECM). Later steps, crucial for the integration of new neurons in existing circuits, have also been covered.

Morales and Mira review the developmental origin of the quiescent pool of adult radial glia-like NSCs with a specific focus on the hippocampal DG niche. They furthermore discuss recent work unveiling the molecular program that regulates the establishment and maintenance of the quiescent NSC state in the rodent brain. ECM signals and adhesion molecules also play a fundamental role in the regulation of the quiescence-proliferation balance and in the maintenance of NSC stemness. These aspects and the functional and structural relevance of "fractones," a specific feature of the V-SVZ niche ECM, are thoroughly discussed in Morante-Redolat and Porlan.



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Urbach and Witte summarize the available evidence on the role of the cell cycle machinery in the integration of extrinsic signals during adult hippocampal neurogenesis. They also revisit the impact of cell cycle length on the proliferation vs. differentiation decisions that take place along the neurogenic process and provide interesting insight into alternative mechanisms of fate regulation during the G1 phase of the cell cycle.

In the context of the newly discovered hypothalamic niche in the mouse, van Lingen et al. examine *Tph2* mutant mice to show that serotonin, a neuromodulator related to several homeostatic functions such as food consumption, is required to maintain appropriate levels of neurogenesis. Thus, they demonstrate an age-dependent decline in cell proliferation in the hypothalamus of Tph2 mutant mice, not observed in control mice.

Rodríguez-Iglesias et al. offer a very integrative view of what is known about the different stages followed by newborn granule cells during their maturation and integration into the hippocampal circuit in rodents and address the main functions of microglial cells in that context. Direct microglia-neuron interactions and the role of released soluble factors in the structural and functional maturation of newborn neurons are discussed.

The elucidation of the general mechanisms of circuit assembly following neurogenesis, benefits from the study of neurogenesis in non-mammalian species, which offer the advantages of tracking the process *in vivo*. Boulanger-Weill and Sumbre review recent findings showing that newborn neurons in the optic tectum of the zebrafish larva require sensory inputs for their integration into local networks and survival, reinforcing the idea that both activity-dependent and hard-wired mechanisms are involved in proper circuit integration.

Understanding the normal physiology of neurogenesis and circuit formation, has important implications in the design of therapeutic strategies aiming to promote neurogenesis and the proper wiring of new neurons in pathological situations associated to neuronal loss, including degenerative diseases and nervous system injuries.

Temporally and spatially dysregulated neuronal activity in the mouse hippocampus upon seizure induction can alter the levels of neurogenesis and induce the conversion of NSCs into reactive-like astroglial cells. However, the methods of induction of seizures that rely on intrahippocampal injections of kainic acid could be directly affecting NSCs. By using kainic acid injection in the amygdala and analyzing the effects in the hippocampus Muro-García et al. show how the reduction of neurogenesis and the reactivity of NSCs upon seizure can occur by a mechanism that depends on the surrounding neuronal circuit activity.

The possibility to manipulate specific signaling cascades to potentiate endogenous neurogenesis in cases of neuronal damage is one of the promising approaches. Geribaldi-Doldán et al. review the evidence for a role of the Protein Kinase C (PKC) pathway in regulating neurogenesis and the identification of compounds that act on specific PKC isoforms that may serve to develop new drugs acting as pro-neurogenic without affecting proliferation of other tissues.

Another pathway that enhances proliferation and neurogenesis in some contexts, including the zebrafish brain and human astrocyte cultures, is the interleukin-4/STAT6 signaling. Mashkaryan et al. explore the possibility that this role can be extended to the dentate gyrus in an Alzheimer's disease mouse model and find that, actually, this endogenous *in vivo* context behaves as a non-permissive environment with a negative effect of IL4 signaling on astroglial survival and neurogenic properties, highlighting the need to consider each context in detail.

Context seems also important in the control of neurite growth during regeneration of functional connections. By following the growth of neurites in retina bipolar cells during regeneration after injury in the adult zebrafish, McGinn et al. identify differences with the process taking place during embryonic development but also leave open the possibility that it may be sufficiently robust to restore visual function.

Finally, environmental enrichment including the combination of increased physical activity, constant cognitive stimulation, and higher social interaction, stands out as a potent regulator of adult neurogenesis in the hippocampus. Moreno-Jiménez et al. identify a major contribution of social stimuli to the promotion of neurogenesis, characterized by an increased number of newborn neurons and higher morphological maturation of their dendritic arbors.

Overall, this special issue provides an updated picture of the field and highlights the importance of the interplay between intrinsic programs and niche signals. Together, they underlie the relative plasticity of neurogenesis and may constitute key targets for regeneration.

## AUTHOR CONTRIBUTIONS

RD and AM were the initial Guest editors of this Research Topic, inviting co-editor HM working with them to define the subjects to be treated. The three of them identified and invited leaders in specific research fields to contribute their work to the Research Topic. They acted as handling editors of manuscripts in the topic (except for Morales and Mira). They wrote the Editorial in coordination.

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REVIEW
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# Rewiring of Memory Circuits: Connecting Adult Newborn Neurons With the Help of Microglia

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New neurons are continuously generated from stem cells and integrated into the adult hippocampal circuitry, contributing to memory function. Several environmental, cellular, and molecular factors regulate the formation of new neurons, but the mechanisms that govern their incorporation into memory circuits are less explored. Herein we will focus on microglia, the resident immune cells of the CNS, which modulate the production of new neurons in the adult hippocampus and are also well suited to participate in their circuit integration. Microglia may contribute to the refinement of brain circuits during development and exert a role in physiological and pathological conditions by regulating axonal and dendritic growth; promoting the formation, elimination, and relocation of synapses; modulating excitatory synaptic maturation; and participating in functional synaptic plasticity. Importantly, microglia are able to sense subtle changes in their environment and may use this information to differently modulate hippocampal wiring, ultimately impacting on memory function. Deciphering the role of microglia in hippocampal circuitry constant rewiring will help to better understand the influence of microglia on memory function.

**Keywords: adult hippocampal neurogenesis, hippocampal circuit, memory, microglia, rewiring**

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## INTRODUCTION

The generation of hippocampal neurons during adulthood has been implicated in critical brain functions, such as memory formation, and pathological conditions, including mood disorders. Therefore, a strong research effort has been put into determining the factors controlling the integration of newly generated neurons into the hippocampus during adulthood, and their impact on brain function. Different memory related functions have been attributed to the new neurons that integrate into the adult rodent hippocampus, which include pattern separation, temporal codification, and memory clearance (Baptista and Andrade, 2018). The

persistence of hippocampal neurogenesis through adulthood in rodents is well established (Ming and Song, 2011), although it decreases exponentially over time (Beccari et al., 2017). In contrast, data on human neurogenesis are more conflicting, and there are recent reports to support either that it persists across human lifespan (Spalding et al., 2013; Boldrini et al., 2018) or, on the contrary, that it is limited to childhood (Sorrells et al., 2018). In any case, adult neurogenesis plays a more critical role during infancy, contributing to the codification and integration of early life experiences, and thus affecting the adaptability and function of the central nervous system (CNS) during adult life (Kempermann et al., 2018).

Adult hippocampal neurogenesis is tightly controlled by both cell-autonomous mechanisms as well as by the interaction with the molecular and cellular niche. In this review, we will focus on the contribution of microglia, the resident immune cells of the CNS, to the incorporation of adult generated neurons into hippocampal memory circuits. Microglia are especially well equipped to sense changes in the brain parenchyma and to interact with other cell types such as neurons and astrocytes (Kettenmann et al., 2011), and thus they are good candidates to act as mediators of the adaptive incorporation of newly generated neurons into hippocampal circuits, although few studies have addressed the role of microglia on neurogenesis directly (Valero et al., 2016, 2017). First, we will describe the main characteristics of hippocampal adult neurogenesis in rodents and how newborn cells incorporate into hippocampal circuitry. Then we will briefly introduce microglia and summarize their main functions in different contexts, such as development, adulthood or pathology, which may be relevant for the integration of new generated neurons into memory circuits. Finally, we will discuss evidence that suggest that microglia effectively play a role in neurite growth as well as in the structural and functional maturation of newborn neurons.

## ADULT HIPPOCAMPAL NEUROGENESIS AND ITS REGULATION

Neurogenesis persists during adulthood in many species from invertebrates to vertebrates, including mammals (Sullivan et al., 2007; Bonfanti and Amrein, 2018), although newly generated neurons are fewer than those generated in embryonic stages and restricted to specific areas of the brain. Adult neurogenesis is initiated by adult neural stem cells, which give rise to functional neurons in the adult CNS (Ming and Song, 2011). Neural stem cells are mainly restricted to two regions of the mammalian adult brain, known as adult neurogenic niches: the subventricular zone of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Altman, 1962; Altman and Das, 1965; Eriksson et al., 1998). In addition, adult neurogenesis has been described in other regions of the brain, such as the hypothalamus in rodents (Sousa-Ferreira et al., 2013) and the striatum in humans (Ernst et al., 2014). Here we will focus on the formation of new granular neurons in the SGZ of rodents, i.e., adult hippocampal neurogenesis.

Adult hippocampal neurogenesis encompasses sequential cellular stages known as the neurogenic cascade (**Figure 1**). The generation of new neurons begins with radial neural stem cells (rNSCs, also known as type 1 cells), which usually divide asymmetrically giving rise to a copy of themselves and an amplifying neuroprogenitor (ANP, also known as type 2 cells) (Bonaguidi et al., 2011; Encinas et al., 2011). ANPs increase their pool rapidly through symmetric divisions and differentiate into neuron-committed cells called neuroblasts. However, not all ANPs and neuroblasts survive, as nearly the 80–90% of newly generated cells die through apoptosis (Sierra et al., 2010; Beccari et al., 2017). The surviving neuroblasts continue differentiating into immature neurons, and they integrate in pre-existing hippocampal circuitries finally becoming mature granule cells (GCs), the most abundant type of excitatory neuron in the DG (Kempermann et al., 2015). The maturation of adult-born granule neurons lasts around 8 weeks (Toda et al., 2018), and it will be described in detail in the next section.

The above described neurogenic cascade is susceptible of modulation at different levels: (1) proliferation of rNSCs and ANPs; (2) survival of ANPs and neuroblasts; (3) differentiation into neurons; and (4) integration of adult-born GCs (Kempermann et al., 2004; Valero et al., 2016, 2017). These steps are regulated by a tightly controlled program that encompasses intrinsic (i.e., signaling cascades and transcription factors), as well as extrinsic factors. Among these later ones stand out circulating hormones and peptides (such as cortisol, growth factors, or inflammatory mediators), extracellular matrix composition, and neighboring cells that compose the cellular niche: adult neurons, astrocytes, blood vessels and microglia (Sierra et al., 2010; Ming and Song, 2011; Valero et al., 2012, 2016, 2017; Aimone et al., 2014). In this review we will specifically focus on how microglia modulates the formation of adult-born GCs exerting their functions on the different steps of the neurogenic cascade, especially on the integration of the new neurons to the hippocampal circuitry (**Figure 1**).

## GCs AND THE HIPPOCAMPAL CIRCUITRY

### GCs Connection to the Tri-Synaptic Circuit

The hippocampal connectivity is characterized by the well-known tri-synaptic circuit, in which pyramidal cells located in layer II of the entorhinal cortex (EC) connect to DG GCs, which send their output signal to the Cornu Ammonis subfield 3 (CA3). Then, the signal runs from CA3 to CA1 pyramidal neurons and from CA1 back to neurons of the EC layer V (**Figure 2**). Nonetheless, hippocampal connectivity is more complex and includes direct connections from EC to CA3 and CA1 (Amaral et al., 2007), as well as direct inputs from the

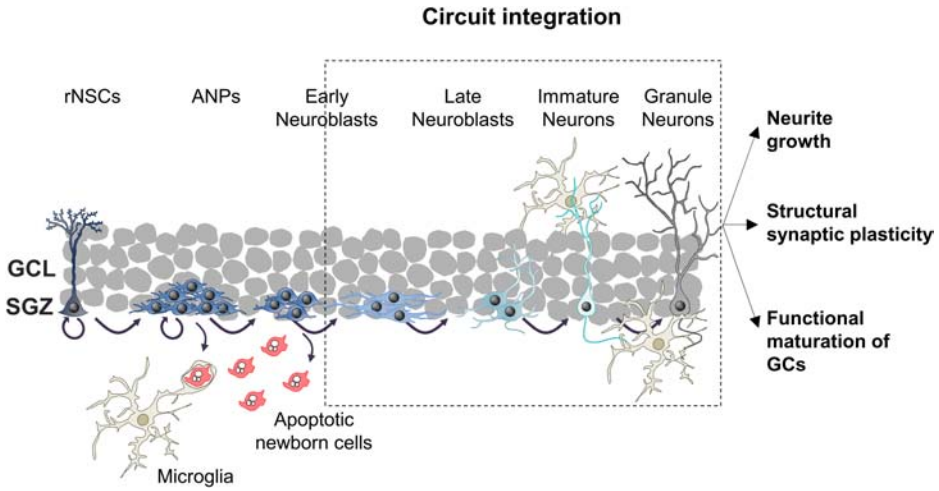


**FIGURE 1 |** Schematic drawing of the neurogenic cascade and the hypothetical roles of microglia on adult neurogenesis. ANPs, amplifying neuroprogenitors; GCs, granule cells; GCL, granular cell layer; rNSCs, radial neural stem cells; SGZ, subgranular zone.

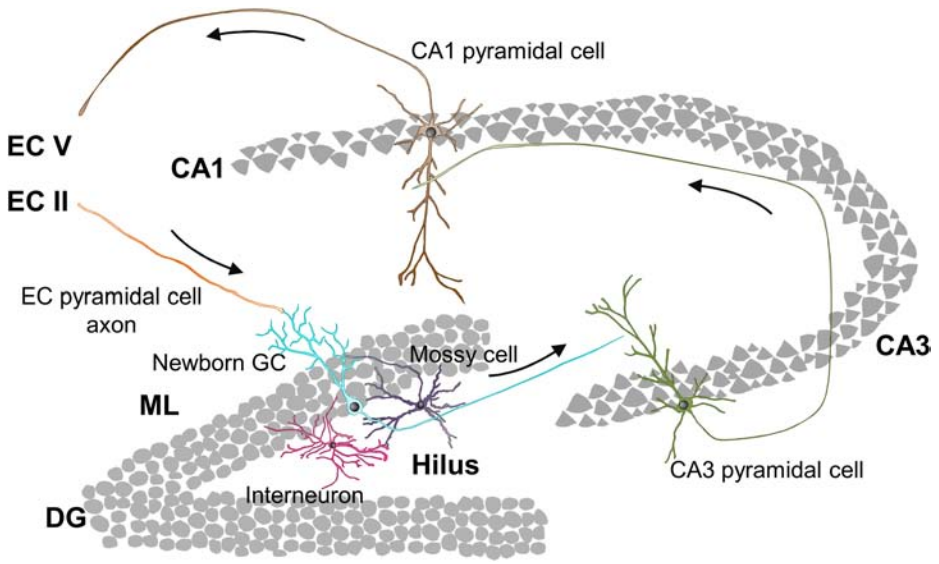


**FIGURE 2 |** Hippocampal tri-synaptic circuit. CA1, Cornu Ammonis subfield 1; CA3, Cornu Ammonis subfield 3; DG, dentate gyrus; EC, entorhinal cortex; GC, granule cell; ML, molecular layer.

perirrhinal cortex (PER) and back projections from CA to the DG (Binicewicz et al., 2016). Within the tri-syanptic circuit, both developmentally and adult generated GCs have similar connectivity: they extend their dendrites into the molecular layer (ML), where they receive glutamatergic synaptic inputs from PER and EC axons; and send their axons (mossy fibers) through the polymorph layer (hilus) to CA3, where they establish highly efficient excitatory multisynaptic contacts with CA3 pyramidal neurons (Toni et al., 2008; Vivar et al., 2012). GCs mossy fibers present axonal boutons, which envelop and contact protuberances (thorny excrescences) from CA3 dendrites which provide multiple synaptic contact regions increasing synaptic efficacy (Faulkner et al., 2008; Toni et al., 2008). In addition to forming the tri-synaptic circuit, GCs are also connected to the local DG circuit, which contributes to the refinement of the information passed to CA3 (**Figure 2**).

## GCs Connection to the Local DG Circuitry

DG local circuits mainly consist in feedback connections between GCs and interneurons. GCs excite GABAergic hilar and CA3 interneurons, which in turn inhibit GCs (feedback inhibition) and CA3 pyramidal cells (feedforward inhibition) (McAvoy et al., 2015; Prince et al., 2016). Furthermore, GCs provide excitatory inputs to glutamatergic interneurons residing in the hilus (mossy

cells), which play a dual role as they directly excite other GCs in the ipsi- and contralateral DG, but also indirectly inhibit GCs by acting on inhibitory interneurons (Scharfman, 2016). This internal excitatory/inhibitory system plays a crucial role in the processing of the input signal, in the refinement of the output sent to CA3, and in the maturation of adult-born GCs, as we will discuss in the following sections.

## INTEGRATION OF ADULT NEWBORN NEURONS INTO THE HIPPOCAMPAL CIRCUITRY

Given the connectivity of GCs, it seems obvious that the integration of newborn neurons into the mature circuit is a complex and highly coordinated process that involves a series of morphological and functional stages. Importantly, the appearance of certain characteristics, such as the maximum complexity of the dendritic tree or number of dendritic spines, may be delayed in older animals and affected by life related factors such as voluntary exercise (Trinchero et al., 2017). In this section we will review the different stages followed by newborn GCs during their maturation and integration into the hippocampal circuit in rodents (in mice, unless otherwise specified), how these stages affect hippocampal function, and summarize the factors that regulate this process.

### Stages of GCs Maturation

At 3 days to 1 week newborn cells have already differentiated into early neuroblasts but are still located at the SGZ and present short processes with no synaptic contacts (**Figure 3A**; Esposito, 2005; Zhao et al., 2006). The first neurotransmitter sensed by these young cells is non-synaptic, tonic GABA (γ-aminobutyric acid), the most abundant inhibitory neurotransmitter in the CNS (Tozuka et al., 2005; Ge et al., 2006; Song et al., 2013). At this stage and up to 3 weeks after cell birth, similarly to what happens during hippocampal development, GABA is excitatory and depolarizes maturing GCs due to their high expression of the sodium-potassium-chloride cotransporter NKCC1, which maintains high levels of intracellular chloride (Tozuka et al., 2005; Ge et al., 2006; Campbell et al., 2010; Spampanato et al., 2012). GABA sensing from new GCs at this stage is crucial for their differentiation and survival (Tozuka et al., 2005; Ge et al., 2006; Song et al., 2013).

One to two weeks after their birth, the dendritic tree of newborn GCs reaches the ML and their axons CA3 (**Figure 3A**). Data about the synaptic contacts they receive are contradictory. Initially, it was shown that at this time point maturing GCs only received synaptic contacts from GABAergic interneurons (Overstreet-Wadiche et al., 2005; Ge et al., 2006; Jagasia et al., 2009; Song et al., 2013; Alvarez et al., 2016). However, a more recent study revealed contacts from glutamatergic, GABAergic, and cholinergic neurons in 1-week old GCs (Sah et al., 2017). At this time point, synaptic GABA is necessary for the morphological maturation, synaptic integration, and survival of newly generated neurons (Ge et al., 2006; Jagasia et al., 2009; Song et al., 2013; Alvarez et al., 2016). Specifically, during a narrow temporal window from 9 to 11 days after their birth the activation of GABAergic parvalbumin interneurons by mature GCs is required for the increase in the complexity of dendrites and number of spines induced by environmental enrichment (Alvarez et al., 2016). The axons of maturing GCs reach the stratum lucidum of CA3 and CA2 1 and 2 weeks after their birth, respectively (Zhao et al., 2006; Faulkner et al., 2008; Llorens-Martín et al., 2015). The initial excitatory synaptic contacts from newborn GCs onto pyramidal cells of the CA3 occur 1 week after their birth, while connectivity into the CA2 has not been analyzed (Ide et al., 2008; Toni et al., 2008). Therefore, by their second week after birth a few maturing GCs are already wired into the hippocampal circuit as they receive excitatory inputs in the DG and contact with CA3 pyramidal cells.

Two to three week-old newborn GCs establish a first circuit, as they receive excitatory inputs in the DG, send projections to CA2 and CA3 pyramidal cells, and are able to fire their first action potentials (**Figure 3B**). The dendritic tree of maturing GCs reaches the inner ML 2 weeks after their birth. Several studies describe that the unique excitatory synaptic contact received by 2 week old GCs is established with hilar mossy cells (Esposito, 2005; Ge et al., 2006; Zhao et al., 2006; Deshpande et al., 2013; Chancey et al., 2014). However, a recent study indicates that other excitatory intrahippocampal neurons, such as mature GCs and CA pyramidal cells, already innervate maturing GCs (Sah et al., 2017). Nevertheless, dendritic spines on newborn GCs are not visible until day 16 after their birth (Zhao et al., 2006). In addition to give a direct excitatory input to maturing GCs, mossy cells also provide indirect depolarization to new GCs through activation of GABAergic interneurons, which combined with the weak excitatory input may help maturing GCs to develop their first action potentials (Chancey et al., 2014). Importantly, during the first 2–3 weeks after cell birth a moderated GABA input is able to induce action potentials in young GCs when paired to the weak glutamatergic input (Heigele et al., 2016). Indeed, at this stage, GABA depolarization is required for synapse unsilencing through activation of NMDA receptors and subsequent recruitment of AMPA receptors to synapses (Chancey et al., 2013). These data suggest that GABAergic mediated excitation of 2–3 week-old GCs may occur during regular hippocampal activity, and that it depends on glutamatergic input. Importantly, as previously indicated, GABAergic input is still essential for the survival of maturing GCs, which may be due to the relevant role of GABA on the firing of the first action potentials. Furthermore, NMDA receptor expression and activation is also crucial for the survival of maturing GCs specifically between weeks 2 and 3 after their birth (Tashiro et al., 2007); suggesting that, at this time point, both inhibitory and excitatory inputs support cell survival. At 17 days after birth, the mossy fiber boutons from newborn neurons form synapses mainly with the dendritic shaft of CA3 pyramidal neurons and dendrites from hilar and CA3 interneurons (Toni et al., 2008; Gu et al., 2012). Hence, at 3 weeks after their birth the majority of maturing GCs receives excitatory inputs in the ML and are connected to CA3 pyramidal cells.

Three to four week-old newborn GCs receive glutamatergic synaptic contacts from PER and EC axons and increase their synapses with CA3 pyramidal cell dendrites (**Figure 3C**). At

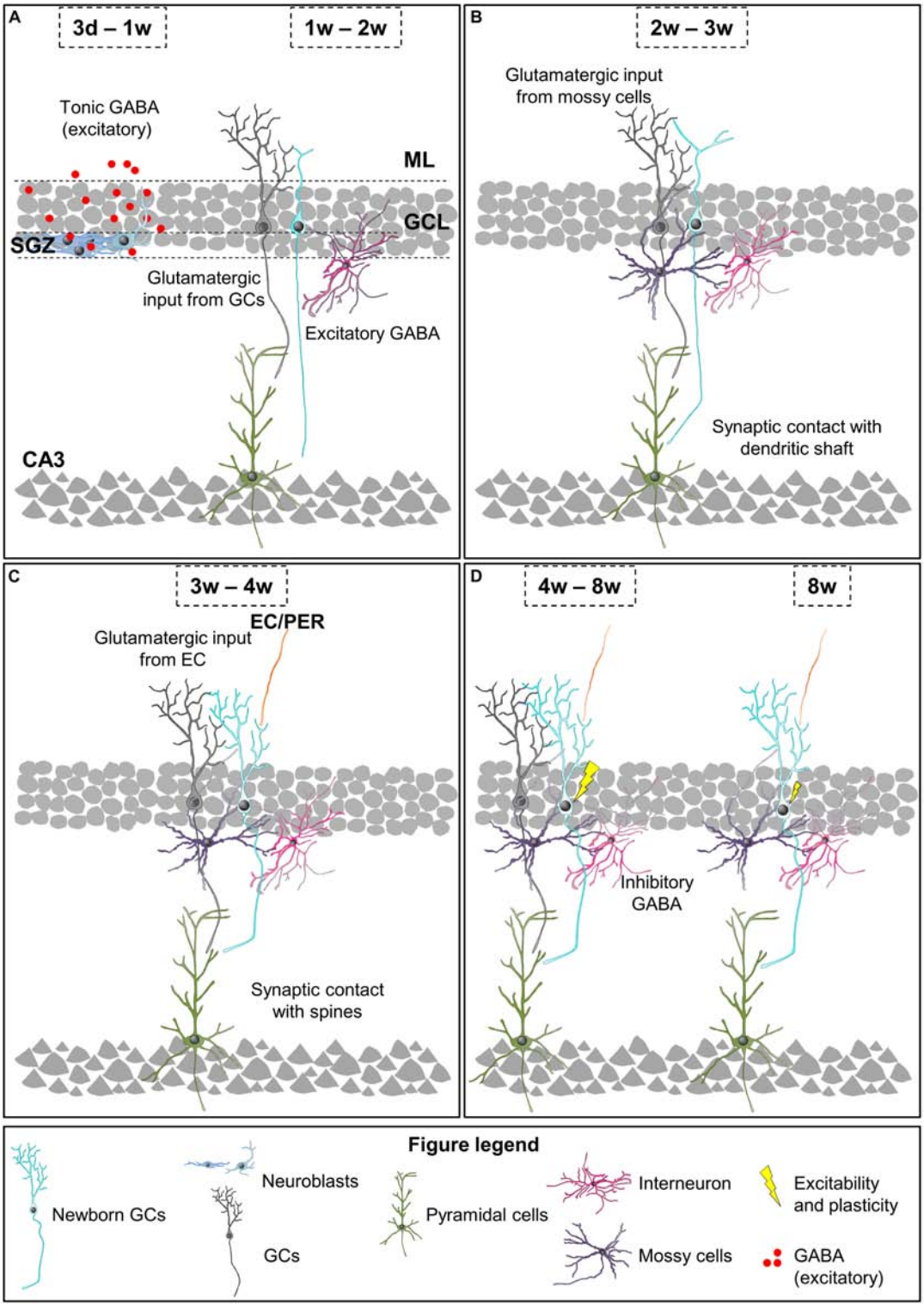


**FIGURE 3 |** Integration stages of adult newborn neurons into the hippocampal circuitry. **(A)** 3 days to 1-week-old neuroblasts sense tonic GABA, which is excitatory. 1–2 weeks after their birth, newborn GCs receive their first glutamatergic input from mature GCs and they are contacted by GABAergic interneurons, while their axons reach the CA3. **(B)** 2–3-week-old newborn GCs receive glutamatergic input from mossy cells and they establish synaptic contacts with the dendritic shafts of CA3 pyramidal cells. **(C)** 3–4-week-old newborn GCs receive glutamatergic inputs from EC and PER, and their axons establish synaptic contacts with dendritic spines of CA3 pyramidal cells. **(D)** 4–8-week-old are fully integrated into the hippocampal circuitry, and show increased excitability and enhanced synaptic plasticity. 8-week-old newborn GCs are fully mature neurons and integrated into the hippocampal memory circuit. EC, entorhinal cortex; GC, granule cell; GCL, granule cell layer; ML, molecular layer; PER, perirhinal cortex; SGZ, subgranular zone.

3 weeks after birth, when the dendritic tree of new neurons has already reached the outer ML and their length and complexity peak, axons from the PER, and EC establish synaptic contacts with new born GCs (Esposito, 2005; Zhao et al., 2006; Vivar et al., 2012; Deshpande et al., 2013; Trinchero et al., 2017). Importantly, at this stage, dendritic spines are few and highly unstable (Toni et al., 2007). Accordingly, glutamatergic inputs are weak and do not induce action potentials but, as previously indicated, may facilitate its generation when combined with moderate depolarizing GABAergic inputs (Heigele et al., 2016). Meanwhile, in CA3 layer, the axonal boutons from newborn GCs already contact with dendritic spines or thorny excrescences from CA3 pyramidal neurons (Faulkner et al., 2008; Toni et al., 2008). The size of the boutons, number of spines they envelop, and active synaptic contacts increase until the fourth week after cell birth (Faulkner et al., 2008). Therefore, 4 week-old GCs are already connecting EC and CA3, thus forming part of the classical tri-synaptic hippocampal circuitry.

Four to eight weeks after their birth newly generated GCs are fully integrated into the adult hippocampal circuitry but still have special electrophysiological features compared to fully mature GCs: increased excitability and enhanced synaptic plasticity (Schmidt-Hieber et al., 2004; Ge et al., 2007; Mongiat et al., 2009; Gu et al., 2012; **Figure 3D**). At this time point, GABA action onto new GCs is already inhibitory, presumably due to a decrease in the chloride importer NKCC1 and an increase in the chloride exporter KCC2 expression (Ge et al., 2006). Importantly, 4–6 week-old GCs are less influenced by feedback inhibition than mature GCs, which contributes to their higher excitability (Marín-Burgin et al., 2012; Dieni et al., 2013). Moreover, the presence of the GluN2B (NR2B) subunit of the NMDA receptor and the T-type calcium channel in these new GCs provides enhanced synaptic plasticity (Schmidt-Hieber et al., 2004; Ge et al., 2007; Gu et al., 2012). All these characteristics favor the activation and subsequent recruitment of 4 week-old newborn GCs to memory engrams, suggesting a relevant involvement in memory function. Indeed, silencing 4 week-old GCs, but not 2 or 8 week-old GCs, interferes with memory retrieval indicating that, at this stage, newborn GCs store relevant memory information (Gu et al., 2012). The density of GC dendritic spines reaches a peak 4 weeks after birth but they are smaller than those from mature neurons, and preferentially reach axonal boutons already contacted by other spines, presumably from mature neurons (Toni et al., 2007; Trinchero et al., 2017). Interestingly, spines from newborn GCs may compete and displace synaptic contacts from mature neurons (Toni et al., 2007). Something similar occurs in the CA3 region, where mossy fiber boutons from new neurons share thorny excrescences or spines with boutons from mature cells. With time, mossy fiber boutons associated exclusively with one thorny excrescence appear, again suggesting that some competition between axonal boutons from mature and newborn neurons may exist (Toni et al., 2008). Relevantly, it has been suggested that this competition may interfere with preexisting memory circuits and contribute to forgetting (Frankland et al., 2013; Akers et al., 2014). Therefore, 4–8 week-old GCs are already connected to the hippocampal circuitry and play a specific role in memory codification.

Eight week-old newborn GCs are generally considered fully mature neurons, perfectly integrated into the hippocampal memory circuit, as they are almost morphologically and electrophysiologically indistinguishable from developmentally generated mature GCs (**Figure 3D**). Therefore, 8 week-old GCs are not hyperexcitable (Mongiat et al., 2009; Dieni et al., 2013) and do not show enhanced synaptic plasticity (Ge et al., 2007; Gu et al., 2012). Nevertheless, newly generated GCs maintain certain particularities compared to developmentally born GCs. Importantly, the inputs from PER and EC to newborn GCs increase, at least, until 12 weeks after the birth of the cells (Vivar et al., 2012). Accordingly, these adult-born GCs still present higher structural plasticity and subtle morphological differences when compared with developmentally generated ones (Lemaire et al., 2012; Kerloch et al., 2018). For instance, memory tasks (Morris Water Maze) increase the complexity and number of spines of the dendritic trees of 8–16 week-old GCs but not developmentally generated GCs in rats (Lemaire et al., 2012). Hence, 8 week-old GCs are fully connected to the hippocampal circuitry and show electrophysiological mature properties while they retain higher structural plasticity compared to developmentally generated GCs.

## Functional Relevance of the Integration of Adult-Born GCs Into Memory Circuits

As we have discussed above, the integration of maturing GCs into the hippocampal circuitry is a slow and continuous process that generates a population of immature neurons in different stages with a range of functional characteristics: from almost silent, passing through a stage of hyperexcitability, to finally become rarely recruited by the incoming inputs (Piatti et al., 2013; Toda et al., 2018). Cells at each different maturation state may contribute to the hippocampal circuitry in particular ways: regulating feedback inhibition of mature GCs (Piatti et al., 2013), rewiring pre-existing connections (Goodwin, 2018), or establishing different memory codes for subtly different incoming signals (pattern separation) (McAvoy et al., 2015; Toda and Gage, 2018). However, the specific contribution of newborn neurons to hippocampal memory codification is a matter of an ongoing and exciting debate (Nakashiba et al., 2012; Frankland et al., 2013; Piatti et al., 2013; McAvoy et al., 2015; França, 2018; Toda et al., 2018).

## Regulation of Maturing GCs Integration Into Memory Circuits

Importantly, the involvement of maturing GCs in memory function indicates that memory may be affected by those factors that regulate the integration of these cells into the hippocampus, such as neuronal activity, which is initially sensed by neuroblasts in the form of tonic GABA and later in the form of inputs from inhibitory and excitatory local circuits (Ge et al., 2006; Jagasia et al., 2009; Song et al., 2013; Alvarez et al., 2016). In addition to surrounding neurons, other resident cells of the hippocampus may play a role in the integration of maturing GCs. This is the case of astrocytes, which have been shown to actively contribute to the incorporation of maturing GCs to the hippocampal circuit

(Krzisch et al., 2015; Sultan et al., 2015). Microglia, the immune cells of the CNS, are also well suited to participate in the wiring of these adult-born cells, as they have been described to participate in the integration of newborn neurons into brain circuits during development, and in the activity dependent modification and pathological modulation of neuronal connectivity. In the next sections we will briefly introduce microglia and then review their role in the wiring of maturing neurons.

## MICROGLIA

Microglia survey the brain parenchyma to maintain brain homeostasis, sensing changes in their environment, removing pathogens and cell debris by phagocytosis, undergoing changes in gene expression and morphology, and mounting the innate immune response when necessary (Sierra et al., 2014). Microglia present specific characteristics that make them unique and clearly distinct from other tissue macrophages. First, microglia have a different embryonic origin when compared with the majority of resident macrophages, as they originate from a myeloid progenitor cell derived from the yolk sac during early embryonic development (Ginhoux et al., 2010). Second, the population of adult microglia does not depend on the supply of peripheral precursor cells (blood monocytes), as it occurs with other adult tissue macrophages (Röszer and Röszer, 2018), but slowly renews itself through adulthood in humans and rodents (Askew et al., 2017; Réu et al., 2017), allowing them a certain degree of "cellular memory" of past events (Cheray and Joseph, 2018). Third, microglial processes are highly motile, and are able to scan the entire brain parenchyma every few hours while their cell body remains immobile (Nimmerjahn et al., 2005; Paris et al., 2018).

Microglia are very dynamic cells with extraordinary functional and morphological diversity. Accordingly, several single cell RNASeq studies have shown that microglia present a great variety of transcriptional responses that are far more complex than the polarization into M1 (pro-inflammatory or classic) and M2 (anti-inflammatory or alternative) states (Chiu et al., 2013; Xue et al., 2014; Gosselin et al., 2017). There is growing evidence that tissue-specific factors from local microenvironment dictate the functional states of developing and adult tissue-resident macrophages. Microglia express a large number of surface receptors, their "sensome," which allows them to sense signals from other cells and the state of their environment, and to detect subtle changes induced by life-style factors, such as cognitive stimulation, physical exercise, diet or stress (Kettenmann et al., 2011; Hickman et al., 2013; Valero et al., 2016; Hanamsagar and Bilbo, 2017). In addition, while surveilling the parenchyma microglia communicate with other cells of the CNS and affect their function (Kettenmann et al., 2013). Some of the sensome receptors contribute to the direct communication between neurons and microglia, such as the receptor of the neuronal chemokine fractalkine (CX3CR1); purinergic receptors (e.g., P2X4, P2Y1, or P2Y12); triggering receptor expressed on myeloid cells 2 (TREM2) and its adaptor DNAX activation protein of 12KDa (DAP12), which participate in phagocytosis; or the CR3 receptor of the immune complement system (Pascual et al., 2012; Schafer et al., 2012; Scianni et al., 2013; Hickman et al., 2013; Eyo et al., 2014; Paolicelli et al., 2014). Their sensome allows microglia to adapt to changing environments and to mount an appropriate response. This communication with other cells allows microglia to shape the CNS playing a role in different developmental relevant processes such as blood vessel formation, myelination, and neurogenesis (Frost and Schafer, 2016).

In the next section we will review the current knowledge about the role of microglia in neurogenesis, focusing on their participation in the integration of maturing GCs into the hippocampal circuitry.

## MICROGLIAL CONTRIBUTION TO THE INTEGRATION OF ADULT GENERATED GRANULE CELLS INTO HIPPOCAMPAL CIRCUITRY

### Microglial Participation in Adult Neurogenesis

Microglia invade the brain during embryonic development and their number peaks postnatally. In the mouse hippocampus microglial density reaches a maximum around postnatal day 15 (Paolicelli et al., 2011), suggesting that they may influence hippocampal neurogenesis since early postnatal days. In the adult neurogenic niche, microglia establish close contacts with all cells in the cascade, from rNSCs to neuroblasts and newborn neurons in physiological conditions (Sierra et al., 2010). Microglia also eliminate apoptotic ANPs and neuroblasts through phagocytosis, maintaining the homeostasis of the neurogenic niche (Sierra et al., 2010). Moreover, microglia may modulate neurogenesis through the secretion of different factors (e.g., cytokines, trophic factors, etc.) that have been suggested to affect proliferation, differentiation, and survival of newborn cells. Importantly, changes in microglia induced by neuroinflammation, aging, and pathology may contribute to the reduction in hippocampal adult neurogenesis and to the concomitant defects in memory function (Valero et al., 2012, 2016, 2017; Sierra et al., 2014). However, there are few data about the possible involvement of microglia on the integration of adult generated GCs into hippocampal circuits. Fortunately, the involvement of microglia in neuronal maturation and integration into brain circuits during development and pathology suggests that they may also participate in the integration of adult generated GCs into hippocampal circuits. We will now review the two major mechanisms by which microglia modulate neuronal integration: direct microglia-neuron interaction and the release of soluble factors (**Table 1**).

### Microglia-Neuron Interaction

Microglial motile processes continuously scan the brain parenchyma, establishing close contacts with neurites and synaptic regions (Kettenmann et al., 2013). While monitoring neurites and synapses, microglia may sense their state through the binding of their receptors to neuronal ligands. The

**TABLE 1 |** Summary table.

| Mechanism | Model | Reference |
|---|---|---|
| **Neurite growth** | | |
| Microglia-neuron interactions | *In vivo* | Squarzoni et al., 2014; Baalman et al., 2015; Clark et al., 2016; Weinhard et al., 2018 |
| | *Ex vivo* | Weinhard et al., 2018 |
| Release of soluble factors | *In vivo* | Guthrie et al., 1995; Batchelor et al., 1999; Batchelor et al., 2002; Liu et al., 2017 |
| | *Ex vivo* | Liu et al., 2017 |
| | *In vitro* | Nagata et al., 1993; Huang et al., 2017 |
| Unknown | *In vivo* | Bolós et al., 2017; Appel et al., 2018 |
| **Structural synaptic plasticity** | | |
| Microglia-neuron interactions | *In vivo* | Wake et al., 2009; Tremblay et al., 2010; Paolicelli et al., 2011; Schafer et al., 2012; Parkhurst et al., 2013; Chen et al., 2014; Kim et al., 2017; Lowery et al., 2017; Schecter et al., 2017; Filipello et al., 2018; Weinhard et al., 2018 |
| | *Ex vivo* | Weinhard et al., 2018 |
| Release of soluble factors | *In vivo* | Parkhurst et al., 2013 |
| | *In vitro* | Lim et al., 2013 |
| Unknown | *In vivo* | Bolós et al., 2017; Appel et al., 2018 |
| **Functional maturation of synapses** | | |
| Release of soluble factors | *In vivo* | Antonucci et al., 2012; Ferrini et al., 2013; Parkhurst et al., 2013; Liu et al., 2017 |
| | *Ex vivo* | Pascual et al., 2012; Scianni et al., 2013; Zhang et al., 2014 |
| | *In vitro* | Antonucci et al., 2012; Pascual et al., 2012; Scianni et al., 2013; Gabrielli et al., 2015; Liu et al., 2017 |
| Unknown | *In vivo* | Roumier et al., 2004, 2008; Hoshiko et al., 2012 |

*Ex vivo, organotypic and acute slices; In vitro, cell cultures; In vivo, fixed tissue and life-imaging experiments.*

interaction (or lack of thereof) of microglial receptors with neuronal ligands, such as fractalkine or the complement system molecules C1q/C3, induce specific responses in microglia that regulate their activity and motility (Kettenmann et al., 2013; Szepesi et al., 2018). However, the functional significance of this monitoring is still widely discussed, but has been suggested to contribute to neuronal connectivity by four mechanisms.

First, microglia are able to engulf small portions of axons, a process named trogocytosis, and contribute to limit axonal outgrowth and to eliminate presynaptic regions (Weinhard et al., 2018). Microglia have been also proposed to phagocytose dendritic spines (Paolicelli et al., 2011; Kim et al., 2017; Filipello et al., 2018); although recent evidence suggests that microglia only partially engulf spines, at least in the hippocampus (Weinhard et al., 2018), as it will be discussed in the next sections. Second, microglia may modulate neuronal connectivity by physically interposing their cell bodies and processes between post and presynaptic regions (Chen et al., 2014). Third, microglial-neuron interaction has been associated with the generation of filopodia from the dendritic shaft and the head of dendritic spines, contributing to the formation and relocation of dendritic spines, respectively (Miyamoto et al., 2016; Weinhard et al., 2018). Finally, microglia also establish close contacts with the axon initial segment (Baalman et al., 2015; Clark et al., 2016), suggesting a yet unexplored mechanism to affect neuronal connectivity. Therefore, through the establishment of close contacts with neurites and synaptic elements microglia participates in the elimination, interruption, and formation of neuronal connections.

## Release of Soluble Factors

In addition to cell-to-cell contact, microglia modulate neuronal wiring through the release of soluble factors. The microglial secretome contains several molecules, such as components of the extracellular matrix, trophic factors, cytokines, endocannabinoids, and microRNAs (miRs) that have been described to influence neuronal connectivity by affecting neurite growth, and structural and functional synaptic plasticity. Importantly, microglia may release these factors either anchored to the surface or encapsulated into extracellular vesicles (EVs) (Szepesi et al., 2018). The effect of these factors may be dose dependent as it occurs with the tumor necrosis factor α (TNFα), which is neuroprotective at low levels but exerts a pro-apoptotic activity at higher concentrations (Bernardino et al., 2008). Additionally, these factors have been postulated to induce effects both at long-distance, and locally while microglial processes are in close proximity to neurons, exerting a global or localized action in brain connectivity (Szepesi et al., 2018).

The data presented here suggest that microglia may modulate the connection of adult-born GCs to the hippocampal circuitry. However, few studies have specifically addressed the role of microglia in the wiring of adult-born neurons. In the next sections we will review the current knowledge about the role of microglia on neuronal wiring during development, activity-dependent synaptic remodeling and pathology by microglia-neuron contact as well as by release of soluble factors. We will focus in three processes that are relevant for neuronal integration into brain circuitry: neurite growth and maintenance, structural synaptic plasticity (formation, elimination, and relocation of synapses),

and functional synaptic plasticity (functional molecular changes in pre- and postsynaptic compartments). Finally, at the end of each section we will discuss the role of microglia in the integration of adult-generated GCs into the hippocampal circuitry.

## MICROGLIAL MODULATION OF NEURITE GROWTH AND MAINTENANCE

Neurite growth involves the initiation, enlargement (including branching), and final stabilization of both dendrites and axons. Neurite outgrowth is highly relevant for adult-born GCs mainly 1–3 weeks after their birth, when these cells extend their dendrites and axons toward the ML and CA3/CA2, respectively. Nevertheless, adult-born GCs may retain the capacity of increasing their dendritic tree upon cognitive stimulation even 16 weeks after their birth (Lemaire et al., 2012).

Direct evidence shows that microglia modulate developmental neurite growth, as their constitutive ablation (by knocking out the transcription factor PU.1) or in pathological conditions (through conditioned expression of the diphtheria toxin receptor in these cells and subsequent administration of the diphtheria toxin) affects both axon sprouting in the striatum and neurite length and complexity in the hippocampus and spinal cord (Squarzoni et al., 2014; Liu et al., 2017). Importantly, the role of microglia in neurite growth differs between different regions of the CNS, as microglia do not participate in the regeneration of retinal ganglion axons after optical nerve crush (Hilla et al., 2017), while they contribute to decrease the complexity of hippocampal dendrites and to increase the complexity of spinal cord dendrites after spinal cord injury (Liu et al., 2017). The effects caused by microglial depletion in neurites during development and in pathological conditions suggest that they may participate in the dendritic and axonal sprouting of newborn GCs that occurs since the early stages of their maturation. In the next sections we will review the different mechanisms proposed for the modulation of neurite growth by microglia and finally discuss whether they may be involved in the integration of adult-born GCs.

### Microglia-Neuron Interaction

Microglia may modulate axonal growth through direct contact with axonal regions. During embryonic and postnatal development in the mouse striatum microglia establish close contacts with dopaminergic axons and engulf them, suggesting that they may limit axonal growth by actively remove axonal fragments (Squarzoni et al., 2014). Direct evidence of microglial engulfment of small axonal regions (trogocytosis) was observed using live imaging in postnatal hippocampal slices and verified using 3D electron microscopy reconstructions, although their contribution to axonal sprouting was not analyzed (Weinhard et al., 2018). In addition, microglia may also contribute to axonal maintenance through physical contact with the axon initial segment of, mainly, excitatory neurons. The interaction of microglia with the axon initial segment was observed both during postnatal development and adulthood; is more prominent in the mouse cortex than in other regions of the brain, such as the thalamus and the striatum; and is preserved across species (mouse, rats and non-human primates) (Baalman et al., 2015). It has been speculated that this contact of microglia with the axon initial segment may provide trophic support. However, data are contradictory as in a model of traumatic brain injury microglial contacts with initial segments diminish suggesting loss of trophic support, while in the experimental model of demyelination by cuprizone and chronic autoimmune encephalomyelitis microglial contacts with axon initial segments increase and precede their axonal breakdown, suggesting that microglial contacts are detrimental for axonal integrity (Baalman et al., 2015; Clark et al., 2016). Therefore, microglial close contact with neurites may restrict their growth through trogocytosis, and be involved in their maintenance.

### Release of Soluble Factors

Microglia have been shown to regulate neurite growth through the release of different soluble factors. First, microglia release components of the extracellular matrix such as plasminogen and thrombospondin, which *in vitro* induce neurite outgrowth (Nagata et al., 1993; Chamak et al., 1994, 1995). Second, several studies indicate that microglia induce neurite growth by releasing different factors after injury such as brain derived neurotrophic factor (BDNF) in the striatum, insulin growth factor-1 (IGF-1) in the hippocampus, and TNF-α in the spinal cord and hippocampus (Guthrie et al., 1995; Batchelor et al., 1999; Batchelor et al., 2002; Liu et al., 2017). TNF-α deserves special attention, as it has been argued to be exclusively expressed by microglia in the CNS (Barres, 2008) and to meditate the effects induced by spinal cord injury in the decrease and increase of the dendrites of hippocampal and spinal cord neurons of mice, respectively (Liu et al., 2017). Accordingly, TNF-α affects neuronal branching *in vitro* in a dose dependent manner. Thus, low levels of TNF-α increase neuronal branching in mouse postnatal SVZ neurospheres, while higher doses have no effects in neurospheres or reduce the branching of cultured neurons from the hippocampus of rat embryos (Bernardino et al., 2008; Keohane et al., 2010). Finally, microglia may affect neurite growth through the release of EVs carrying modulatory molecules; this is the case for pre-micro RNA miR-124-3p, which is released via exosomes by the microglial cell line BV2 (Huang et al., 2017). BV2 cells treated with brain extracts from experimental mouse models of traumatic brain injury secrete exosomes enriched in miR-124-3p that, *in vitro*, induce neurite outgrowth (Huang et al., 2017). Importantly, miR-124 is also found in microglia acutely purified from the adult brain (Ponomarev et al., 2011) although its activity is exclusively detected in neurons (Åkerblom and Jakobsson, 2014). Thus, it is possible that the inactive form of miR-124 may be released by microglia encapsulated in exosomes, reach neurons, and then become activated promoting neurite outgrowth, although this mechanism remains to be demonstrated. Hence, microglia release different factors that have been demonstrated to contribute to the neurite changes that occur during development and in pathological conditions.

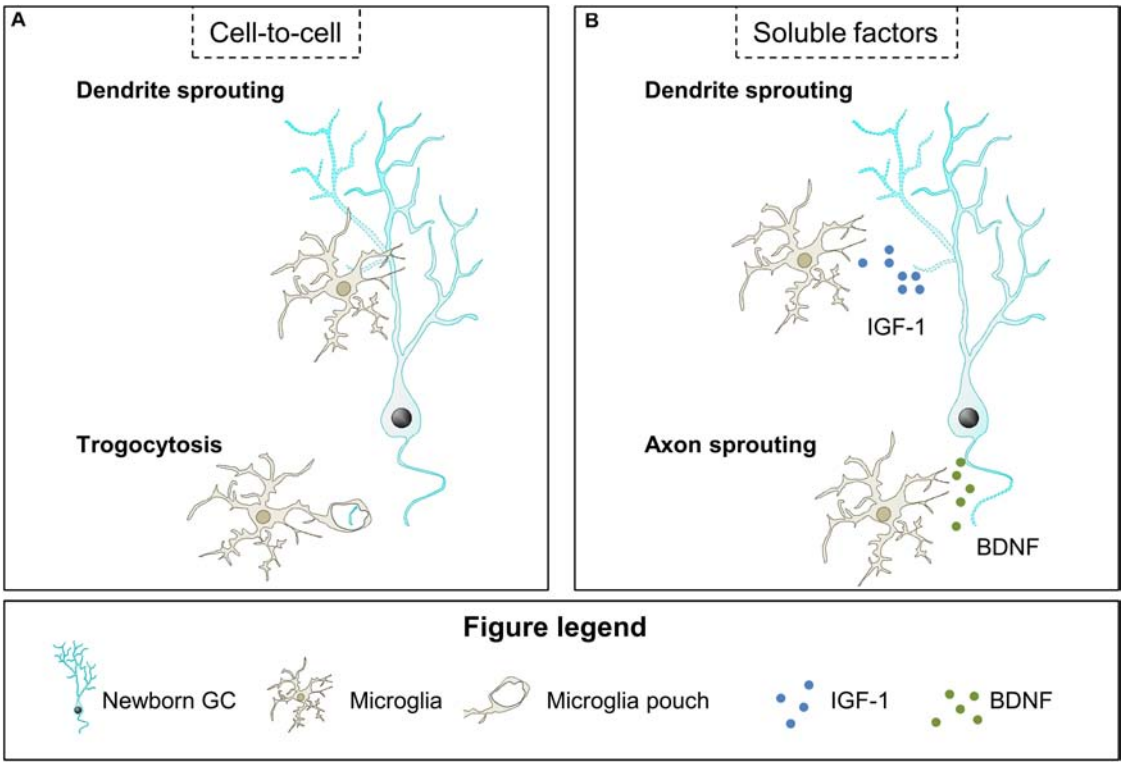


**FIGURE 4 |** Hypothetical contribution of microglia to neurite growth. **(A)** Microglia contact newborn GC dendritic tree and may modulate dendrite sprouting through an unknown mechanism. Also, microglia may engulf small segments of the newborn GC axon and restrict neurite growth. **(B)** Microglia have been shown to secrete soluble factors that may contribute to dendrite and axon sprouting, such as IGF-1 and BDNF, respectively. BDNF, brain derived neurotrophic factor; IGF-1, insulin growth factor-1.

## Microglial Modulation of Neurite Growth in Adult Neurogenesis

The described effects of microglia on neurite outgrowth during development and pathologies may be relevant in the context of adult neurogenesis. We speculate that microglial contacts may modulate neurite sprouting in maturing GCs during the first 3 weeks after their birth, when these cells extend their dendrites toward the ML and axons toward the CA3/CA2 regions; or affect axonal integrity afterward, once initial neurite growth has concluded (**Figures 4A,B**).

Importantly, several studies using mouse models in which microglia are dysfunctional have suggested their involvement in dendritic tree sprouting of adult-born GCs (**Figure 4A**). In these studies both mice with microglial-specific depletion of the vacuolar sorting protein 35 (VSP35), involved in endosomal trafficking to the Golgi, and mice lacking CX3CR1 show a reduction in length and complexity of newborn GCs dendritic tree and concomitant alterations in microglial morphology and density (Rogers et al., 2011; Pagani et al., 2015; Bolós et al., 2017; Appel et al., 2018). Although these data indicate that microglia play a role in neurite growth of maturing GCs the underlying mechanisms have not been explored. These effects may be mediated by direct microglia-neuron contact, as microglia establish stable and transient contacts with the dendrites of 3–7 week-old newborn GCs (Chugh and Ekdahl, 2016). The functional relevance of these contacts is unknown and they may modulate dendritic sprouting but also the dynamics of dendritic spines, as we will review in the next section. In addition, microglia may also control axonal sprouting through trogocytosis of newborn mossy fiber axons on their way to CA3, a process in which microglia have been involved during development (Squarzoni et al., 2014; Weinhard et al., 2018). Although there is no evidence of the effects of the close contacts between microglia and neurites of maturing GCs, there are some studies indicating that microglia may act on neurite sprouting of maturing GCs by releasing soluble factors, as it will be next discussed.

Microglial action on neurite growth of maturing GCs may also be mediated by local release of soluble factors such as IGF-1 and BDNF (**Figure 4B**). IGF-1 and BDNF are well-known promoters of neurite growth (Bondy and Cheng, 2004; Guo et al., 2018) and they are overexpressed by microglia after voluntary exercise (Kohman et al., 2012; Littlefield et al., 2015), which is known to increase the complexity of the dendrites of newborn GCs in mice (Dostes et al., 2016; Sah et al., 2017; Trinchero et al., 2017). However, in both normal and voluntary exercise conditions, microglial BDNF effects on dendritic sprouting of maturing GCs are probably small, as it mainly depends on the autocrine production of BDNF (Wang et al., 2015). Interestingly, in the same study lack of autocrine secretion of BDNF did not

affect axonal sprouting, which leaves open the possibility that microglial BDNF may act on axons. Indeed, it is plausible that microglial BDNF promotes axonal formation by binding to the neurotrophin receptor p75NTR, whose activation is necessary for the initiation of the axon of adult-born GCs (Zuccaro et al., 2014). Altogether, the data presented here indicate that microglia may be involved in the sprouting of newborn GCs neurites by contacting them and releasing neurotrophic factors under conditions such as voluntary exercise, although this possibility should be directly tested in future studies.

## MICROGLIAL INVOLVEMENT IN STRUCTURAL SYNAPTIC PLASTICITY

Structural synaptic plasticity involves changes in the morphology of pre- and postsynaptic elements: synapse elimination, formation, and remodeling. Newborn GCs transverse critical periods of structural synaptic plasticity from 2 to 8 weeks after birth (Zhao et al., 2006; Toni et al., 2007; Trinchero et al., 2017). Several factors, such as exercise and cognitive stimulation, potentiate the formation of dendritic spines; while others, such as aging, delay their formation (Tronel et al., 2010; Alvarez et al., 2016; Trinchero et al., 2017). Microglia may participate in this modulation of GCs structural synaptic dynamics; as they are able to sense life-related factors and contribute to structural synaptic plasticity during development, pathology, and physiological activation of neurons. Several pieces of evidence indicate that microglia exert their action on synaptic remodeling through the establishment of microglia-neuron contacts with synaptic elements and through the release of soluble factors, as we will review here. Finally, the possible role of microglia in structural synaptic plasticity of adult-born GCs will be discussed in the last part of this section.

### Microglia-Neuron Interaction

Microglia establish close contacts with pre- and postsynaptic regions that may result in their engulfment and elimination, the loss of synaptic contacts by physical interposition, and the formation of filopodia that induce the generation and/or relocation of dendritic spines. Here we will review previous studies suggesting the existence of these three possible mechanisms of action.

Microglia have a crucial role in the physiological removal of the excess of synapses produced during development, a phenomenon called "synaptic pruning" (reviewed in Kettenmann et al., 2013; Salter and Beggs, 2014; Mosser et al., 2017). In this regard, microglia have been described to be involved in the elimination of presynaptic and postsynaptic (dendritic spines) elements. For instance, visual cortex microglia contact dendritic spines in an activity-dependent manner, indicating that these contacts may be relevant for the function of the dendritic spines and their dynamics (Wake et al., 2009; Tremblay et al., 2010). The role of microglia in the elimination of dendritic spines was suggested by data showing that delayed microglial invasion of the mouse hippocampus, induced by the lack of CX3CR1, leads to delayed decrease in dendritic spines (Paolicelli et al., 2011). Furthermore, microglial processes have been shown to contain puncta positive for classical postsynaptic markers, such as the postsynaptic density protein 95, *in vitro* and *in vivo* in the mouse cortex and hippocampus (Paolicelli et al., 2011; Kim et al., 2017; Appel et al., 2018; Filipello et al., 2018). However, although microglial trogocytosis of axonal portions has been demonstrated, phagocytosis of spines has not been directly observed. Indeed, a recent study indicated that postsynaptic elements are not phagocytosed by microglia, at least in the postnatal (P15) hippocampus, where apparently engulfed dendritic spines are always found connected to the dendrite through the spine neck (Weinhard et al., 2018). Importantly, microglial contacts with synaptic elements are prominent during the peak of plasticity of the visual cortex (P28) and have been related to the elimination of synapses through engulfment of presynpatic but not postsynaptic regions, as CX3CR1 KO mice show a reduction in the number of microglial contacts with axon terminals and a concomitant increase in axonal density (Lowery et al., 2017; Schecter et al., 2017). Relevantly, microglia eliminate presynaptic elements in an activity-dependent manner in the P5 dorsal lateral geniculate nucleus (dLGN) of mice as reduced and increased activity of retinal ganglion cells (RGCs) potentiates and decreases, respectively, axon terminals engulfment by microglia (Schafer et al., 2012). In the dLGN, the complement receptor CR3 is necessary for microglia engulfment of axon terminals, as CR3 KO mice have increased axon density and decreased axon colocalization with microglial staining, suggesting decreased engulfment of axon terminals (Schafer et al., 2012). However, CR3 is involved in the elimination of presynaptic regions only in some regions of the brain as the hippocampus of CR3 KO mice shows similar levels of trogocytosis when compared with control mice (Weinhard et al., 2018). Therefore, the elimination of axonal terminals may be mediated by trogocytosis, while the mechanism of dendritic spine elimination is not known. We speculate that dendritic spines disappearance may be related to the lack of contact with a presynaptic terminal, which may be induced by both the uncompleted engulfment of the spine or the elimination of the presynaptic terminal performed by microglia.

In addition to engulfing synaptic regions, microglia interfere with synapses by physically interposing their cell body and processes between pre- and postsynaptic elements. This mechanism of synaptic interference has been described in inhibitory synapses in the mouse cortex after the induction of systemic inflammation by intraperitoneal administration of LPS (gram negative bacteria lipopolysaccharide), when microglia displace inhibitory synaptic contacts from the surface of the soma of pyramidal neurons (Chen et al., 2014). The microglial surrounding of the pyramidal neuron soma is speculated to decrease inhibitory input and thus to increase neuronal firing and neuronal synchronicity (Chen et al., 2014). Additionally, the partial engulfment of dendritic spines described by Weinhard et al. in the postnatal mouse hippocampus may also dislodge excitatory pre- and postsynaptic regions (Weinhard et al., 2018), although this is highly speculative, as interruption of excitatory synapses by microglial processes has not been analyzed properly. These data indicate that microglial direct interposition between pre- and postsynaptic elements may interfere with synapses.

Finally, the microglial close contact with neurons may lead to the formation and/or relocation of dendritic spines during postnatal development by promoting the growth of dendritic shaft or spine head filopodia. Direct evidence of the role of microglia in dendritic spines formation comes from their genetic depletion by diphtheria toxin mice in the early postnatal barrel cortex and the adult motor cortex (Parkhurst et al., 2013; Miyamoto et al., 2016). Importantly, Miyamoto et al., indicated that microglia promote dendritic spine formation by contacting dendrites and propitiating the generation of dendritic shaft filopodia (early stage dendritic spines) (Yoshihara et al., 2009), presumably through the induction of transient local rises in calcium levels and subsequent actin polymerization in the contacted region (Miyamoto et al., 2016). Spine head filopodia induction by microglia has also been proposed to relocate dendritic spines in the hippocampus of P15 mice, as their contacts with the head of hippocampal dendritic spines is followed by the formation of spine head filopodia and the relocation of the dendritic spines (Weinhard et al., 2018). In summary, previous studies indicate that contact of microglia with dendrites during postnatal development contributes to the remodeling of synapses through the induction of elimination of dendritic spines and trogocytosis of presynaptic regions, the interruption of synaptic communication, and the formation and relocation of dendritic spines by filopodia.

## Release of Soluble Factors

Microglia release several soluble factors which may contribute to the formation and elimination of pre- and postsynaptic terminals. One key factor seems to be BDNF, a well-known pro-survival factor controlling synaptic plasticity (Leal et al., 2014). BDNF is required for the formation of spine head filopodia in dendritic spines and the relocation of spines to multisynaptic boutons in the mouse hippocampus and olfactory bulb *in vivo* (Breton-Provencher et al., 2016; Niculescu et al., 2018), although the cellular origin of this BDNF is unclear. Importantly, microglia BDNF contributes to activity-dependent formation of dendritic spines in the motor cortex (Parkhurst et al., 2013), possibly by inducing spine head filopodia emerging from the dendritic shafts (Miyamoto et al., 2016) although the actual mechanism remains unknown. Another soluble microglial mediator involved in synaptic structural plasticity is interleukin-10 (IL-10), which increases the number of pre- and postsynaptic terminals in neuronal cell cultures from rat hippocampus, an effect that is antagonized by interleukin-1β (IL-1β) (Lim et al., 2013). TNF-α may also contribute to dendritic spine remodeling, at least in pathological conditions, as after spinal cord injury TNF-α contributes to the decrease and increase of dendritic spines in the hippocampus and spinal cord, respectively (Liu et al., 2017). Therefore, microglia release several soluble factors that modulate structural synaptic plasticity.

## Microglial Modulation of Structural Synaptic Plasticity in Adult Neurogenesis

The data above suggest that microglia may exert a role on structural synaptic plasticity of newborn GCs. Thus, microglia may participate in the formation, relocation and elimination of the excess of synaptic contacts established by maturing GCs from 2 to 8 weeks after birth, when newborn GCs transverse critical periods of structural synaptic remodeling. Microglia may act using several of the mechanisms described above based in close microglia-neuron contact (elimination, interposition, or formation and relocation of synaptic contacts) or the release of soluble factors (**Figures 5A,B**).

Microglia may modulate maturing GCs connectivity through the elimination of pre- and postsynaptic regions. Although this hypothesis has not been directly analyzed, a recent study suggested that microglia may regulate the shape of axon terminals of maturing GCs in mice, as lack of CX3CR1 decreased their area (Bolós et al., 2017). Microglia may also interfere with synaptic regions avoiding synaptic communication. Indeed, microglia may interfere with the GABAergic input received by maturing GCs, as it occurs with inhibitory synapses in the mouse cortex after systemic inflammation induction by intraperitoneal administration of LPS (Chen et al., 2014), suggesting that this interference may have relevant and different consequences for maturing GCs depending on the time in which it occurs. Therefore, microglial interference with GABAergic synapses might decrease maturation and survival of maturing GCs if it takes place when GABA is excitatory (until 4 weeks after their birth); or decrease the inhibitory input into GCs when GABA inhibits them (before 4 weeks after their birth), thus contributing to their higher excitability. It should be taken into account that the microglial interference with GCs GABAergic input is highly speculative and, as far as we know, there are no studies analyzing this hypothesis. Another possibility is that microglia contribute to the formation and relocation of dendritic spines from 2 to 8 week-old maturing GCs. Data from previous studies suggested that microglia are involved in structural synaptic remodeling of maturing GCs but did not indicated whether they participate in synaptic elimination, formation, or both (Bolós et al., 2017; Appel et al., 2018). The density of dendritic spines of newborn GCs is reduced in the adult hippocampus of mice lacking microglial specific proteins relevant for their function such as the fractalkine receptor CX3CR1 (Bolós et al., 2017) or the VSP35 (Appel et al., 2018). These data suggest that microglia may regulate structural synaptic plasticity of maturing GCs through microglia-neuron interaction (**Figure 5A**).

Finally, although there is no direct evidence of microglial release of soluble factors that contribute to the dynamics of synapses in maturing GCs, the formation of spine head filopodia has been related to BDNF levels in adult-born neurons of the mouse olfactory bulb (Breton-Provencher et al., 2016), suggesting that BDNF may also exert a similar effect in adult-born hippocampal neurons (**Figure 5B**). We speculate that microglia may be the source of this BDNF, as microglial BDNF is known to contribute to the formation of dendritic spines (Parkhurst et al., 2013) in the mouse cortex; and microglia have been described to participate in the formation of dendritic spines by inducing the generation of dendritic shaft filopodia in the mouse somatosensory cortex (Miyamoto et al., 2016). Hence, microglia may contribute to the characteristic location of

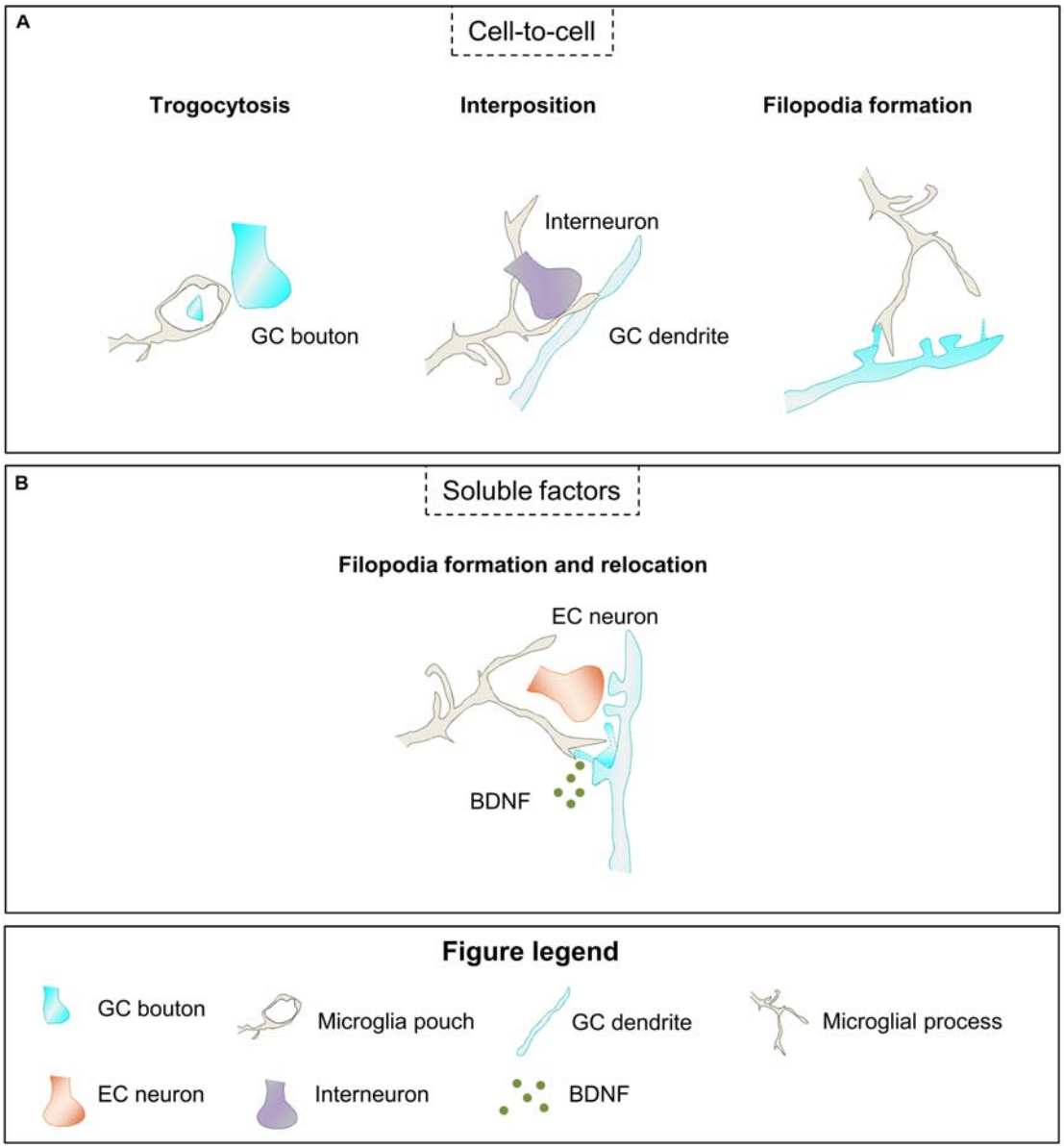


**FIGURE 5 |** Hypothetical contribution of microglia to structural synaptic plasticity of newborn GCs. **(A)** Microglia may modulate synaptic structural plasticity by eliminating small parts of the GC bouton (trogocytosis), interfering with GABAergic synapses (interposition), and inducing the formation of new filopodia in GC dendrites or spines. **(B)** Microglia secrete BDNF which may participate in filopodia formation and relocation to multisynaptic boutons. BDNF, brain derived neurotrophic factor; EC, entorhinal cortex; GC, granule cell.

maturing GCs dendritic spines to multisynaptic boutons (Toni et al., 2007) by inducing the formation of spine head filopodia, which has been related to the relocation of dendritic spines to multisynaptic regions in the postnatal mouse hippocampus (Weinhard et al., 2018). While there is no direct evidence for this hypothesis, we suggest that microglia may act in GCs synaptic regions by establishing close contacts with them to induce their elimination, interrupt synaptic communication, or contribute to their formation and relocation; and by releasing soluble factors such as BDNF. Altogether, these data point toward a possible role of microglia during the development of synaptic contacts that integrate adult-born GCs into the hippocampal circuitry.

## MICROGLIAL REGULATION OF FUNCTIONAL MATURATION OF SYNAPSES

Microglia may exert a role in the functional maturation of synapses through the release of soluble factors that induce changes in the molecular composition of pre- and postsynaptic

compartments (microglia-neuron contact has not been involved in the functional maturation of synapses). These mechanisms are relevant for the integration of newborn GCs into the hippocampal circuitry (2–4 weeks after their birth), and their recruitment to memory engrams and final maturation (4–8 weeks after their birth). Therefore, we will discuss the participation of microglia on functional synaptic maturation of adult-born GCs by modulation of GABA-mediated inhibition and excitatory synaptic efficacy during development and pathology.

## Microglial Modulation of GABA-Mediated Inhibition

Microglia may modulate GABA mediated inhibition through the release of soluble factors that may affect pre- and postsynaptic terminals. For instance, microglial BDNF modulates the inhibitory effect of GABA in neurons by affecting the expression of the contransporter KCC2 (Ferrini and De Koninck, 2013; Ferrini et al., 2013). KCC2 normally increases during neuronal maturation, contributing to the change in the action of GABA from excitatory to inhibitory in maturing neurons (Rivera et al., 2005). Evidence of the role of microglia in the modulation of KCC2 expression comes from animal models of pathological pain, in which microglial BDNF was shown to bind to TrkB receptor expressed by neurons, promoting its phosphorylation, and triggering a decrease in KCC2, which subsequently reduces GABA mediated inhibition, thus sensitizing and inducing pathological pain (Ferrini and De Koninck, 2013). In these models of pathological pain, microglial BDNF release is induced by ATP acting on microglial P2X4, which is nonetheless expressed at very low levels in physiological conditions (Ferrini and De Koninck, 2013; Ferrini et al., 2013). In addition to affect GABA sensing in the postsynaptic region, microglia may also affect presynaptic transmission in GABAergic neurons by releasing EVs. Rat primary microglia exposed to ATP release EVs that carry on their surface the endocannabinoid N-arachidonoylethanolamine (AEA), which *in vitro* activates CB1 cannabinoid receptors of GABAergic neurons decreasing inhibitory transmission (Gabrielli et al., 2015). Therefore, microglia may modulate GABAergic transmission acting in the postsynaptic element through the release of BDNF and in the presynaptic region through the activation of the endocannabinoid system.

## Microglial Modulation of Excitatory Synaptic Efficacy

Microglia have been suggested to participate in functional synaptic maturation through the modulation of excitatory synaptic efficacy. We will review in this section the role of microglia in the modulation of postsynaptic regions through their influence in AMPA/NMDA ratio and GluN2B content of NMDA receptors. In addition, microglia collaborate with astrocytes to potentiate glutamatergic transmission through a mechanism that involves adenosine or ATP release by microglia, after fractalkine or LPS action, respectively (Pascual et al., 2012; Scianni et al., 2013). However the microglia-astrocytes crosstalk is not strictly considered a mechanism of synaptic functional plasticity and it is out of the scope of this review. Finally, although data about microglial effect on the composition of the presynaptic regions is scarce we will also briefly discuss this possibility.

Microglia have been involved in the control of synaptic efficacy through the modulation of relative levels of activity of AMPA and NMDA receptors at postsynaptic regions. The increase in synaptic AMPA receptors is relevant for synapse transformation from silent into functional, and to increase synapse efficacy (Petralia et al., 1999; Renger et al., 2001). Intact microglial function is required for the adequate control of the postnatal rise in AMPA/NMDA ratio, as it is abolished in the barrel cortex of P9 mice lacking CX3CR1 expression (Hoshiko et al., 2012) while it is increased in the hippocampus of P18-25 mice lacking or wearing a non-functional mutation of DAP12, specifically expressed by microglia in the brain (Roumier et al., 2004, 2008). In addition, microglia may affect AMPA/NMDA ratio in opposite directions in different regions of the CNS. Evidence arises from a mouse model of spinal cord injury in which microglia are required for the increase in AMPA/NMDA ratio occurring in the hippocampus after injury, and for the decrease of this ratio in the spinal cord, effects that are suggested to be mediated by microglial TNF-α (Liu et al., 2017). Finally, microglia may induce AMPA receptor endocytosis in postsynaptic terminals of hippocampal slices through the production of superoxide after simultaneous exposition to hypoxia and LPS, a mechanism that is dependent in CR3 activation and that induced long term depression (Zhang et al., 2014). These data indicate that microglia modulate postnatal and pathological changes in AMPA/NMDA ratio.

Another mechanism by which microglia may influence excitatory synaptic efficacy is by affecting the composition of NMDA receptors. The implication of microglia in functional maturation of postsynaptic sites is suggested by the analysis of mice with dysfunctional microglia due to the lack of DAP12 or CX3CR1. The brain of these mice shows increased proportion of the GluN2B subunit of NMDA receptors (Roumier et al., 2004, 2008; Hoshiko et al., 2012). In CX3CR1 KO mice this effect is transient and coincides with a time of delayed migration of microglia into the barrel cortex, suggesting that the presence of microglia is required for synaptic maturation (Hoshiko et al., 2012). Importantly, microglia may exert a different effect in the composition of extra-synaptic and synaptic NMDA receptors, as genetic depletion of microglia using the diphtheria toxin leads to increased levels of GluN2B in the whole brain but decreases levels in synaptosomes (Parkhurst et al., 2013). These data imply that microglia may increase the proportion of synaptic immature NMDA receptors, i.e., those containing GluN2B instead of GluN2A subunits, and thus increase the strength of the synapses (for further information about the role of GluN2B in synapses check: Shipton and Paulsen, 2014; Volianskis et al., 2015). Importantly, microglial BDNF is required for functional maturation of synapses, as mice lacking microglial expression of BDNF also show decreased levels of synaptic GluN2B (Parkhurst et al., 2013). Therefore, microglia may be involved in the modulation of the efficacy of synapses through their influence in synaptic levels of GluN2B containing NMDA receptors.

While the data above show that microglia modulates functional plasticity of postsynaptic regions, their involvement

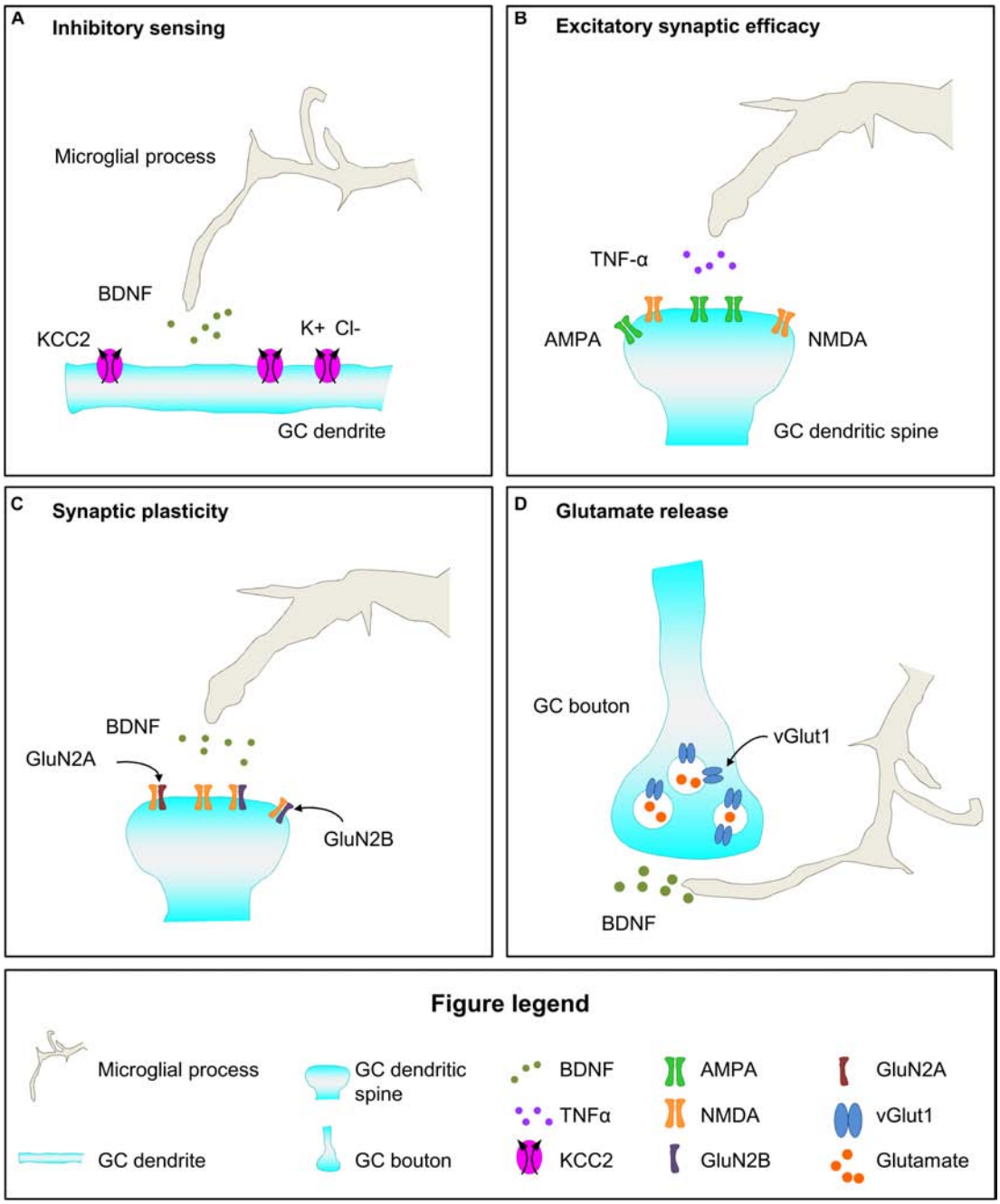


**FIGURE 6 |** Hypothetical mechanisms of microglial regulation of functional maturation of synapses of newborn GCs. **(A)** Microglia may secrete BDNF, which upregulates the expression of the cotransporter KCC2 in newborn GCs, and increases their inhibitory sensitivity to GABA. **(B)** Microglia derived TNF-α may increase synaptic efficacy by increasing the AMPA/NMDA ratio. **(C)** Microglial BDNF may contribute to the enhanced synaptic plasticity of newborn GCs by raising the proportion of GluN2B subunit in NMDA receptors. **(D)** Microglia secreted BDNF may increase glutamatergic transmission by upregulating the expression of vGlut1 in presynaptic vesicles. BDNF, brain derived neurotrophic factor; Cl, chloride; GC, granule cell; K, potassium; TNF-α, tumor necrosis factor-α; vGlut1, vesicular glutamate transporter 1.

in the maturation of presynaptic regions is not well known. In the diphtheria toxin mouse model, depletion of microglia or lack of microglial BDNF leads to decreased levels of the presynaptic protein vesicular glutamate transporter 1 (vGlut1), suggesting alterations in the presynaptic compartment that may be related to a decrease in glutamate release (Parkhurst et al., 2013). In addition, microglial released EVs may also increase presynaptic glutamatergic transmission through the induction of sphingosine synthesis, which favors exocytosis and subsequent glutamate release, as it has been observed in primary neuronal

cell cultures and the rat visual cortex (Antonucci et al., 2012; Gabrielli et al., 2015). Although further analyses are required to understand the involvement of microglia in functional maturation of postsynaptic regions, current data indicate that microglial release soluble factors such as BDNF, TNF-α, and EVs, that modulate functional synaptic maturation of both pre- and postsynaptic elements.

## Microglial Modulation of Functional Synaptic Plasticity in Adult Neurogenesis

The data discussed above indicate that microglia participate in different aspects of functional synaptic maturation in development and pathological conditions, and thus suggest that they may contribute to functional synaptic maturation of newborn GCs. First, microglia may affect the excitatory and inhibitory effect of GABA in maturing GCs as indicated by the role of microglial BDNF release on neuronal KCC2 content in pathological conditions (**Figure 6A**; Ferrini and De Koninck, 2013; Ferrini et al., 2013). Importantly, final maturation of newborn GCs involves the increase in the expression of the KCC2 transporter, which is necessary to sense GABA as inhibitory, a fundamental change in GCs maturation (Ge et al., 2006). Second, microglia may participate in the maturation of glutamatergic excitatory contacts of adult-born GCs. A relevant step of GCs maturation is the incorporation of AMPA receptors to excitatory synapses to make them functional and increase their efficacy, a process in which microglia have been involved during development (**Figure 6B**; Roumier et al., 2004, 2008; Hoshiko et al., 2012). Furthermore, microglia may participate in the maintenance of the enhanced synaptic plasticity that defines 4–8 weeks maturing GCs by releasing BDNF and contributing to the high content in GluN2B that characterize these cells (**Figure 6C**; Schmidt-Hieber et al., 2004; Ge et al., 2007; Gu et al., 2012; Parkhurst et al., 2013). Regarding the presynaptic compartment, microglial BDNF may favor glutamate release by increasing vGlut1 levels in mossy fiber presynaptic vesicles (**Figure 6D**), as it has been shown during development (Parkhurst et al., 2013). Microglia have been demonstrated to modulate functional synaptic maturation in pathological conditions and during development, leading to the provocative hypothesis that microglia are involved in the functional maturation of adult-born GC, ultimately impacting on memory function.

## CONCLUSION

The integration of maturing GCs into the adult hippocampus is a continuous process that generates a range of immature neurons with different structural and functional characteristics. Young neurons at each different maturation state may contribute to the hippocampal circuitry in particular ways. Therefore, the study of the mechanisms involved in the integration of newborn GCs into the hippocampal circuit is necessary to fully understand memory function and its regulation. Several lines of evidence lead us to propose that microglia may participate in the integration of adult generated GCs into hippocampal memory circuits by controlling neurite growth, formation and elimination of synapses, and changing the molecular composition of synapses. The role of microglia in the wiring of newborn GCs may be particularly relevant for the effects that life factors and disease exert in the integration of newborn GCs into memory circuits, as microglia are able to detect subtle changes in their environment such as those induced by cognitive stimulation, physical exercise, diet, stress, inflammation or pathology (Valero et al., 2016, 2017). The development of experimental animal models that allow the depletion of microglia during a specific temporal window or the inhibition of the expression of soluble factors exclusively in microglia (e.g., BDNF) have been crucial to demonstrate the involvement of microglia in the incorporation of neurons into brain circuits. These mouse models offer an excellent opportunity to investigate the role of microglia in the incorporation of adult newborn GCs into hippocampal circuits and memory function. Elucidating the nature of the intricate relationships between microglia, integration of adult newborn GCs into the hippocampal circuit, and behavior is highly relevant to properly understand the contribution of microglia to memory function in physiological and pathological conditions.

## AUTHOR CONTRIBUTIONS

NR-I created the schemes included in this manuscript. JV wrote the initial draft of the manuscript with input from all authors. AS and NR-I critically revised the manuscript. All authors reviewed and approved the manuscript.

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# Protein Kinase C: Targets to Regenerate Brain Injuries?

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Acute or chronic injury to the central nervous system (CNS), causes neuronal death and irreversible cognitive deficits or sensory-motor alteration. Despite the capacity of the adult CNS to generate new neurons from neural stem cells (NSC), neuronal replacement following an injury is a restricted process, which does not naturally result in functional regeneration. Therefore, potentiating endogenous neurogenesis is one of the strategies that are currently being under study to regenerate damaged brain tissue. The insignificant neurogenesis that occurs in CNS injuries is a consequence of the gliogenic/non-neurogenic environment that inflammatory signaling molecules create within the injured area. The modification of the extracellular signals to generate a neurogenic environment would facilitate neuronal replacement. However, in order to generate this environment, it is necessary to unearth which molecules promote or impair neurogenesis to introduce the first and/or eliminate the latter. Specific isozymes of the protein kinase C (PKC) family differentially contribute to generate a gliogenic or neurogenic environment in injuries by regulating the ADAM17 mediated release of growth factor receptor ligands. Recent reports describe several non-tumorigenic diterpenes isolated from plants of the *Euphorbia* genus, which specifically modulate the activity of PKC isozymes promoting neurogenesis. Diterpenes with 12-deoxyphorbol or lathyrane skeleton, increase NPC proliferation in neurogenic niches in the adult mouse brain in a PKCβ dependent manner exerting their effects on transit amplifying cells, whereas PKC inhibition in injuries promotes neurogenesis. Thus, compounds that balance PKC activity in injuries might be of use in the development of new drugs and therapeutic strategies to regenerate brain injuries.

**Keywords: protein kinase C, neurogenesis, ADAM17/TACE, brain injury, neuroregeneration**

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## NEUROGENESIS IN THE ADULT BRAIN UNDER PHYSIOLOGICAL CONDITIONS

Cell replacement in several mammalian organs is an orchestrated process that may lead to the regeneration of a completely functional organ. Unfortunately, this is not the case of the adult central nervous system (CNS). Neurogenesis, the process of generation of new neurons that occurs during development of the CNS and remains during the infant and adult stages is built up on the capacity of neural stem cells (NSC) to produce neurons and glial cells. Yet quiescent NSC are distributed ubiquitously along the adult CNS (Magavi et al., 2000), once development is ended, neurogenesis is

restricted to a few specific regions. In these areas NSC produce neurons because they are situated in a context of signaling molecules that induce their transition to an activated state, from which they produce a progeny mainly comprised of cells with a neuronal phenotype. Two neurogenic regions have been thoroughly described in the adult mammalian brain, the subventricular zone (SVZ) and the dentate gyrus of hippocampus (DG) (Kuhn et al., 1996; Doetsch et al., 1997). Within these regions an environment of extracellular signaling molecules creates a neurogenic niche that preserves the necessary conditions to support neurogenesis during a lifetime. Different cell types derived from the NSC progeny can be distinguished within these niches: undifferentiated neural progenitor cells (NPC) produced by activated NSC, and neuronal progenitor cells (neuroblasts) that differentiate into mature neurons. Since the potentiality of NPC is almost identical to that of NSC, they can produce either neuronal progenitors or glial progenitors (Reynolds and Weiss, 1992; Doetsch et al., 1999; Torroglosa et al., 2007). However, once in the niche, extracellular, matrix-bound and membrane-bound signals determine their fate toward a neuronal phenotype (Codega et al., 2014).

## INJURY-INDUCED NEUROGENESIS

Adult neurogenesis has generated a great deal of attention in the context of designing cell replacement therapies following neuronal loss. Focal traumatic or cerebrovascular brain injuries cause acute damage, induce neuronal death and irreversible cognitive deficits or sensory-motor alterations (Blennow et al., 2012). No effective treatment is currently available to compensate neuronal loss in these patients, however, the potential of the CNS to generate new neurons that replace the lost ones has opened a new perspective in the development of therapies to treat this type of lesions. One of the strategies, currently under study, to enable neuronal replacement is facilitating the recruitment of endogenous NPC and neuroblasts within the injured tissue (Saha et al., 2012). Two different sources of neuroblasts facilitate neuronal replacement in injuries: NPC generated from NSC activated at the site of injury (Magavi et al., 2000; Chen et al., 2004; Geribaldi-Doldan et al., 2018) and NPC or actual neuroblasts that are generated in neurogenic regions as a reaction to the damage, which migrate in the direction of the injury (Parent et al., 1997; Arvidsson et al., 2002; Kandasamy et al., 2015; Geribaldi-Doldan et al., 2018). However, cells from both sources would need an adequate environment to survive and to lead their destiny toward mature neurons that would integrate into existing neuronal circuits (Romero-Grimaldi et al., 2011). Following an injury, neurogenic regions react activating different steps of the neurogenic process: activation of quiescent NSC, induction of NPC proliferation, differentiation, and alteration of neuroblast migration patterns (Liu et al., 1998; Fallon et al., 2000; Jin et al., 2001) in the direction of the injured area. However, this attempt to repair the damaged tissue is generally unsuccessful and most of the originated NPC lead the destiny of their progeny toward astroglial cells (gliogenesis) rather than neurons (neurogenesis) (Romero-Grimaldi et al., 2011; Susarla et al., 2014; Geribaldi-Doldan et al., 2018). The lack of significant neurogenesis in damaged brain areas, even when endogenous NPC are available, may be due to the absence of molecules necessary for neuronal differentiation and/or to the presence of molecules that favor the differentiation of NPC toward a glial phenotype (Benner et al., 2013). Inflammatory molecules released mainly by glial and microglial cells create a gliogenic/non-neurogenic environment that facilitates the generation of glial progenitor cells, which differentiate into glial cells (Buffo et al., 2008). Thus, it may be possible that these glial cells activate a paracrine positive feedback loop that favors gliogenesis from NPC over neurogenesis.

## NEURONAL REPLACEMENT STRATEGIES: PROMOTION OF ENDOGENOUS NEUROGENESIS

Potentiating endogenous neurogenesis is one of the strategies that are currently being under study to regenerate damaged brain tissue. Four important stages of the neurogenic process can be modulated in order to promote endogenous neurogenesis: (1) to stimulate NSC activation and proliferation of NPC within the lesion (Luzzati et al., 2014; Barker et al., 2018), (2) to generate an environment that favors neurogenesis in neurogenic regions to lead the fate of NPC into a neuronal phenotype (Parent et al., 1997; Grade and Gotz, 2017), (3) to promote migration of neuroblasts toward the damaged areas, as well as survival (Geribaldi-Doldan et al., 2018), and (4) to stimulate differentiation of neuroblasts into mature neurons (Geribaldi-Doldan et al., 2018) facilitating the posterior integration of the newly generated neurons into preexisting circuits. In summary, replacement of dead neurons in an injured CNS region requires the modification of the extracellular environment to generate a neurogenic niche in which the progeny of endogenous NSC is predominantly mature functional neurons. However, it is first necessary to uncertain which molecules promote or impair neurogenesis and introduce the first and/or eliminate the latter. Recent reports aimed to understand the cellular and molecular mechanisms involved in the generation of this gliogenic/non-neurogenic background highlight the key role of neurogenic signaling molecules such as Noggin (Lim et al., 2000) or Neuregulin 1 (NRG1) that promotes neuroblast migration, and gliogenic signaling pathways such as those initiated by Notch (Benner et al., 2013), or the epidermal growth factor receptor (EGFR) (Kuhn et al., 1997; Gonzalez-Perez et al., 2009; Romero-Grimaldi et al., 2011; Geribaldi-Doldan et al., 2018) among other signals.

It is worth mentioning the essential role of EGFR activation in the neurogenic processes. EGFR signaling participates in SVZ neurogenesis promoting proliferation of undifferentiated transit amplifying progenitors (TAPs) (Doetsch et al., 2002; Torroglosa et al., 2007). In NPC cultures under differentiation conditions, EGFR activation favors gliogenesis over neurogenesis (Romero-Grimaldi et al., 2011). In brain injuries, the shedding of EGFR ligands such as TGFα activate EGFR and prevent the generation of neurons facilitating gliogenesis and contributing

to the generation of gliogenic niches in areas of brain damage (Romero-Grimaldi et al., 2011; Geribaldi-Doldan et al., 2018). Regulating EGFR activity might be relevant when developing strategies to promote endogenous neurogenesis in brain injuries and understanding the mechanisms that activate this receptor may lead to the identification of molecular targets to regenerate brain injuries. Thus, in the subsequent paragraphs, we will discuss molecular mechanisms involved in the regulation of EGFR activity.

## ADAM17/TACE DEPENDENT EGFR LIGAND RELEASE

As commented above, epidermal growth factor (EGF)–induced EGFR activation, in cultures of NPC isolated from the SVZ, promotes glial cell differentiation whereas EGFR inhibition facilitates the differentiation of these cells toward a neuronal phenotype (Romero-Grimaldi et al., 2011). The effect of the EGFR inhibition can be mimicked *in vitro* by the inhibition of the tumor necrosis factor alpha-converting enzyme (ADAM17/TACE), a metalloprotease of the A Disintegrin And Metalloproteinase (ADAM) family. These metalloproteases catalyze the cleavage of the extracellular domains (ectodomains) of several EGFR ligands. These ligands are synthesized as membrane-anchored precursor proteins (pro-ligands). The active soluble ligands are detached from the pro-ligands and released to the extracellular medium in a proteolytic reaction catalyzed by convertases of the ADAM family. In particular TGFα release is mediated by ADAM17 (Blobel, 2005), a membrane-bound peptidase, which is a limiting step in regulating signaling through EGFR. ADAM17 is the main convertase involved in the release of the EGFR ligands TGFα, HB-EGF, Epiregulin and Amphiregulin (Sunnarborg et al., 2002; Lee et al., 2003). Nevertheless, ADAM17 also catalyzes the release of other ligands of the ErbB family of receptors (**Figure 1A**). The selectivity of this enzyme for each ligand is mediated by phosphorylation reactions in the C-terminal domain of the pro-ligand, catalyzed by kinases of the protein kinase C (PKC) family (Dang et al., 2013).

## PKC ISOZYMES STRUCTURE AND CLASSIFICATION

Kinases of the PKC family are enzymes composed of regulatory and catalytic domains (**Figure 1B**), which phosphorylate a great variety of substrates. These proteins remain in an inactivated state that can be reverted upon the binding of their regulators diacylglycerol (DAG), calcium and phosphatidyl serine (PS) to the regulatory domains. They are characterized by a conserved kinase domain, which undergoes a conformational change and activates itself to enable catalysis. As shown in **Table 1**, kinases of the PKC family play an essential role in transducing signals related with cell cycle entrance, differentiation, apoptosis or autophagy among other functions (Watanabe et al., 1992; Dempsey et al., 2000; Black and Black, 2012). This family of proteins consists of ten serine-threonine kinases, which based on their regulatory domains and physiological activators, are classified in three subfamilies (Mellor and Parker, 1998): the classical, the novel and the atypical. The classical PKCs (α, β, and γ), depend on calcium, DAG, and PS for their activation. The novel PKCs (δ, ε, θ, and η), are calcium independent kinases but they still require DAG and PS for their activation. The atypical PKC (λ and ζ) do not depend on calcium or DAG for their activation and are regulated by protein-protein interactions (Rosse et al., 2010; **Figure 1B**).

## PKC AND THERAPEUTIC CONSIDERATIONS

The above-mentioned role of PKC isozymes in regulating ADAM17 activity may lead to hypothesize that modulating PKC activity might be of use as a treatment to promote endogenous neurogenesis in lesions. However, therapeutic considerations may be taken into account because of the strong association of these kinases in cancer or neurodegenerative diseases such as Alzheimer's disease (AD) (reviewed in Newton, 2018a; **Table 1**). Almost all PKC isozymes have been associated with tumor progression and also with the metastasis process. Classical PKC are involved in tumorigenicity, for example PKCα regulates cell motility in some cancer models and some authors described a relationship between up or down-regulation of these isozyme depending of the type of cancer (Konopatskaya and Poole, 2010). PKCβ II induces endothelial cells proliferation and stimulates tumor angiogenesis in breast cancer, whereby some inhibitors of this isozyme have been postulated as a therapeutic treatment (Sledge and Gokmen-Polar, 2006) for this type of cancer. Novel PKCδ has been associated with pro-apoptotic signaling, in fact it is involved in tumor suppression inhibiting cell cycle progression (Basu and Pal, 2010). PKCε is one of the most studied isozymes in cancer research with a significant role in lung cancer (Baxter et al., 1992). PKCθ has been implicated is gastrointestinal cancer (Ou et al., 2008). Atypical PKC as PKCζ is involved in breast cancer development and in promoting glioma initiating cells proliferation, invasion and migration (Huang et al., 2012; Malla et al., 2012). It is important to clarify that in general, a reduced PKC activity and protein expression has been associated to different types of cancer.

This reduced activity in tumors contrasts with the enhanced PKC activity and expression found in models of neurodegenerative diseases such as AD or stroke (**Table 1**) (reviewed in Sun and Alkon (2014), Newton (2018a)). Acute and chronic changes in PKC activity can be found in models of AD, stroke and age-dependent neurodegeneration with different effects depending on the disease and the stage of the disease. As the body ages, activity and proper translocation of PKC isozymes is critical for memory, and injury repair (**Table 1**) (reviewed in Lucke-Wold et al., 2015). Gain of function mutations of classical PKCα activity has been specifically involved in the reduction of synaptic activity caused by AD (Alfonso et al., 2016) contributing to cognitive decline. In a similar fashion, inhibition of novel PKCδ reduces amyloid ß and reverses AD (Du et al., 2018). Alteration on PKC expression can also be observed as aging

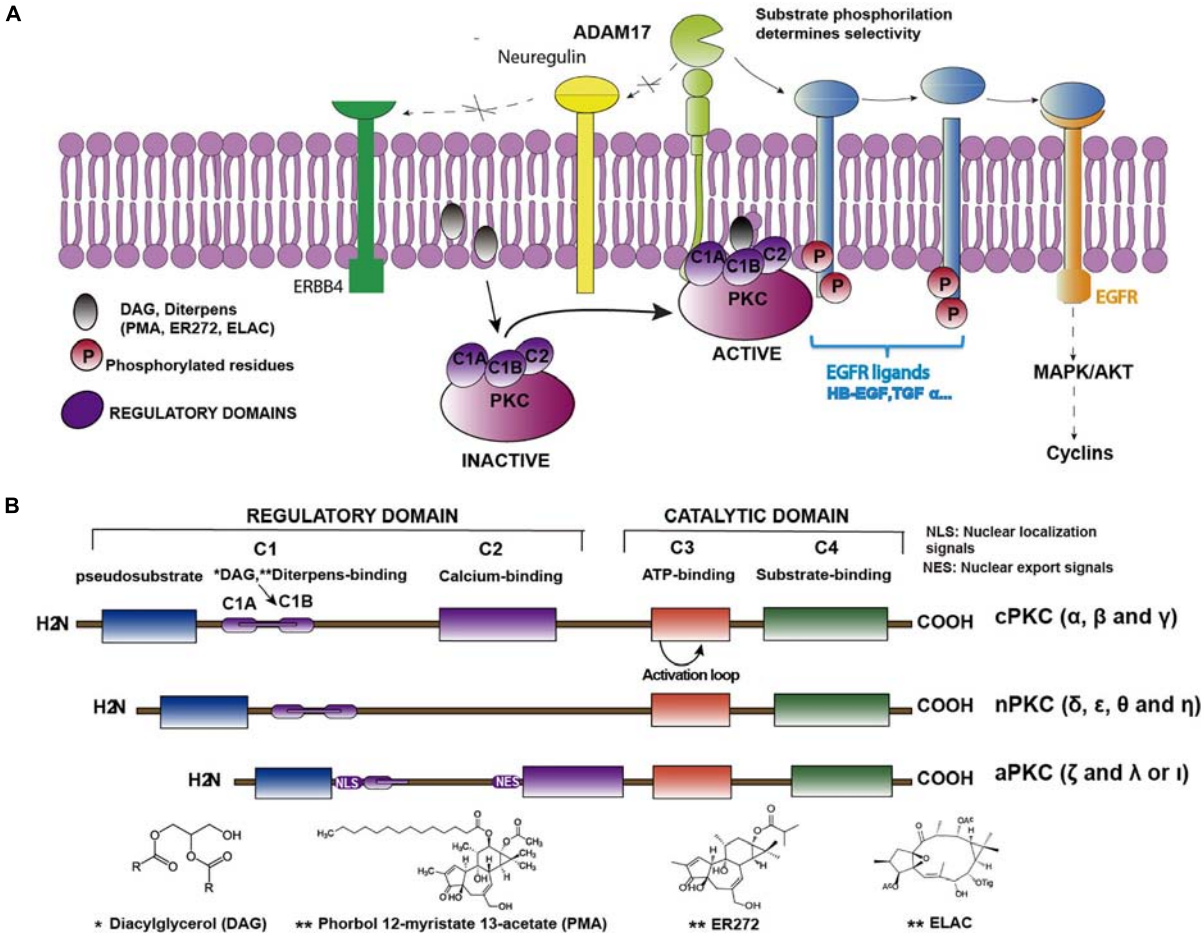


**FIGURE 1 |** Structure of PKC isozymes and their indirect role in EGFR activation. **(A)** Cartoon representing the sequence of PKC-ADAM17-TGFα-EGFR pathway. Binding of DAG or non-physiological diterpenes to the regulatory domains of PKC activates the enzymes. Upon activation, enzymes are translocated close to the plasma membrane where they catalyze the phosphorylation of membrane bound EGFR pro-ligands or ligands of other receptors of the ERBB family (i.e., neuregulin) or ligands that activate other receptors, (i.e., neuregulin, which activates ERBB4). Only phosphorylated pro-ligands are selected by ADAM17 as substrates over other non-phosphorylated ones. ADAM17-mediated shedding occurs on the phosphorylated pro-ligands, releasing the soluble ligand and activating the receptor. **(B)** Classification of PKC isozymes according to their structure and regulatory properties. Regulatory domains (C1 and C2) and binding sites for regulatory molecules (DAG, $Ca^{2+}$, and PS) are shown as well as the conserved catalytic domains (C3 and C4). *See structure of Diacylglicerol below; **See structures of different diterpenes (PMA, ER272 and ELAC) below.

occurs and a downregulation in PKC expression is found in different models probably as a consequence of epigenetic modifications (Pascale et al., 2007). Whether these alterations are the basis of the disease or a homeostatic response to the disorders remains to be clarified.

## PKC, ADAM17 AND EGFR: ROLE IN ADULT NEUROGENESIS

Protein kinase C isozymes phosphorylate several downstream substrates including EGFR ligands as well as other ErbB receptor ligands such as NRG1. Specific activation of PKC isozymes determines ADAM17 selectivity for its different substrates (**Figure 1A**). Thus, PKCα activated by Phorbol-12-myristate-13-acetate (PMA) catalyzes the phosphorylation of TGFα, Amphiregulin and HB-EGF precursors facilitating their shedding mediated by ADAM17 and releasing the soluble growth factor outside the cell (Dang et al., 2013). On the contrary, activation of novel PKCδ is required for ADAM17 mediated secretion of NRG1. Phosphorylation of serine 286 in the cytoplasmic domain of NRG1 catalyzed by PKCδ facilitates the scission of its ectodomain (Watanabe et al., 1992; Dang et al., 2011) releasing NRG1 into the extracellular medium. Overall, ADAM17 substrate specificity and selectivity is mediated by the activation of different PKC isozymes, which play a key role in the secretion of different types of ligands (Dang et al., 2011, 2013) governing several steps of adult neurogenesis. As examples, autocrine secretion of TGFα in brain injuries leads NPC toward a glial fate preventing the generation of neurons; on the contrary, inhibition of ADAM17 dramatically increases the generation of neurons (Romero-Grimaldi et al., 2011; Geribaldi-Doldan et al., 2018). NRG1

**TABLE 1 |** Pathophysiological role of PKC isozymes.

| PKC isoforms | Tissue expression | Functions | Reference |
|---|---|---|---|
| **Classical PKCs** | | | |
| α | Ubiquitous | • Cell proliferation and metastasis Heart failure, decreased contractility.<br>• Apoptosis, tumorigenecity, cell adhesion, differentiation, migration.<br>• Synaptic defects in AD.<br>• Role in learning and memory. | Konopatskaya and Poole, 2010; Liu and Molkentin, 2011; Sun and Alkon, 2014; Alfonso et al., 2016; Singh et al., 2017 |
| β | Ubiquitous | • Cancer development: vasculogenesis and cell invasion.<br>• Diabetes: vascular complications. | Sledge and Gokmen-Polar, 2006; Geraldes and King, 2010 |
| γ | Brain and spinal cord | • Pain modulation in dorsal root ganglia.<br>• Long term potentiation, long term depression, modulation of receptors, neurological disorder. | Saito and Shirai, 2002; Sweitzer et al., 2004 |
| **Novel PKCs** | | | |
| δ | Ubiquitous | • Proliferation, immune function, apoptosis, and cell migration.<br>• Fertility.<br>• Cancer development: angiogenesis.<br>• Regulation of amyloid-β degradation pathway.<br>• Neuronal loss in animal models of Parkinson's Disease | Zhang et al., 2007; Reyland, 2009; Basu and Pal, 2010; Ma et al., 2015; Du et al., 2018 |
| ε | Ubiquitous | • Heart failure, increased fibrosis, ischemia, mitochondria protection.<br>• Pain modulation in spinal cord.<br>• Bipolar diseases: neuronal transmission malfunction.<br>• Role in learning and memory.<br>• Ischemic tolerance | Sweitzer et al., 2004; Inagaki et al., 2006; Einat et al., 2007; Liu et al., 2012; Sun and Alkon, 2014 |
| η | Ubiquitous | • Acquired resistance to radiation.<br>• Epithelial cell growth and differentiation. | Kraft et al., 1982; Cabodi et al., 2000 |
| θ | Ubiquitous | • Gastrointestinal stromal tumor, cell proliferation and antiapoptosis.<br>• T cell responses, inflammation. | Ou et al., 2008; Zanin-Zhorov et al., 2011 |
| **Atypical PKCs** | | | |
| λ | Ubiquitous | • Glioblastoma cell invasion. | Baldwin et al., 2008 |
| ζ | Ubiquitous | • Breast cancer cell metastasis.<br>• Glioma, cell proliferation, survival, invasion, and migration. | Van Kolen and Slegers, 2006; Guo et al., 2009; Huang et al., 2012 |

mediated activation of ErbB4 promotes neurogenesis in the adult SVZ increasing NPC proliferation and organizing migration of neuroblasts from the SVZ toward the olfactory bulb (Anton et al., 2004; Ghashghaei et al., 2006). These evidences point out at the mission of specific kinases of PKC family on stimulating the production of signaling molecules such as TGFα or NRG1 (Dang et al., 2011, 2013), which may have a decisive role in leading NSC and NPC toward gliogenesis or neurogenesis, respectively (Ghashghaei et al., 2006; Romero-Grimaldi et al., 2011).

## PKC ISOZYMES IN ADULT NEUROGENESIS

Several members of the PKC family are present in neurogenic regions (Minami et al., 2000) and participate in distinct signaling cascades initiated by growth factors (GF), often determining GF specificity (Corbit et al., 2000). Activation of classical PKCß promotes proliferation of NPC *in vitro* and induces the expression of cyclins E and D in the absence of EGFR. *In vivo* in the SVZ and DG of mice, PKC activation promotes proliferation (Geribaldi-Doldan et al., 2016; Murillo-Carretero et al., 2017) mainly of $EGFR^+$ transit amplifying cells. In addition, atypical PKC have been involved in the NSC-to-neuron transition during development, and in the adult brain (Wang et al., 2012). On the contrary, novel PKCε, activation is crucial for the astrocytic differentiation of NPC (Steinhart et al., 2007). Phosphorylation of the CREB binding protein (CBP) by atypical PKC promotes hippocampal neurogenesis as well as memory and learning in mature adult mice in which CREB activity is reduced as a consequence of age (Gouveia et al., 2016). Metformin-induced activation of atypical PKC in mice promotes hippocampal neurogenesis and enhances spatial reversal learning in the Morris Water Maze task (Wang et al., 2012). Furthermore, local treatment of mechanical brain injuries with a pan-PKC inhibitor, promotes neuroblast enrichment facilitating differentiation of NPC toward neurons (García-Bernal et al., 2018). Overall, activation of classical PKC

isozymes promotes neurogenesis in neurogenic regions whereas its inhibition facilitates neurogenesis in injuries.

## PKC ACTIVATION BY DITERPENES

The physiological activator of PKC is DAG; this molecule binds to the C1B domain of classical and novel PKC isozymes (**Figure 1B**) inducing a conformational change that results in the activation of the protein. The affinity of PKC isozymes for DAG is higher in novel PKC than in classical PKC isozymes (reviewed in Newton, 2018b). Non-physiological molecules such as phorbol esters can activate classical and novel PKC isozymes. These tetracyclic diterpenoids activate PKC because they mimic the action of diacylglycerol (DAG) (Newton, 1995) binding to the same regulatory domain with different affinities. The most commonly used diterpene with phorbol ester structure is PMA. This phorboid has been extensively tested as a potent activator of PKC (Deleers and Malaisse, 1982). It binds to the C1B domain in PKC and promotes its translocation to the plasma membrane (Newton, 1995); unfortunately, it lacks of clinical use due to being a very potent tumor promoter (Szallasi and Blumberg, 1991). Its tumor-promoting activity seems to be associated to the nature and extent of the reversibility of PKC activation (Newton, 2018b), which in parallel is associated to the affinity of this molecules for the C1B domain and the effect of these molecules on the translocation of PKC to the plasma membrane. Thus, phorbol esters with more lipophilic substituents have a higher affinity for the C1B domain and lock PKC in an open (active) conformation on the membrane. This results in their dephosphorylation and subsequent degradation of the protein, a process referred to as down–regulation (Hansra et al., 1999). However, other diterpenes with a more hydrophobic character (Wang et al., 1999; Wang et al., 2000; Braun et al., 2005) reversibly activate PKC and this translates into signaling events that generate different kinds of cell responses (Murphy et al., 1999). This is the case of prostratin (13-*O*-acetyl-12-deoxyphorbol) a commercially available 12-deoxyphorbol initially isolated from the plant *Homalanthus nutans* (Márquez et al., 2008) or other 12-deoxyphorbols isolated from plants of the *Euphorbia* genus like ER272 (12-deoxyphorbol-13-isobutyrate) (Schmidt and Evans, 1977; Fatope et al., 1996; Kirby et al., 2010). The mechanisms underlying tumor promoting activity of PKC remain unknown, although it is possible that this downregulation is responsible for the tumor promoting activity (Newton, 2018b). Therefore, as it has been previously proposed by Newton (2018a,b), phorbol esters with more hydrophobic side chains like prostratin or other 12-deoxyphorbols (Schmidt and Evans, 1977; Fatope et al., 1996; Kirby et al., 2010) may lead to balance PKC activity generating the desired biological responses in the absence of tumorigenic activity.

Protein kinase C inhibitors have also been used in the literature to treat specific CNS injuries (Wang et al., 2014; García-Bernal et al., 2018; Tang et al., 2018). Specific inhibitors that target one PKC isozyme can only be found for classical PKCß (Lesyk et al., 2015) and atypical PKCζ (Puls et al., 1997; Bogard and Tavalin, 2015). On the contrary the majority of commercially available PKC inhibitors target a group of isozymes showing a smaller IC50 for classical PKCα and β. No specific inhibitors target novel PKCs alone without affecting other classical isozymes. Classical PKC inhibitors have been used to repair cervical dorsal spinal hemisection (Wang et al., 2014) and mechanical cortical injuries (García-Bernal et al., 2018), whereas peptide induce inhibition of novel PKCδ has been effective in the treatment of damage of the blood brain barrier (Tang et al., 2018). The molecular mechanisms underlying the effects of the inhibitors are not clear in some cases, because of the lack of specificity.

## DITERPENES, PKC AND NEUROGENESIS

Non-tumor promoting diterpenes with 12-deoxyphorbol or lathyrane structure exert a proliferative effect on NPC cultures *in vitro* (Geribaldi-Doldan et al., 2016; Murillo-Carretero et al., 2017). Interestingly, whereas 12-deoxyphorbols promote NPC proliferation *in vitro* independently of their structure, not all lathyranes are able to exert this effect and only 3,12-di-O-acetyl-8-O-tigloilingol (ELAC) induces a classical PKC dependent effect on NPC proliferation. Treatment of NPC cultures with 12-deoxyphorbols in the absence of EGF increases proliferation promoting cyclin D and E expression mimicking the effects of EGF signaling. This sustains the hypothesis of a PKC dependent release of EGFR ligands (Geribaldi-Doldan et al., 2016; Murillo-Carretero et al., 2017). Additionally, intracerebroventricular administration of diterpenes such as the 12-deoxyphorbols like prostratin or ER272, and the diterpene with lathyrane skeleton ELAC, increase NPC proliferation in neurogenic niches in the adult mouse brain in a PKC dependent manner (Geribaldi-Doldan et al., 2016; Murillo-Carretero et al., 2017). The cellular and molecular mechanisms underlying the proliferative effect of these compounds have also been investigated and it is the specific activation of classical PKCβ what promotes the proliferation of $EGFR^+$ transit amplifying cells in the SVZ (Murillo-Carretero et al., 2017).

## CONCLUSION

Regulating EGFR activity might be relevant when developing strategies to promote endogenous neurogenesis in brain injuries. A limiting step in this activation is the metalloprotease ADAM17, which is regulated by PKC. We have discussed in here how different PKC isozymes govern different steps of the neurogenic process in different niches, concluding that PKC might be a target to promote neurogenesis in injuries. Non-tumorigenic diterpenes with 12-deoxyphorbol or lathyrane skeleton activate PKC and increase NPC proliferation in adult neurogenic niches by activating classical PKC. Diterpenes with the capacity to activate classical PKC might be the active principle of useful drugs to treat disorders that require potentiation of neurogenesis (aging, AD, or Huntington's disease among other). Because of their capacity to reversibly activate PKC, diterpenes are better

drugs than other PKC activators that promote tumor growth. However, specific activators of each PKC isozymes need to be discovered in the short future that could be used to treat diverse CNS disorders in which PKC isozymes are differentially expressed and regulated. On the contrary, inhibition of classical PKC in injuries, thus maintaining novel PKC activities may lead to a limited release of EGFR ligands favoring the generation of new neurons over glial cells. Therefore, finding non-tumorigenic activators of each specific PKC isozyme, which facilitate adjustment of the homeostatic balances of PKC activity, will definitely lead to the development of new drugs and therapeutic strategies to regenerate brain injuries.

## AUTHOR CONTRIBUTIONS

CC, NG-D, and PN-A contributed to conception and design of the review. NG-D wrote the first draft of the manuscript and created the cartoon in **Figure 1**. CC, NG-D, PN-A, RG-O, and SD-G wrote sections of the manuscript. RG-O organized all the information included in the table. All authors contributed to manuscript revision, read and approved the submitted version.

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# Functional Integration of Newborn Neurons in the Zebrafish Optic Tectum

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Neurogenesis persists during adulthood in restricted parts of the vertebrate brain. In the optic tectum (OT) of the zebrafish larva, newborn neurons are continuously added and contribute to visual information processing. Recent studies have started to describe the functional development and fate of newborn neurons in the OT. Like the mammalian brain, newborn neurons in the OT require sensory inputs for their integration into local networks and survival. Recent findings suggest that the functional development of newborn neurons requires both activity-dependent and hard-wired mechanisms for proper circuit integration. Here, we review these findings and argue that the study of neurogenesis in non-mammalian species will help elucidate the general mechanisms of circuit assembly following neurogenesis.

**Keywords: neurogenesis, newborn neurons, zebrafish, activity-dependent development, optic tectum, visual system**

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## INTRODUCTION

Neurogenesis is the process by which new neurons are generated from neural progenitor cells. This process starts during embryonic development, where an initial scaffold is generated and populated by massive waves of newborn neurons. In mammals, this process occurs primarily before birth (Götz and Huttner, 2005), except for two brain regions in which neurogenesis remains active during adulthood: the subgranular zone of the dentate gyrus in the hippocampus and the subventricular zone of the lateral ventricles (Ernst and Frisén, 2015). In mice, embryonic neurogenesis begins with the transformation of neuroepithelial cells (around E10) that are located in the ventricular zone and subventricular zone into radial glial cells. Radial glial cells are neuronal progenitor cells that can generate neurons by symmetric (forming two neurons) or asymmetric division (forming a radial glial cell and a neuron), or by the production of intermediate progenitor cells that later undergo symmetric neurogenic divisions (Götz and Huttner, 2005; Yao et al., 2016). Neurons then migrate, acquire electrical excitability, neurotransmitter identity, and develop axons and dendrites (Spitzer, 2006). Eventually, embryonic neurogenesis sets the global morphology of the nervous system. After birth and during adolescence, extensive axonal, dendritic and synaptic pruning is achieved by a phagocytic-dependent process (Riccomagno and Kolodkin, 2015). In humans, this process is likely to be protracted as synaptic elimination continues in the prefrontal cortex until early adulthood (Rakic et al., 2011).

Adult neurogenesis niches contain quiescent radial glia-like neural stem cells that generate intermediate progenitor cells capable of producing neuroblasts. In mice, approximately 30,000

neuroblasts migrate along the rostral migratory stream every day and populate the olfactory bulb, where they differentiate into interneurons (Alvarez-Buylla et al., 2001). Once mature, they participate in olfactory discrimination, and olfactory short- and long-term associative memory (Lepousez et al., 2015). In the hippocampus, ∼9,000 neuroblasts differentiate daily into granule cells and populate the hippocampus (Cameron and Mckay, 2001). To populate the already-functional neuronal scaffold, newborn neurons compete with the existing circuitry to integrate into the network (Toni et al., 2007, 2008; McAvoy et al., 2016). This integration process lasts ∼8 weeks and plays a critical role in long-term spatial learning (Kee et al., 2007), pattern separation (which permits discrimination between two similar inputs) and affective behaviors (Kempermann et al., 2015).

Zebrafish neurogenesis has traditionally been separated into primary and secondary neurogenesis (Chapouton and Bally-cuif, 2004). Primary neurogenesis takes place before 2 days post-fertilization (dpf), and generates a scaffold of large and mostly transient neurons that mediate spontaneous coils and reflexive motor responses to touch stimuli (Kimmel et al., 1991; Wullimann, 2009). These initial neurons pioneer the major brain components and axon tracts of the embryo (Korzh et al., 1993; Chapouton and Bally-cuif, 2004). Secondary neurogenesis, also termed "post-embryonic," becomes dominant in the hatching larva (Mueller and Wullimann, 2003; Wullimann, 2009) and massively adds neurons to the initial scaffold. At 4 dpf, the nervous system is already mature and the larva already displays a large repertoire of visually induced behaviors (Muto and Kawakami, 2013). Newborn neurons are then added to this functional scaffold in a fashion similar to mammals: neuroepithelial progenitors generate radial glia that serve as progenitor cells with life-long neurogenic potential (Dirian et al., 2014; Galant et al., 2016). In teleosts, up to 16 neurogenesis regions have been described, conferring widespread brain regenerative potential. This capacity may reflect a growing demand of sensory-input processing associated with lifelong body growth (Grandel and Brand, 2013). These qualities make zebrafish an emerging complementary model for the study of newborn neuron integration into mature circuits (Boulanger-Weill et al., 2017; Hall and Tropepe, 2018b).

The optic tectum (OT) has provided important insights into the initial development of post-embryonic neurons (Niell and Smith, 2005; Zhang et al., 2011; Avitan et al., 2017; Pietri et al., 2017). At 5 dpf, the larva is capable of engaging in tectum-dependent complex behaviors such as prey capture (Muto and Kawakami, 2013), and tectal neurons are already responsive to visual stimuli of specific sizes, orientations, and directions (Hunter et al., 2013; Barker and Baier, 2015; Dunn et al., 2016). Due to the small size of the zebrafish larva, its transparency and its genetic accessibility, calcium dynamics of both newborn and mature neurons in the OT can be recorded simultaneously using two-photon microscopy (Boulanger-Weill et al., 2017). These characteristics make the zebrafish OT a complementary and advantageous model to study the mechanisms underlying the incorporation of newborn neurons into the pre-existing circuitry.

Here, we describe the neurogenic niche of the OT and present recent findings on the functional maturation, integration and survival of newborn tectal neurons (Boulanger-Weill et al., 2017; Hall and Tropepe, 2018b). When possible, we provide a comparative description with mammalian neurogenesis. These new studies highlight the power of the zebrafish larva for neurogenesis research and open up exciting new avenues to gain further insights on the general principles underlying the incorporation of newborn neurons into established neuronal circuits.

## NEUROGENESIS AND CELL DIVERSITY IN THE ZEBRAFISH OPTIC TECTUM

At larval stages, the OT neurogenic niche forms a continuous and superficial crescent spanning from its dorso-medial to caudo-lateral margins, where it folds ventrally and connects to the torus semicircularis. This peripheral midbrain layer (PML, **Figure 1A**) contains a discrete *her5*-positive population arranged as a polarized mono-layer of cuboidal cells bound by tight junctions (Galant et al., 2016). They express markers of apico-basal polarity including zona occludens protein 1 (ZO-1), γ-tubulin and aPKC, typical of neuroepithelial cells, and are mosaically positive for Proliferating Cell Nuclear Antigen (PCNA, a marker of the G1 phase of the cell cycle and indicator of cell proliferation, Ito et al., 2010; Galant et al., 2016). Using an elegant genetic tracing approach from post-embryonic to adult stages, Galant et al. (2016) demonstrated that these progenitors generate a transit amplifying pool at the posterior domain of the OT (TPZ, tectal proliferation zone, **Figures 1A,B**). The TPZ subsequently gives rise to neurons by two distinct mechanisms: directly (minor route) or through transiently neurogenic *her4*-positive glia (major route, **Figure 1C**). Such bimodal neurogenic activity seems to be present during early post-embryonic neurogenesis (from 5 dpf) (Galant et al., 2016; Hall and Tropepe, 2018b), but their relative contribution still needs to be assessed. This neuroepithelial neurogenesis is also present in the lateral pallium of zebrafish (Dirian et al., 2014) and closely resembles mammalian embryonic neurogenesis, where neuroepithelial cells are generated first and later give rise to neurogenic glia, eventually becoming the major source for the generation of neurons (Götz and Huttner, 2005). It is noteworthy that neurogenic neuroepithelial cells are absent in the adult mammalian brain, where newborn neurons are formed solely by the division of radial glia-like cells (Bond et al., 2015).

In the OT, newborn periventricular neurons do not migrate but are rather pushed away from the TPZ in a conveyor belt manner (Zupanc et al., 2005; Devès and Bourrat, 2012; Boulanger-Weill et al., 2017), while a small population of reelin-expressing interneurons may actively migrate to the neuropil (Del Bene et al., 2010). In the larva's OT, most neurons become glutamatergic and GABAergic while a minority becomes cholinergic (Robles et al., 2011). Several morphological types have been observed: non-stratified GABAergic or glutamatergic periventricular interneurons (Robles et al., 2011; Barker and Baier, 2015), bi-stratified GABAergic or glutamatergic interneurons (Gabriel et al., 2012), GABAergic periventricular projection neurons (Robles et al., 2011),

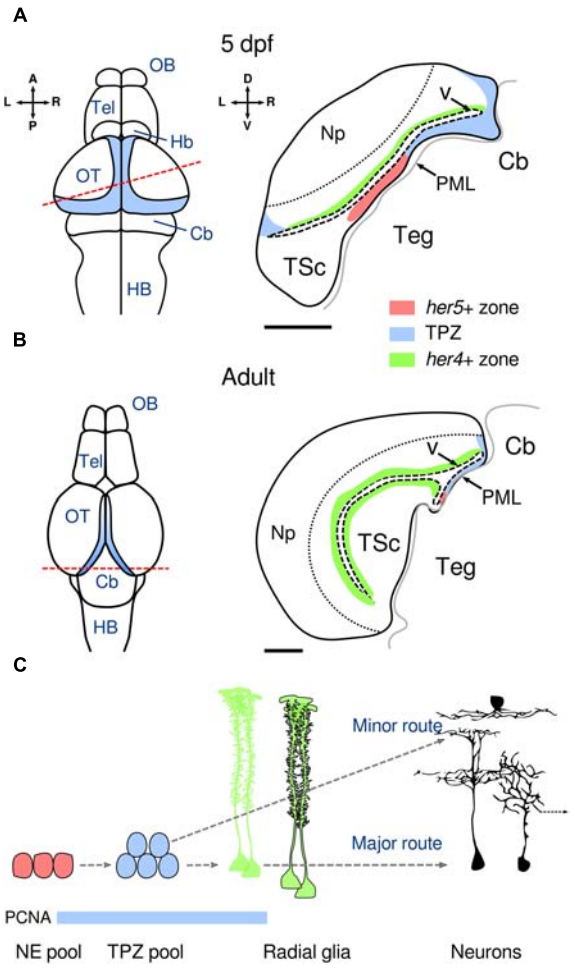


**FIGURE 1 |** Neuroepithelial neurogenesis in the post-embryonic OT. **(A)** Organization of the neurogenesis niche in the OT at 5 dpf. Left panel: dorsal view of the brain representing the main sub-divisions. The blue shaded region represents the TPZ. The dashed red line indicates the cross-section plane in the right panel. Right panel: schematic cross-section of one OT hemisphere showing in red the neuroepithelial *her5*-positive territory, in blue the TPZ and in green the radial glia *her4*-positive territory. The PML provides the developing tectum with progenitor cells that are progressively pushed upward toward the OT. The thin dashed line represents the separation between the neuropil and the periventricular zone containing most of the neuronal cell bodies. The scale bar represents 50 μm. **(B)** Organization of the neurogenesis niche in the OT in the adult zebrafish (older than 3 months). Left panel: dorsal view of the brain representing the main sub-divisions. The high-rate proliferation area is depicted in blue according to Ito et al. (2010). Orientations as in **(A)**. Right panel as in **(A)** showing the reduction of the *her5*-positive territory. The scale bar represents 100 μm. **(C)** Neurogenic sequences in the post-embryonic OT. The *her5*-positive neuroepithelial pool divides at low rates and generates progenitors in the TPZ with high mitotic activity, which in turn generate *her4*-positive radial glia (major route) or neurons (minor direct route). Radial glia are transiently neurogenic in the OT (non-neurogenic radial glia are outlined in black). Although both routes contribute to the neuronal lineage, their relative contribution to neuronal diversity and their dynamics in post-embryonic stages are unknown. Three representative neuronal subtypes are shown: a superficial interneuron (top), a peri-ventricular projection neuron (right, output to other brain regions indicated by the dashed arrow) and a bi-stratified interneuron (left). PCNA expression is indicated by the blue bar. Dashed arrows indicate lineage relationships. L: left, R: right, A: anterior, P: posterior, D: dorsal, V: ventral, OB: olfactory bulb, Tel: telencephalon, Hb: habenula, Cb: cerebellum, HB: hindbrain, Np: neuropil, TSc: torus semicircularis, Teg: tegmentum, V: ventricle, PML: peripheral midbrain layer, TPZ: tectal proliferation zone. Adapted with permission from Galant et al. (2016).

GABAergic or glutamatergic superficial interneurons (Del Bene et al., 2010; Preuss et al., 2014) and mono-stratified interneurons (**Figure 1C**). Taken together, these results suggest that tectal neurons are generated by a protracted post-embryonic mechanism involving neuroepithelial progenitors. Future work will help delineate how these common progenitors give rise to such a diverse neuronal population.

## FUNCTIONAL MATURATION OF NEWBORN NEURONS

The transparency of the larva and its rapid development enable monitoring of the morphological and functional maturation of virtually all neurons from the onset of spontaneous and sensory-induced activity (Niell and Smith, 2005; Warp et al., 2012; Pietri et al., 2017), up to several weeks of development (Boulanger-Weill et al., 2017; Bergmann et al., 2018). As the primary neuronal scaffold is established around 2 dpf, it is possible to monitor both the initial functional assembly of the tectum (Niell and Smith, 2005; Pietri et al., 2017) and the addition of newborn neurons into this scaffold, from their differentiation until their maturity (Boulanger-Weill et al., 2017; Hall and Tropepe, 2018b). Already at 66 hours post-fertilization (hpf), soon after the initial innervation of the tectum by retinal ganglion cells (48 hpf) (Stuermer, 1988), the first post-embryonic neurons display visually induced responses (Niell and Smith, 2005; **Figure 2A**). Surprisingly, retinotopic organization of the visual responses is already evident at 72 hpf, and only slight modifications occur thereafter (up to 9 dpf) (Niell and Smith, 2005). A few direction-selective neurons are also present at 72 hpf, but their population doubles over the course of 6 days. A recent report showed no changes in the tuning curves of direction-selective tectal neurons between 4 and 7 dpf (Nikolaou and Meyer, 2015). These results suggest that direction selectivity is a cardinal property of tectal neurons that is acquired rapidly after innervation by retinal inputs. For a comprehensive review of direction selectivity in the tectum, see Gebhardt et al. (2013).

Neuronal maturation has also been characterized by measuring the development of the visual spatial receptive fields (RFs) of tectal neurons. These RFs represent the area of the visual field of the larva that triggers activity of a given neuron. In Xenopus tadpoles, a distinctive property of mature tectal neurons is the temporal correlation between inhibitory and excitatory inputs, wherein the latter precede the former (Akerman and Cline, 2006). This delay has been suggested to arise from local feed-forward inhibition, and probably serves to enhance the temporal fidelity of visually induced responses, which is vital during prey capture or predator avoidance (Tao and Poo, 2005; Akerman and Cline, 2006). By using whole-cell

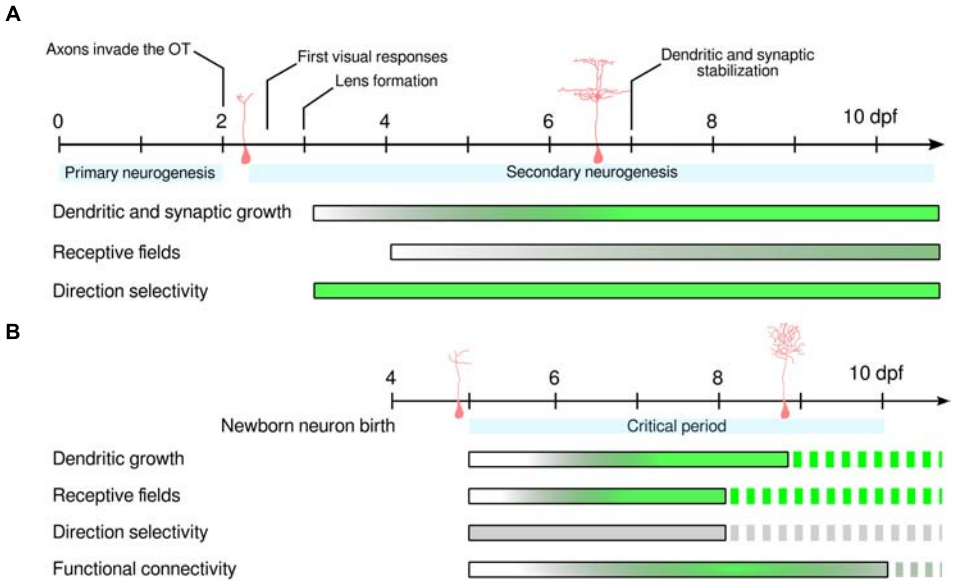


**FIGURE 2 |** Functional development of OT neurons. **(A)** Maturation time-course of initial tectal neurons from 0 to 10 dpf. After primary neurogenesis has set the initial neuronal scaffold, retinal axons invade the OT, the first visual responses in tectal neurons are observed and the eye's lens starts forming. Then, secondary neurogenesis continuously supplies the OT with newborn neurons. Neurons born in the early phase of secondary neurogenesis show rapid structural development, acquiring mature dendritic arborization and post-synaptic densities in 4 days (Niell et al., 2004). RF development is a longer process requiring the temporal synchronization of inhibitory and excitatory RFs, which is complete at 9 dpf. This refinement persists in juvenile stages with the reduction of RF width at up to ∼18 dpf (Bergmann et al., 2018). Direction selectivity is already present in early tectal responses, indicating that this property does not require any pruning of initial erroneous connections in order to emerge. This suggests that activity-independent and hard-wired mechanisms enable the formation of direction-selective neuronal responses in the OT. **(B)** Maturation time-course of newborn neurons born at 4 dpf. During an initial sensitive period following newborn neuron generation (blue shade, from 5 to 10 dpf), sensory experience is required to promote neuronal survival. During this initial period, retinal inputs are also required for functional integration into local networks. Newborn neurons acquire mature dendritic arborization and receptive fields during the first 4 days of development. However, the newborn neurons studied in Boulanger-Weill et al. (2017) show weak direction selectivity, suggesting either slower functional development or biased labeling of non-direction-selective neurons. Functional connectivity increased until 8 dpf and decreased thereafter, suggesting pruning of connections among nearby neurons with different receptive fields. Dashed bars indicate lack of information in the literature. Shaded bars indicate the maturation time-course. The length of each bar is indicative of the measurements performed in the literature. Gray indicates immature stage while green indicates complete maturation. For **(A)**, complete maturation indicates that no further refinement has been observed. For **(B)**, complete maturation indicates that newborn neurons have developed similar properties as mature tectal neurons. dpf: days post-fertilization.

voltage-clamp recordings (Zhang et al., 2011) demonstrated that the RFs of tectal neurons first undergo a period of growth from 4 to 6 dpf and then refinement from 6 to 9 dpf. During this period, excitatory inputs to tectal neurons first increase and then decrease, while the size of the inhibitory RFs, initially larger, decrease to match those of the excitatory RFs at later stages (Zhang et al., 2011). A more recent study (Bergmann et al., 2018) suggests that RF size continues to decrease at least until 18 dpf, indicating that tectal circuits show a high degree of plasticity at juvenile stages (**Figure 2A**). This fine-tuning of visual properties is likely to underlie the continuous improvement in prey capture until adulthood (Westphal and O'Malley, 2013). Therefore, RF maturation is a gradual process that extends at least through the first weeks of larval development.

Apart from the constitutive increase in the number of neurons to adapt to the ever-growing organism, post-embryonic neurogenesis might also promote an increase in visual acuity and efficiency of downstream motor transformations. Indeed, together with the reduction of receptive field sizes, the growth of the tectum could provide higher decoding capabilities in terms of object positions (Avitan et al., 2016). To understand how newborn neurons acquire their mature properties, Boulanger-Weill et al. (2017) monitored the development of their receptive fields over the course of 4 consecutive days, from 5 to 8 dpf as they integrate into the existing functional scaffold. At 1 day old (at 5 dpf), newborn neurons were not visually responsive, indicating that at that developmental stage, they either receive no retinal inputs or lack intrinsic excitability. Two-day-old neurons showed weak and highly variable visual responses, and their RFs were not tuned to any specific region of the field of view. Three-day-old neurons had stronger and less variable visual responses, and their spatial receptive fields were beginning to emerge. Just 1 day later (4-day-old newborn neurons), the neurons displayed mature RFs (**Figure 2B**).

The pair-wise correlations between the spontaneous activity of newborn and mature neurons can be used as a measure of their functional connectivity (Miller et al., 2014; Romano et al., 2015). Analysis of these correlations in the zebrafish OT showed that for the first 2 days of development, newborn neurons were functionally isolated from the neighboring circuitry. Later on, when the neurons reached 3 days old, they displayed correlated activity with mature neurons at close physical distances. Later (at

10 days old), the number of correlations with other neurons in the circuit significantly decreased, probably reflecting a pruning mechanism to remove initially generated erroneous connections between nearby neurons with different visual receptive fields (**Figure 2B**). This process also takes place in the visual cortex of the developing mouse, in an activity-dependent manner (Rochefort et al., 2009; Van Der Bourg et al., 2017). Our results show that the functional integration of newborn neurons is a rapid process, lasting just 4 days. During this process, newborn neurons first receive retinal input, acquire tuned visual receptive fields and correlate their spontaneous activity with that of neighboring neurons. Since recordings in juvenile animals have been technically challenging, the terminal stage of functional maturation of tectal neurons remains to be assessed. Future studies will shed light on the full time-course of functional maturation in the OT and describe how it is affected by sensory-motor experience.

## ACTIVITY-DEPENDENT MATURATION AND INTEGRATION OF NEWBORN NEURONS

Activity-dependent mechanisms play a critical role in mammalian embryonic brain development (Ganguly and Poo, 2013; Pratt et al., 2016). In lower vertebrates, genetically programmed mechanisms are more prevalent, enabling the initial formation of functional circuits even in the absence of sensory stimulation (Niell and Smith, 2005; Pietri et al., 2017). However, sensory experience can later prune and fine-tune initially coarse circuits. For example, exposing Xenopus tadpoles to only 4 h of darkness triggered changes in morphology and excitability in inhibitory neurons, which were rescued by subsequent visual stimulation (He et al., 2016). Similarly, temporary dark rearing of zebrafish larvae significantly reduced the long-term ability of the larvae to hunt prey (Avitan et al., 2017 and see Marachlian et al., 2018 for a review).

In the zebrafish OT, spontaneous activity is organized in topographically compact neuronal assemblies, grouping neurons with strong pair-wise correlations and functionally similar properties (e.g., spatial tuning curves). These neuronal assemblies show attractor-like dynamics and are predictive of directional motor behaviors (Romano et al., 2015; Pietri et al., 2017). Under sensory-deprived conditions (enucleations performed just before newborn neurons start to respond to visual stimuli), newborn neurons in the OT of the zebrafish larva failed to generate functional connectivity with mature neighboring neurons (Boulanger-Weill et al., 2017). Despite enucleation, the mature part of the tectal circuitry remained unaffected, as the spatial and temporal correlations between these neurons were similar to non-enucleated control larvae (Romano et al., 2015; Boulanger-Weill et al., 2017). This result supports the necessity of retinal inputs for the integration but not the maintenance of the initial local functional connectivity. Hall and Tropepe (2018b) carefully examined the survival of newborn neurons when larvae were reared in dim light. They first observed that newborn neuron survival was reduced while proliferation of progenitors and terminal differentiation into mature neuronal subtypes remained unaffected. Second, they demonstrated that exposure to dim light during only the first 5 days (5 to 10 dpf, 0 to 5 days after neuronal birth) of newborn neuron development reduced their survival (**Figure 2B**). Indeed, later exposure to dim light (5 to 10 days after neuronal birth) did not affect neuronal survival, indicating that a critical period constrains neuronal survival in the OT.

## DISCUSSION AND FUTURE DIRECTIONS

In the zebrafish larva, the maturation of newborn neurons and their incorporation takes ∼4 days. In mammals, the maturation process during adult neurogenesis is significantly slower. Indeed, in mice, newborn neurons take around 8 weeks to reach maturity in the olfactory bulb circuit (Livneh et al., 2014). During this maturation process, newborn neurons display a period of increased sensory responsiveness at 4 weeks and then recede to the specificity observed among the resident neurons (Livneh et al., 2014). Newborn hippocampal neurons, similar to embryonic neurons, are first tonically activated by GABA, which is critical for synaptic integration and morphological development (Ge et al., 2006). Later on, after the GABA switch (transition from excitation to inhibition), neurons undergo a transient period of enhanced pre-synaptic excitability (Marín-Burgin et al., 2012). This process might facilitate the strengthening of weak synaptic inputs and therefore facilitate their integration into local networks. These differences are likely to reflect the challenge newborn neurons face when incorporating into adult neuronal circuits, which are probably less plastic than those observed during development. Indeed, in the mammalian hippocampus, newborn neurons compete with the existing circuitry (McAvoy et al., 2016) for sensory inputs, and this enhanced excitability might facilitate their incorporation. In the zebrafish larva, the conveyor belt organization of the OT imposes an order in which newborn neurons are in direct contact with slightly older neurons, thus forming a smooth functional, molecular and morphological maturity gradient. This arrangement may facilitate newborn neurons' incorporation without an increase in their excitability. Recent advances in connectomics both in mammals and fish (Wanner et al., 2016; Schmidt et al., 2017) will enable probing the developing synaptic connectivity in these areas and reveal the global dynamics of circuit assembly.

In zebrafish, sensory experience plays a critical role in the survival and incorporation of newborn neurons. In mice, survival of newborn neurons in the adult hippocampus and olfactory bulb has long been thought to be an activity-dependent process (Rochefort et al., 2002; Song et al., 2013; Alvarez et al., 2016). However, a recent report has suggested that under normal conditions, cell death is absent in the olfactory bulb. They suggest that the cell death observed in previous studies was instead induced by the toxicity of the BrdU labeling (Platel et al., 2018). Still, other studies support that acquisition of functional properties and subsequent integration are constrained by sensory inputs in both regions (olfactory bulb and hippocampus).

In the mouse olfactory bulb, newborn neurons that were exposed to an olfactory-enriched environment during their increased sensitivity period (from 2 to 5 weeks after neuronal birth), displayed enhanced tuning for odors presented during the enrichment (Livneh et al., 2014). The mature neurons only showed these specific responses to odors enriched during their development and not to control odors. These results indicate that newborn neurons can modify their olfactory tuning curves to adapt to environmental conditions. In the mouse hippocampus, physical exercise or exploration of novel environments can also influence the production, maturation, survival and connectivity of adult-born granule cells (Kempermann et al., 1997; Bergami et al., 2015; Alvarez et al., 2016; Trinchero et al., 2017). Indeed, in a recent study, Alvarez et al. (2016) demonstrated that mouse newborn hippocampal neurons are sensitive to an enriched environment during an early critical window lasting 48 h, 9 days after birth (over an 8-week development period to reach maturity). Three weeks after birth, newborn neurons exposed to this environment displayed longer dendrites and spine densities, suggesting quicker maturation and enhanced integration. Taken together, these results suggest that survival and integration of newborn neurons in the mammalian hippocampus, olfactory bulb and zebrafish OT depend on sensory-induced activity. They highlight the existence of critical periods during which the fate of newborn neurons can be modified: enhancing their synaptic integration and morphological maturation (Alvarez et al., 2016), sensory tuning (Livneh et al., 2014), survival (Alvarez et al., 2016; Hall and Tropepe, 2018b) and functional integration (Boulanger-Weill et al., 2017). However, depending on the brain region or animal model, sensory experience may not always affect the development of newborn neurons. For example, in the mammalian developing cortex, the morphology and migration patterns of vasoactive intestinal peptide (VIP)-positive interneurons are not affected when their excitability is altered (De Marco García et al., 2011). Also, in the zebrafish forebrain, neurogenesis is regulated by motor activity but not by visual inputs (Hall and Tropepe, 2018a).

Newborn neurons are constantly added to the vertebrate brain during adulthood. This observation has sparked the idea that endogenous or exogenous newborn neurons could be used to repair damaged or diseased parts of the brain. In a recent work, Falkner et al. (2016) have demonstrated that embryonic neurons injected in the lesioned visual cortex of adult mice can acquire morphology and functional responses that closely match those of the lost neurons. However, these approaches have shown overall minimal improvement in preclinical trials targeting neurodegenerative diseases (see Barker et al., 2018 for review). Therefore, understanding the mechanisms that enable the survival and integration of newborn neurons into already-developed circuits has been a long-standing goal in neurogenesis research. In recent years, functional integration has been assessed by multiple means: electron microscopy of input or output synapses (Toni et al., 2007, 2008), calcium imaging (Marín-Burgin et al., 2012; Boulanger-Weill et al., 2017) and electrophysiology of evoked neuronal responses (Livneh et al., 2014; Alvarez et al., 2016). The zebrafish larva, suitable for the implementation of all these approaches (Gabriel et al., 2012; Wanner et al., 2016) may open the door for a comprehensive and dynamic characterization of the integration of newborn neurons into established circuits.

## AUTHOR CONTRIBUTIONS

JB-W and GS wrote the manuscript.

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REVIEW
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# Divide or Commit – Revisiting the Role of Cell Cycle Regulators in Adult Hippocampal Neurogenesis

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The adult dentate gyrus continuously generates new neurons that endow the brain with increased plasticity, helping to cope with changing environmental and cognitive demands. The process leading to the birth of new neurons spans several precursor stages and is the result of a coordinated series of fate decisions, which are tightly controlled by extrinsic signals. Many of these signals act through modulation of cell cycle (CC) components, not only to drive proliferation, but also for linage commitment and differentiation. In this review, we provide a comprehensive overview on key CC components and regulators, with emphasis on $G_1$ phase, and analyze their specific functions in precursor cells of the adult hippocampus. We explore their role for balancing quiescence versus self-renewal, which is essential to maintain a lifelong pool of neural stem cells while producing new neurons "on demand." Finally, we discuss available evidence and controversies on the impact of CC/$G_1$ length on proliferation versus differentiation decisions.

**Keywords: dentate gyrus, neural stem cells, cyclins, cyclin-dependent kinases, proliferation, differentiation, fate determination, $G_1$**

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## INTRODUCTION

The presence of NSC capable of generating new neurons throughout life provides adult mammals with an exceptional level of brain plasticity. aNSC are multipotent, have the capacity to self-renew and generate progenitor cells which give rise to neurons that functionally integrate into pre-existing networks (Zhao et al., 2006; Suh et al., 2007; Toni et al., 2008; Zhao et al., 2008; Bonaguidi et al., 2011; Encinas et al., 2011; Mongiat and Schinder, 2011; Pilz et al., 2018). Under physiological conditions, adult neurogenesis is mainly restricted to two brain regions: the SVZ of the lateral ventricles and the SGZ of the DG (Doetsch, 2003; Alvarez-Buylla and Lim, 2004). Persuasive evidence suggests that adult-born neurons participate in specific brain functions, including learning and memory, mood regulation, and pheromone-related behaviors (Ming and Song, 2011). Adult neurogenesis is a dynamic, finely tuned process that involves repeated fate decisions, including the proliferation and differentiation of stem and progenitor cells, or survival and maturation of newborn neurons (**Figure 1**). Sustaining the balance between such fate decisions is critical for maintaining homeostasis in the system, as excessive proliferation may induce stem cell exhaustion followed by premature depletion of their pool (Kippin et al., 2005; Sierra et al., 2015). On the other hand, disordered generation of new neurons might disturb the function of the circuit in which they integrate and contribute to neurological disorders (Zhao et al., 2008; Ruan et al., 2014; Jessberger and Parent, 2015). Hence, every step of adult

**Abbreviations:** aNSC, adult neural stem cells; CC, cell cycle; DG, dentate gyrus; IPC, intermediate or transit-amplifying progenitors; NPC, neural precursor cells (undistinguished pool comprising NSC, IPC, and neuroblasts); NSC, neural stem cells; SGZ, subgranular zone; SVZ, subventricular zone; VZ, ventricular zone.

neurogenesis must be tightly regulated by a complex interplay of extrinsic and intrinsic genetic factors to facilitate proper circuit adaption to changing environmental demands (Zhao et al., 2008; Ming and Song, 2011; Faigle and Song, 2013; Opendak and Gould, 2015). In a wide array of cellular contexts, including neural cells, cell fate decisions are closely linked to the CC. In particular the $G_1$ phase opens a time window through which cells can respond to mitogens and specification signals to execute their decision to divide, differentiate or exit the CC (Blomen and Boonstra, 2007; Salomoni and Calegari, 2010; Boward et al., 2016).

We will herein discuss recent progress in our understanding of how the CC integrates extrinsic signals to regulate processes leading to the birth of a new neuron, with emphasis on the $G_1$ phase. Since particularly the hippocampal newborn neurons play critical roles in learning and memory, with disturbances in their generation being associated to neurologic diseases and aging-related cognitive decline, we focus our review on the adult DG. Beyond that, we will discuss relevant findings obtained in the embryonic brain for which more comprehensive data exist, and draw comparisons to the SVZ, where applicable.

## NEUROGENESIS IN THE ADULT DENTATE GYRUS

Neural stem cells residing in the hippocampal SGZ, a narrow area between the dentate granule cell layer and the hilus, are a source of lifelong neurogenesis. They generate intermediate progenitors (IPCs) which give rise to neuroblasts that exit the CC and convert into immature granule neurons (**Figure 1**; Kempermann et al., 2004; Ming and Song, 2011). These precursor stages can be distinguished by their specific morphologies, expression profiles, mitotic activity and their potential to generate distinct progeny. Prototypical aNSCs (also termed radial glia-like cell or type 1) have an apical process which extends radially through the granule cell layer to the molecular layer, and express glial and stem cell markers like GFAP, nestin, BLBP, and Sox2 (Seri et al., 2001; Filippov et al., 2003; Seri et al., 2004; Suh et al., 2007; Berg et al., 2018). Besides neurons, aNSCs can generate astrocytes and daughter NSCs, suggesting multipotency and self-renewal capacity (Suh et al., 2007; Bonaguidi et al., 2011; Encinas et al., 2011; Pilz et al., 2018). In contrast to embryonic neurogenesis, aNSCs are mostly quiescent with only a few progressing through the CC at any time (Lugert et al., 2010; Encinas et al., 2011). Upon activation, aNSCs predominantly divide asymmetrically to produce another NSC and a type 2 cell (Bonaguidi et al., 2011; Encinas et al., 2011). Type 2 progenitors represent an important stage of clonal expansion and linage choice: They are transit-amplifying, prevalently using a symmetric division mode (Encinas et al., 2011; Pilz et al., 2018), and comprise cell states that mark the transition from a glial/stem-like phenotype (type 2a) to a neuronal phenotype (type 2b; Filippov et al., 2003; Steiner et al., 2006). Phenotypically, all type 2 cells are characterized by an irregularly shaped cell body with short horizontal processes, expression of nestin and Tbr2/Eomes, but no longer GFAP (Filippov et al., 2003; Hodge et al., 2008; Bonaguidi et al., 2011; Encinas et al., 2011). Whereas type 2a cells still express BLBP and Sox2, type 2b cells instead show first signs of neuronal commitment, including the expression of NeuroD1, Prox1, and DCX (Steiner et al., 2006; Kempermann et al., 2015). Committed IPCs then give rise to slowly proliferating neuroblasts that, after migrating a short distance into the granule cell layer, exit the CC to become an early postmitotic neuron (Ming and Song, 2011). New neurons then pass through a continuous process of morphological and functional maturation to fully integrate into the hippocampal network (terminally differentiated stage; Zhao et al., 2006; Toni et al., 2008; Mongiat et al., 2009; Mongiat and Schinder, 2011). However, only a small subset of the newborn cells in the SGZ will eventually develop into a mature granule neuron (Biebl et al., 2000; Tashiro et al., 2006; Sierra et al., 2010; Encinas et al., 2011). The majority of these cells are eliminated through controlled cell death, and studies suggest that two critical periods exist for their survival (Sierra et al., 2010). The main critical period is settled around the transition from IPCs to neuroblasts, when cells exit the CC and most of them die. A later critical period exists at the immature neuron stage which is only survived by neurons that are properly integrated (Tashiro et al., 2006; Sierra et al., 2010). At this time, around 4 weeks of age, adult-born neurons are highly excitable whereas inhibitory input is not yet fully established, and exhibit enhanced synaptic plasticity (Zhao et al., 2006; Mongiat et al., 2009; Marín-Burgin et al., 2012). Owing to these unique features, the young granule neurons are considered to enhance hippocampal information processing. In fact, experimental and computational evidence suggest contributions of new neurons to learning, memory, pattern discrimination, and mood regulation (reviewed in Ming and Song, 2011; Toda and Gage, 2017). Whether or not adult neurogenesis is relevant also in humans is still an ongoing debate (Kempermann et al., 2018). In consideration of two recent reports that came to opposing conclusions regarding the neurogenic potential in adult humans (Boldrini et al., 2018; Sorrells et al., 2018), it definitely deserves further efforts to elucidate the significance of this process for human DG plasticity.

### Trophic Factors and Morphogens Regulating Adult Hippocampal Neurogenesis

The adult SGZ is a specialized microenvironment that provides a wide range of extrinsic signals to preserve the self-renewing population of aNSCs and to ensure on-demand production of new granule cells. Besides the NPCs, the niche comprises a dense vascular network and several cell types, including astrocytes, endothelial cells, microglia and neurons (Massirer et al., 2011; Ming and Song, 2011; Faigle and Song, 2013; Licht and Keshet, 2015). NSCs in turn ensheath synapses and blood vessels and adhere to adjacent astrocytes with their fine processes to sense their local environment (Moss et al., 2016). As extensively reviewed by others (Suh et al., 2009; Faigle and Song, 2013; Choe et al., 2015; Toda and Gage, 2017), the effects of niche components on NSC/NPC are mediated by direct cell contacts and soluble molecules such as growth factors, morphogens, hormones, and neurotransmitters. These cues are concurrently

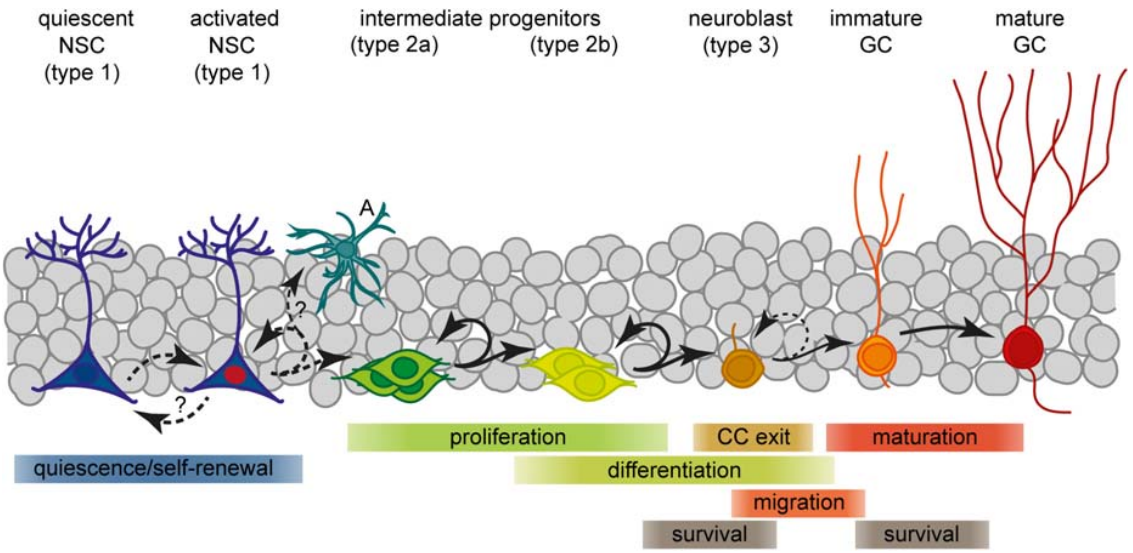


**FIGURE 1 |** Scheme of the current view of lineage progression and fate decisions during adult hippocampal neurogenesis. A, astrocyte; GC, granule cell; NSC, neural stem cell.

acting on adult SGZ precursors not only to coordinate their self-renewal, expansion and fate decisions, but also to maintain NSCs in a quiescent state to prevent their premature exhaustion. Within this section we briefly recapitulate the pathways most vitally involved in CC control of NPCs and summarize their functions in adult hippocampal neurogenesis.

Brain-derived neurotrophic factor (BDNF) is known to promote adult neurogenesis through acting as an autocrine factor for dendritic maturation, functional integration and long-term survival of newborn granule cells (Bergami et al., 2008; Chan et al., 2008; Wang et al., 2015). BDNF and its receptor complex TrkB/p75NTR are also expressed by dividing SGZ progenitors (Chan et al., 2008; Li et al., 2008; Bernabeu and Longo, 2010). As shown *in vitro* and *in vivo*, increased levels of BDNF stimulate the proliferation of hippocampal NPCs, and enhance neurogenesis (Katoh-Semba et al., 2002; Li et al., 2008). It was further observed that NPC-specific deletion of TrkB impairs proliferation of DG progenitors, demonstrating that BDNF and TrkB are required to maintain basal levels of their proliferation (Li et al., 2008). Studies also suggest that increased BDNF signaling serves as mechanism through which exercise, environmental enrichment and antidepressant treatment improve hippocampal neurogenesis and cognition (Rossi et al., 2006; Li et al., 2008).

Fibroblast growth factor 2 (FGF-2) has been extensively studied *in vitro* where it is required for maintaining adult NPCs in a proliferative state (Gage et al., 1995; Gritti et al., 1996). *In vivo* studies revealed FGF-2 as potent modulator of proliferation and differentiation. For example, intraventricular administration of FGF-2 caused a strong increase in proliferation and neurogenesis in the SGZ (Jin et al., 2003; Rai et al., 2007). Moreover, the newborn neurons exhibited enhanced dendritic growth, indicating additional roles in neuronal differentiation and maturation (Rai et al., 2007; Werner et al., 2011). Increased astrocytic release of FGF-2 has recently been identified as requirement for the proliferative effects of acute stress (Kirby et al., 2013).

Insulin-like growth factor-1 (IGF-1) regulates various steps of adult SGZ neurogenesis, including proliferation, differentiation and maturation of neurons, perhaps in a dose-dependent manner (Aberg et al., 2003). IGF-1 directly stimulates proliferation and neurogenesis, both *in vitro* and *in vivo* (Aberg et al., 2000; Yuan et al., 2015). Peripheral administration of IGF-1 induces an increase of NPC proliferation through activation of their IGF-I receptors (Trejo et al., 2001; Aberg et al., 2003; Yuan et al., 2015). Moreover, the study of Trejo et al. (2001) showed that blocking brain uptake of IGF-1 completely abolishes the neurogenesis-promoting effect of voluntary exercise, suggesting that circulating IGF is an important determinant of exercise-induced changes in DG plasticity.

Vascular endothelial growth factor (VEGF) released from endothelial cells exerts direct mitogenic effects on hippocampal NPCs, as shown after intraventricular infusion of VEGF (Jin et al., 2002; Cao et al., 2004). VEGF activates quiescent aNSCs through an autocrine mechanism and VEGF signaling through VEGFR3 controls the response of aNSCs to voluntary exercise (Han et al., 2015). Congruently, blockade of VEGF signaling abolishes the neurogenic actions of running, environmental enrichment or antidepressant treatment (Cao et al., 2004; Warner-Schmidt and Duman, 2007). Altogether, previous investigations on the role of growth factors in the SGZ support a model in which they act as important mediators linking changes in environmental conditions with the processes of adult neurogenesis.

Morphogens play essential roles for neural patterning, proliferation and fate specification in the developing central nervous system. Many of these factors, like sonic hedgehog

(Shh), bone morphogenetic proteins (BMPs), Wnts, and Notch continue to regulate adult NPCs. Their actions often span multiple steps of neurogenesis and differ depending on the specific cellular context. Moreover, many of these morphogen signaling cascades have been shown to cooperate with each other, adding an additional level of complexity to the control of adult neurogenesis (Shimizu et al., 2008; Antonelli et al., 2018; Armenteros et al., 2018).

Bone morphogenetic proteins released by granule neurons and NSCs are essential for maintaining the pool of undifferentiated aNSCs (Mira et al., 2010; Porlan et al., 2013). Beyond that, BMP4 signaling also decelerates the tempo of neurogenesis in later stages of the linage, by directing the transition between activation and quiescence in IPCs (Bond et al., 2014). This and other findings suggest that inhibition of BMP signaling likely represents a mechanism for rapid neuronal expansion in response to behavioral stimulation (Gobeske et al., 2009). Consistently it has been found that endogenous expression of the BMP antagonist Noggin releases NSCs from quiescence to support their proliferation, self-renewal and precursor production (Bonaguidi et al., 2008; Mira et al., 2010). Others discovered that augmented Noggin and BMP4 downregulation mediate the neurogenic and behavioral effects of antidepressants (Brooker et al., 2017). Besides that, BMPs have been shown to control glial fate decisions, having dual functions as promotor of astrogliogenesis and inhibitor of oligodendrogliogenesis (Cole et al., 2016). Accordingly, overexpression of BMP4 in the adult SGZ induces the generation of astrocytes from NSC at the expense of neurogenesis (Bonaguidi et al., 2005).

Notch signaling is reiteratively used to control cell fates during adult neurogenesis in a cell-type specific manner (Ables et al., 2011). It is well established that Notch effector genes Hes1 and Hes5 inhibit differentiation in the CNS by repressing proneural genes (Ohtsuka et al., 1999). Notch1 and Hes5 are highly expressed by aNSC, are absent from neuroblasts to become re-expressed in immature neurons (Stump et al., 2002; Breunig et al., 2007; Ehm et al., 2010; Lugert et al., 2010). Their ligands in turn are found on local astrocytes, IPCs and NSCs (Lavado et al., 2010; Lavado and Oliver, 2014). Inhibition of Notch signaling through manipulation of Notch1, Jagged1 or RBPJκ triggers the activation of quiescent NSC and the production of committed IPCs and neuroblasts, but ultimately exhausts aNSC resulting in premature depletion of their pool (Ables et al., 2010; Ehm et al., 2010; Imayoshi et al., 2010; Lugert et al., 2010; Lavado and Oliver, 2014). This demonstrates crucial roles of Notch signaling for maintaining a reserve of quiescent and undifferentiated NSC throughout life (Chapouton et al., 2010). More specifically, depletion of Jagged1-expressing committed IPCs revealed that Notch signaling serves as homeostatic feedback mechanism that links aNSC maintenance to neuronal differentiation of their progeny (Lavado et al., 2010). Later in the linage, Notch regulates the survival and dendritic morphology in the new, maturing neurons (Breunig et al., 2007).

Wnt ligands are well-established regulators of adult hippocampal neurogenesis (Varela-Nallar and Inestrosa, 2013). Secreted by local astrocytes and NPCs, Wnts act both as autocrine and as paracrine factors on the NSC niche (Lie et al., 2005; Wexler et al., 2009; Qu et al., 2010). Astrocyte-derived Wnt3 promotes neuroblast proliferation and neuronal differentiation via the canonical Wnt pathway (Lie et al., 2005). In IPCs, Wnt can directly induce the proneural gene NeuroD1 (Kuwabara et al., 2009). Accordingly, canonical Wnt signaling is required for neuronal differentiation *in vivo*, i.e., the transition of Sox2-expressing precursors into neuroblasts. In addition, Wnt signaling emerged as important pathway promoting multipotency and self-renewal of aNSCs (Mao et al., 2009). This is probably achieved through an autocrine signaling loop that has been identified *in vitro* (Wexler et al., 2009). Under conditions of low network activity, this pathway is repressed through tonic release of the inhibitor sFRP3 from granule cells, which keeps aNSCs quiescent (Jang et al., 2013). Neuronal activity decreases sFRP3 levels in the DG, resulting in aNSCs activation and accelerated maturation of new neurons, providing a mechanism to produce neurons on demand (Jang et al., 2013).

Sonic hedgehog is a member of the hedgehog family of secreted glycoproteins that acts through the patched-smoothened receptor complex to trigger the expression of GLI transcription factors (Ruiz I Altaba et al., 2002). Previous studies found that NSCs residing in the adult SGZ originate from Shh responsive cells in the ventral hippocampus and that Shh signaling is essential to establish their population (Han et al., 2008; Li et al., 2013). Although it is known that quiescent NSCs and their transit-amplifying progeny respond to Shh activity (Lai et al., 2003; Ahn and Joyner, 2005), the source of Shh in the adult DG is still obscure. Li et al. (2013) identified hilar mossy cells and neurons in the medial entorhinal cortex as principal sources of Shh in the postnatal DG. As they analyzed the mice at postnatal day 15, when the SGZ niche is almost fully established, it is conceivable that these neuronal sources sustain their activity far beyond postnatal development to provide Shh for adult SGZ precursors. Several recent reports emphasize the importance of Shh for balancing aNSC maintenance and proliferation (Lai et al., 2003; Machold et al., 2003; Bragina et al., 2010). Activation of Shh signaling elicits a strong, dose-dependent proliferative response, both *in vitro* and *in vivo* (Lai et al., 2003; Machold et al., 2003). Functional disruption of primary cilia, which are required for Shh signaling, increases CC exit in aNSCs and decreases the production of IPCs (Breunig et al., 2008; Amador-Arjona et al., 2011). A recent study showed that single copy deletion of patched1 results in deregulation at multiple steps of linage progression that accumulates with aging, including depletion of radial NSC, accumulation of IPCs, and a decrease in neurogenesis (Antonelli et al., 2018).

## BASIC PRINCIPLES AND ESSENTIAL PLAYERS OF THE MAMMALIAN CELL CYCLE

The eukaryotic CC can be divided into two major periods composed of four consecutive phases: The interphase, in which cells typically spend most of their lifetime, comprising $G_1$ phase, S phase, and $G_2$ phase, and the M phase in which mitosis and cytokinesis occur (**Figure 2**). The $G_1$ phase is the longest phase

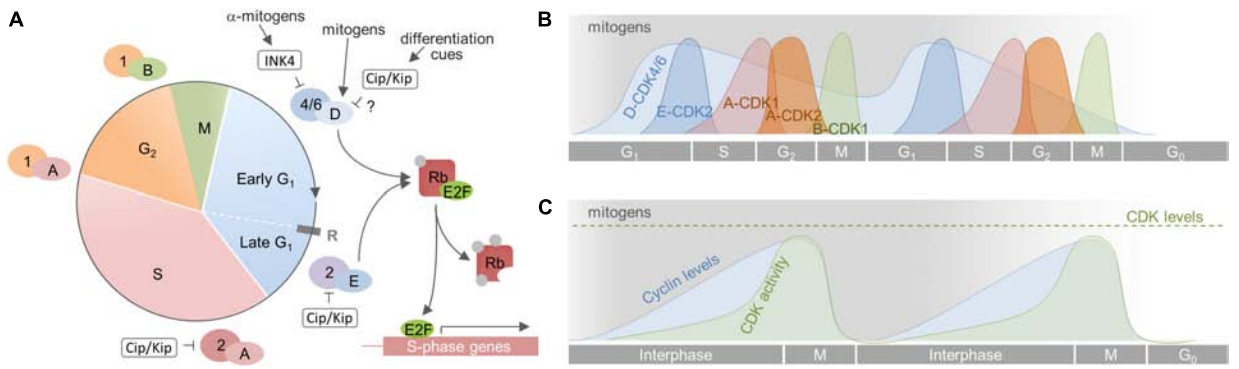


**FIGURE 2 |** Cell cycle progression in mammalian cells. **(A)** The CC is divided into 4 phases ($G_1$, S, $G_2$, and M). The $G_1$ phase is further divided in an early mitogen-dependent, and a late mitogen-independent phase, that are separated by the restriction point (R). Progression through the CC is driven by the sequential activation of CDKs by cyclins. The CC is initiated by mitogens [indicated by gray shading in **(B)** and **(C)**] that induce synthesis of D-cyclins, which in turn activate CDK4/6. This initiates phosphorylation of the tumor suppressor Rb that subsequently liberates E2F. This transcription factor transcribes genes necessary for the transition to S phase, including cyclin E which associates to CDK2 to fully inactivate Rb. All subsequent steps are independent from mitogen supply. **(B)** Activity dynamics of distinct cyclin-CDK complexes throughout the CC. Cyclin D-CDK4/6 complexes are active for as long as mitogens are present. Their upregulation induces expression of cyclin E and a subsequent autonomous sequence of cyclin expression and CDK activation. If mitogens are withdrawn, cyclin D-CDK4/6 complexes become inactive and the cell cannot transit through the next $G_1$ Rather, and depending on the cellular context, it becomes quiescent, senescent or terminally differentiates ($G_0$). **(C)** Cumulative cyclin expression and CDK activity throughout the CC. A, B, D, E – cyclins A, B, D, E; 1, 2, 4/6 – CDKs 1, 2, 4/6.

and the main period of cell growth. $G_1$ prepares the cell for DNA replication and cell division before it can pass into the S phase, in which the cell duplicates its chromosomes. The S phase is followed by the $G_2$ phase, in which the cell proceeds growing and prepares for entering the M phase that eventually results in the generation of two daughter cells that again enter $G_1$. These cells may immediately commence a new round of the CC, as is the case for rapidly dividing progenitor cells. Other cells, like slowly dividing aNSCs or terminally differentiated neurons, may exit the CC to enter a temporary (quiescent) or permanent (post-mitotic/differentiated) $G_0$ state.

Unidirectional progression through the CC is driven by the coordinated activation and inactivation of cyclin-dependent kinases (CDKs) through association with cyclins and cyclin-dependent kinase inhibitors (CKIs; **Figure 2**). CDKs are serine/threonine kinases that require the binding of regulatory subunits, i.e., cyclins, and phosphorylation by CDK-activating kinases (CAKs) to exert their catalytic function (Sherr, 1995; Johnson and Walker, 1999; Lolli and Johnson, 2005; Malumbres, 2014). CDKs and CAKs are abundantly expressed and their levels remain fairly stable throughout the CC, consequently they are not rate-limiting for its progression (Lees, 1995; Morgan, 1995). Cyclin levels in turn fluctuate through synthesis and degradation and, in this way, periodically activate CDKs (Matsushime et al., 1994; King et al., 1996; Udvardy, 1996). The activated cyclin-CDK complexes phosphorylate multiple downstream targets, ensuring appropriate ordering of cell-cycle events (Swaffer et al., 2016). Depending on the activity of cyclin-CDK complexes in the $G_1$ phase, cells will commit to divide or withdraw from CC (Blagosklonny and Pardee, 2000–2013, 2002). This decision depends on extracellular signals like mitogens and growth factors that induce the expression of D-cyclins (Matsushime et al., 1994; Sherr, 1994a; Ekholm and Reed, 2000). The D-cyclins assemble with CDK4 or CDK6 to drive progression through the $G_1$ phase. Cyclin D-CDK complexes initiate the phosphorylation of Rb proteins and thus weaken their growth suppressive effect by releasing E2F transcription factors. E2Fs then start to transcribe various genes required for $G_1$/S phase transition, including DNA polymerase α, cyclins E and A (Sherr, 1995; Lundberg and Weinberg, 1998; Julian and Blais, 2015). Assembly of cyclin E with CDK2 during later $G_1$ further phosphorylates Rb, forming a positive feedback loop that fully activates the transcription of genes essential for DNA replication and onset of S phase (Lundberg and Weinberg, 1998; Johnson and Walker, 1999). Activation of cyclin E/CDK2 complexes demarks the point at which commitment occurs and cells do no longer require mitogens to complete division. This point, termed the restriction point, represents a point of no return and divides $G_1$ into an early, mitogen-dependent phase and a late, mitogen-independent phase (Johnson and Walker, 1999; Blagosklonny and Pardee, 2000–2013, 2002). Soon thereafter, cyclin A interacts with CDK2 to turn off E2F-mediated transcription and drive the cell through S phase (Krek et al., 1994; Xu et al., 1994). In late S phase, cyclin A associates with CDK1 to facilitate transition to and progression through $G_2$ and to prepare the cells entry into the M phase (Yam et al., 2002; Lindqvist et al., 2009). Finally, cyclin B builds an active complex with CDK1, termed M phase promoting factor, which coordinates the onset of M phase and the reorganization of cell structures required for mitosis and cytokinesis (Nigg, 2001; Gavet and Pines, 2010). As the newly born cells exit mitosis, cyclin B is degraded and the CC is reset, allowing the establishment of a new replication-competent state, i.e., $G_1$ (King et al., 1996; Sherr and Roberts, 1999). Important to note, cyclins, CDKs and CKIs carry out important functions beyond CC regulation, either complexed or individually, such as transcription, stem cell self-renewal, differentiation, neuronal function, cell death, or metabolism (Lim and Kaldis, 2013; Pestell, 2013; Hydbring et al., 2016).

## D-Cyclins

D-cyclins are the first cyclins induced when quiescent cells become stimulated to enter the CC (Matsushime et al., 1994; Sherr, 1994a,b). Unlike other cyclins that are periodically expressed during the CC, D-cyclins are induced by extracellular mitogens, including the above mentioned niche factors (Bottazzi and Assoian, 1997; Perry et al., 1998; Ekholm and Reed, 2000; Baek et al., 2003; Frederick and Wood, 2004; Campa et al., 2008; Shimizu et al., 2008; Chen et al., 2018), and hence are regarded as crucial direct link between the extracellular environment and the CC machinery (Matsushime et al., 1994). They comprise a family of three homologous proteins (cyclin D1, D2, and D3) that bind to and thereby activate either CDK4 or CDK6 (Sherr and Roberts, 1999, 2004). Extensive studies in knockout mice uncovered that D-cyclins are, to a great extent, functionally redundant but that each has unique tissue-specific functions (Wianny et al., 1998; Sherr and Roberts, 2004; Satyanarayana and Kaldis, 2009). Intriguingly, not all cells require D-cyclins/cyclin D-CDK complexes for proliferation and embryos lacking all three D-cyclins develop normally until midgestation (Kozar et al., 2004). D-cyclins drive cells through the $G_1$ restriction point, after which mitogen stimulation is no longer required to complete the cycle. They are unstable proteins that become rapidly degraded via the ubiquitin/proteasome pathway if mitogens are withdrawn (Diehl et al., 1997; Ekholm and Reed, 2000). According to the classical model of $G_1$ progression, active cyclin D/CDK complexes inactivate the Rb tumor suppressor through gradual hypo-phosphorylation to liberate E2F transcription factors required for CC progression (Mittnacht et al., 1994; Sherr and Roberts, 1999). This view has been recently challenged by a study demonstrating that cyclin D-CDK complexes do not inactivate, but instead activate Rb during early $G_1$ through mono-phosphorylation (Narasimha et al., 2014). Hyper-phosphorylation of Rb through Cyclin E/CDK, which inactivates Rb to ensure E2F-dependent transcription in later $G_1$, seems to be independent from that mono-phosphorylation. Instead, Narashima et al. (2014) propose that Rb-mono-phosphorylation by cyclin D-CDK complexes keeps cells in an "alert" state, priming them for CC entry and preventing CC exit. The debate was fueled again by subsequent work that suggests that cyclinD-CDK4/6 complexes control the timing of onset of E2F activity, indicating that D-cyclins indeed are the main regulators of $G_1$ length, rather than being the initiator of CC entry (Dong et al., 2018). Despite this controversy on being drivers or modulators of $G_1$ progression, D-cyclins are consistently regarded as crucial link between extracellular growth-stimulating signals and the CC machinery. In addition to their role in modulating Rb activity, cyclin D-CDK complexes have also kinase-independent CC functions in titrating away inhibitors $p21^{cip1}$ and $p27^{kip1}$ from CDK2-containing complexes to facilitate cyclin E-CDK activation and coordinated CC progression in later $G_1$ (Sherr and Roberts, 1999; Kozar and Sicinski, 2005).

## Cyclin-Dependent Kinases (CDKs)

To this day, eleven different CDKs have been related to the mammalian CC, out of which at least five (CDK1, 2, 3, 4, and 6) are directly involved in its progression (Satyanarayana and Kaldis, 2009; Malumbres, 2014). Contrasting many of the initial *in vitro* studies that showed specific requirements for each interphase CDK in cycle progression, mice lacking a single CDK survive, indicating profound functional redundancy among CDKs (Sherr and Roberts, 2004; Satyanarayana and Kaldis, 2009; Malumbres, 2014). Even mouse embryos lacking all interphase CDKs undergo organogenesis and develop until midgestation. This is possible because CDK1, by complexing with all necessary cyclins, can execute all crucial events required for cell division (Santamaria et al., 2007). Nevertheless, single-CDK knockout mice display more or less narrow, tissue-specific deficits demonstrating that CDKs have different roles and cannot fully compensate for each other (Sherr and Roberts, 2004; Kozar and Sicinski, 2005; Malumbres, 2014). In addition to cyclin binding, different mechanisms are engaged in the control of cyclin-CDK complexes, including phosphorylation through constitutively active CAKs and binding of CKIs (Lees, 1995; Morgan, 1995; Ekholm and Reed, 2000; Malumbres, 2014). Importantly, cyclin binding determines the timing of activation and contributes to substrate specificity of CDKs (Morgan, 1995; Satyanarayana and Kaldis, 2009).

## CDK Inhibitors (CKIs)

CDK Inhibitors are generally assumed as negative CC regulators that constrain the activities of CDKs in response to anti-mitogenic factors or starvation. Thus, CKIs in addition to D-cyclins confer a second layer of CC control by extracellular signals. They comprise two families of inhibitory proteins that differ in structure and substrate specificity (Arellano and Moreno, 1997; Sherr and Roberts, 1999; Besson et al., 2008).

Members of the INK4 (INhibitors of CDK4) family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) act as brakes of $G_1$ progression in response to mitogenic withdrawal, differentiation signals and other growth inhibiting cues (Koff et al., 1993; Kato et al., 1994; Polyak et al., 1994). They bind exclusively to and inhibit CDK4 and CDK6 to prevent their interaction with cyclin D (Guan et al., 1994; Hirai et al., 1995; Sherr and Roberts, 1999; Jeffrey et al., 2000; Pei and Xiong, 2005), but with different preferences. $P18^{INK4c}$ preferentially interacts with CDK6, whereas $p16^{INK4a}$ associates with both CDKs (Guan et al., 1994; Noh et al., 1999). INK4 proteins differ also in their regulation and function. $P15^{INK4b}$ is induced by growth-inhibitory factors such as TGFβ and contributes to their ability to induce growth arrest (Reynisdottir et al., 1995; Sherr and Roberts, 1999). $P16^{INK4a}$ is ubiquitously expressed at low levels in most tissues and accumulates as cells age. It becomes induced during cellular senescence, in response to oncogenic stimuli or inactivation of Rb (Serrano, 1997; Sherr and Roberts, 1999). $P18^{INK4c}$ and $p19^{INK4d}$, even if sensitive to extrinsic signals, oscillate throughout the CC with maximum levels during S phase (Hirai et al., 1995; Roussel, 1999). Both are found in proliferating cells in which they facilitate CC exit during terminal differentiation (Hirai et al., 1995; Roussel, 1999). The *INK4A* locus is unique in that it transcribes another protein, $p19^{ARF}$ that is also associated with cellular senescence (Lowe and Sherr, 2003). Instead of binding CDKs, $p19^{ARF}$ confers its role through both,

activation of p53 and inhibition of c-Myc (Quelle et al., 1995; Roussel, 1999; Qi et al., 2004).

In contrast, Cip/Kip proteins ($p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$) act more broadly by modulating the activities of cyclin D-, E- and A-dependent kinases. Cip/Kip proteins bind to preformed cyclin-CDK complexes and block their substrate access (Russo et al., 1996; Sherr and Roberts, 1999). They most effectively inhibit complexes containing CDK2 and thereby prevent S phase entry and transition (Sherr and Roberts, 1999). Discordance exists regarding their effects on cyclin D-CDK complexes. In addition to reports arguing in favor of a pan-CDK-inhibitory role of Cip/Kip proteins (Bagui et al., 2003; Pei and Xiong, 2005; Cerqueira et al., 2014), others suggest that they act as assembly and stabilization factors of cyclin D-CDK4/6 to facilitate cyclin D-dependent CC events (LaBaer et al., 1997; Cheng et al., 1999; Parry et al., 1999; Sherr and Roberts, 1999). Despite their general importance for restraining cell proliferation, each Cip/Kip protein has unique functions that distinguish it from the other family members. Expression and functional studies suggest that $p21^{cip1}$ has a predominant role in DNA-damage-induced CC arrest, while $p27^{kip1}$ inhibits cell growth and maintains cells in a quiescent state, and $p57^{kip2}$ regulates growth and differentiation (Deng et al., 1995; Nakayama et al., 1996; Zhang et al., 1997; Besson et al., 2008). Unlike its siblings, $p57^{kip2}$ expression is more restricted and under control of morphogen pathways such as Notch and BMP (Besson et al., 2008). A unique feature of $p21^{cip1}$ and $p57^{kip2}$ is their ability to inhibit PCNA (Zhang et al., 1993; Besson et al., 2008). This allows them to coordinately arrest the CC by both, preventing E2F-dependent transcription and inhibiting PCNA-dependent replication (Cayrol et al., 1998). During the past years Cip/Kip proteins have emerged as versatile factors with functions beyond cycle regulation, including direct transcriptional regulation (e.g., E2F and STAT3), fate determination and cell death control (Besson et al., 2008).

## Rb Proteins and E2F

Rb and its family members p107 and p130, collectively known as "pocket proteins," are placed at the core of the molecular machinery that regulates the $G_1$ restriction point (Dick and Rubin, 2013). They impose a constitutive barrier on CC progression through sequestration of E2F transcription factors, for which each Rb protein displays distinct binding preferences (Hurford et al., 1997). Rb interacts specifically with "activator" E2Fs 1–3, whereas p107 and p130 interact with "repressor" E2Fs 4 and 5 (Mulligan and Jacks, 1998; Popov and Petrov, 2014). Importantly, growing evidence suggests that E2F activities are context-dependent, e.g., switching from activators in progenitors to repressors in differentiating cells (Chong et al., 2009). Another characteristic distinguishing Rb proteins is their allocation to different CC states: while Rb is constitutively expressed in all cells, acting as *bona fide* tumor suppressor, p107 predominates in cycling cells, and p130 is most prevalent in quiescent and differentiated cells (Mulligan and Jacks, 1998; Dick and Rubin, 2013). Moreover and in contrast to Rb, p107 and p130 confer growth suppression through another mechanism involving direct inhibition of CDK2-containing complexes (Lees et al., 1992; Woo et al., 1997). Beyond their direct role in CC progression, Rb proteins can interact with many other proteins to retain cells in $G_0/G_1$, such as histone deacetylases 1 and 2, histone methyltransferases, cyclins and complexes of the SWI/SNF nucleosome remodeling complex (Brehm et al., 1998; Mulligan and Jacks, 1998; Robertson et al., 2000; Strobeck et al., 2000). In addition to their CC regulatory function, Rb and E2Fs are capable of regulating genes and processes with much broader range of function, many of which directly instruct cell fate decisions (Julian and Blais, 2015). Accordingly, Rbs and E2Fs emerged as essential regulators of stem cell fate in a number of lineages (Sage, 2012; Julian and Blais, 2015). Rb deletion is sufficient to induce CC re-entry in various cell types, suggesting that it helps to maintain quiescence also in aNSCs (Sage et al., 2003; Sage, 2012). Furthermore Rb's capacity to modulate chromatin structure has been proposed as important regulator of stem cell plasticity (Chinnam and Goodrich, 2011; Sage, 2012).

# SPECIFIC ROLES OF CELL CYCLE COMPONENTS IN REGULATING ADULT HIPPOCAMPAL NEUROGENESIS

## D-Cyclins

All three D-cyclins are dynamically expressed during neurulation (Wianny et al., 1998). Later in brain development, D3 becomes gradually lost, whereas D1 and D2 activities are maintained to regulate the generation of distinct progenitor populations (Sun et al., 1996; Bryja et al., 2004; Glickstein et al., 2007a; Komada et al., 2013). Recent studies suggest that, beside their role in proliferation, D-cyclins and D1 in particular may directly control stem cell fate decisions to induce neuronal differentiation (Lukaszewicz and Anderson, 2011; Pauklin and Vallier, 2013; Pauklin et al., 2016). Apparently, this is accomplished by two complementary mechanisms: D-cyclins cross-talk with the Activin/Nodal–Smad2/3 signaling pathway through CDK4/6 and also directly bind to transcription factors that suppress endoderm and activate neuroectoderm genes in stem cells (Pauklin and Vallier, 2013; Pauklin et al., 2016). Further, D1 has been identified as critical component of FGF-2 and Wnt signaling that inhibits astroglial differentiation of NSCs (Bizen et al., 2014).

During *embryogenesis*, D1 and D2 appear to have complementary roles in proliferation and differentiation of NPCs (Glickstein et al., 2007a,b, 2009; Lukaszewicz and Anderson, 2011). Initially it was shown that D1 and D2 define separate progenitor pools in the embryonic neocortex (Ross, 1996; Glickstein et al., 2007a). Whereas D1 is predominantly expressed in the VZ, D2 is localized mainly to the SVZ (Glickstein et al., 2007a). More detailed studies revealed that D1 and D2 co-label subsets of Pax6 expressing radial glia/apical precursors, which are the stem cells in the embryonic brain (Glickstein et al., 2009). As these cells commit to the neuronal linage, D1 is gradually downregulated and D2 becomes induced (Glickstein et al., 2009; Lange et al., 2009). The selective expression of D2 in Tbr2-positive IPCs/basal precursors and their loss upon genetic deletion of D2 suggest that D2 is selectively used for

the expansion of the IPC pool (Glickstein et al., 2009). Withal, the knockout also diminishes the radial glia population. The same study revealed severe disturbances in CC dynamics in progenitors of D2 mutants, such as lengthening of $G_1$, decreased in-cycle and increased cycle-exit fraction of IPCs, consistent with premature terminal differentiation. Interestingly, deletion of D2 not only altered $G_1$ length but it also shortened the S phase. In contrast, CC parameters and numbers of Pax6 and Tbr2-positive precursors in the cortical neuroepithelium remained unchanged in D1 mutants (Chen et al., 2005; Glickstein et al., 2007a, 2009). This shows that D2, perhaps in conjunction with other CC proteins, can compensate for the loss of D1 but not vice versa (Carthon et al., 2005; Chen et al., 2005). Collectively, these findings show that D2 is more essential for cortical development than D1. They fit a concept in which D2 is required for expansive divisions of radial glia and IPCs whereas D1 drives asymmetric stem cell divisions to maintain their pool (Glickstein et al., 2009). Supporting the progenitor-specific requirement of D2 in brain formation, D2 mutant mice also display a disproportionate loss of parvalbuminergic interneurons in the neocortex and hippocampus, as well as loss of half of cerebellar granule neurons and virtually all cerebellar stellate interneurons (Huard et al., 1999; Glickstein et al., 2007b; Leto et al., 2011). Direct *in vivo* evidence for a CC-independent proneurogenic function of cyclin D1, as originally discovered in human pluripotent stem cells (Pauklin and Vallier, 2013; Pauklin et al., 2016), comes from loss- and gain-of-function experiments in the embryonic spinal cord, where D1 is expressed by differentiating NPCs and newly generated neurons (Lukaszewicz and Anderson, 2011). Knockdown of D1, but not that of D2, decreased neuronal fate specification and differentiation through activating the non-canonical Notch effector Hes6, whereas overexpression had the opposite effect. D2, on the other hand, promotes proliferation and maintains the undifferentiated progenitor pool, probably through regulation of the canonical Notch effector Hes5 (Lukaszewicz and Anderson, 2011). Strikingly, studies in the embryonic cortex have shown that asymmetrically inherited D2 may act as fate determinant in radial glia of the VZ (Tsunekawa et al., 2012). During division, these cells allocate D2 mRNA to their basal end-feet (Glickstein et al., 2007a; Tsunekawa et al., 2012). The daughter inheriting the basal process and thus D2 upon asymmetric mitosis will become another radial glia cell that maintains self-renewal capacity, while its sibling will differentiate into an IPC or a neuron (Tsunekawa et al., 2012). The molecular mechanisms are not fully understood, but the biased localization of D2 to one daughter might shorten her $G_1$ phase. Alternatively, D2 might control cell fate independent from its direct action on CC progression. D2 might advance proliferation and inhibit neurogenesis through elimination of the CKI $p27^{kip1}$, which promotes neuronal differentiation (Nguyen et al., 2006; Susaki et al., 2007; Tsunekawa et al., 2012). In opposition to neocortical development, both D-cyclins are expressed in the VZ of the early hippocampal primordium (Glickstein et al., 2007a). At perinatal ages, they are also found in the dentate anlage, but D1 predominates in the secondary and tertiary matrices, while more D2-positive neuroblasts are found in the dentate migratory stream. Postnatally, D1 and D2 are found in the tertiary matrix and the emerging SGZ. In the adult DG, D2 expression is restricted to the SGZ whereas D1 shows a more widespread, scattered expression throughout all layers of the DG (Kowalczyk et al., 2004; Glickstein et al., 2007a).

In accordance with the apparently distinct roles of cyclins D1 and D2 during development, absence of either cyclin is associated with strikingly different outcomes in the *adult brain*. As mentioned before, mice lacking D1 have essentially normal brain sizes with no selective deficits in particular regions, except a cerebellar hypoplasia (Chen et al., 2005; Pogoriler et al., 2006; Glickstein et al., 2007b, 2009). The gross architecture of D2 mutant brains is also normal, but these mice are microcephalic (about 25% reduced size), and especially the cortex, cerebellum, olfactory bulb and hippocampus are smaller in size (Kowalczyk et al., 2004; Glickstein et al., 2009; Ansorg et al., 2012). The lack of apparent deficits in D1 knockout mice may result from compensatory mechanisms, including the upregulation of other $G_1$ cyclins, i.e., cyclin D2 and cyclin E (Carthon et al., 2005; Chen et al., 2005; Glickstein et al., 2007a).

**Cyclin D1** is expressed in the adult SGZ. Some of the D1 cells proliferate as indicated by incorporation of the S phase label bromodeoxyuridine (Glickstein et al., 2007a). Studies evaluating the consequences of D1 deletion on hippocampal cell proliferation came to conflicting results, with one showing no effect on proliferation, and another reporting >40% reduction of BrdU incorporation into the adult SGZ (Kowalczyk et al., 2004; Ma et al., 2010). In support of a role of D1 in aNPC proliferation, Ma et al. (2010) found that cultured precursor cells of D1 knockout mice become arrested in $G_0/G_1$. In addition, mutated precursors displayed increased apoptosis and disturbed differentiation into astrocytes, while neuronal differentiation remained intact (Ma et al., 2010). Yet, the overall picture of D1 regulation of adult neurogenesis is far from clear and needs further investigation. For instance, none of the previous studies had looked on the specific requirements of D1 in distinct precursor types as has been published for the embryonic brain. Evaluating such roles is complicated through the fact that D2 becomes upregulated and might compensate in the SGZ of D1 mutant mice (Carthon et al., 2005; Glickstein et al., 2007a).

Available data on the role of **cyclin D2** in adult hippocampal neurogenesis are more consistent. The SGZ of adult D2-deficient mice is virtually devoid of proliferating cells and neuroblasts (Kowalczyk et al., 2004; Ansorg et al., 2012). We showed that the requirement of D2 for adult neurogenesis builds up progressively during postnatal development. With full maturity of the DG/SGZ around 4 weeks of age, mutant mice are virtually devoid of newly born neurons in this region (Altman and Bayer, 1990; Ansorg et al., 2012; Nicola et al., 2015). Moreover, this impairment in adult neurogenesis cannot be overcome through exposure to physiological or pharmacological stimuli that normally increase adult hippocampal neurogenesis. For instance, numbers of cells incorporating BrdU remained negligible in response to environmental enrichment or fluoxetine treatment (Kowalczyk et al., 2004; Jedynak et al., 2012). So far it is not clear whether this deficit results from the inability of mutant SGZ precursors to divide or from a lack of precursors that can respond to such neurogenic stimuli (Ansorg et al., 2012). Interestingly,

despite of their severe impairments in adult neurogenesis, D2KO mice are able to master hippocampus-dependent tasks, including contextual or trace fear conditioning, object recognition, and spatial learning in Barnes or water mazes (Jaholkowski et al., 2009; Jedynak et al., 2012; Ben Abdallah et al., 2013; Urbach et al., 2013). However, impairments became apparent in tasks requiring a certain degree of flexibility for integrating novel information into previously learned contexts (Garthe et al., 2014). Clinically relevant, loss-of-function mutations affecting the *ccnD2* locus have been linked to brain pathologies like microcephaly and epilepsy (by loss of interneurons; Glickstein et al., 2009). In humans, *de novo ccnD2* mutations stabilizing D2 have been identified as cause of the megalencephaly polymicrogyria-polydactyly hydrocephalus syndrome (Mirzaa et al., 2014).

In summary, available data suggest that D1 and D2 have discrete, non-overlapping functions in adult neurogenesis. Considering recent observations in human embryonic or pluripotent stem cells, available data suggest that the main function of D2 is controlling adult precursor proliferation, whereas D1 seems to be rather involved in neuronal differentiation (Lukaszewicz and Anderson, 2011; Pauklin and Vallier, 2013; Pauklin et al., 2016).

## Cyclin-Dependent Kinases (CDKs)

All three $G_1$ CDKs have been detected in apical and basal precursors of the embryonic VZ/SVZ (Telley et al., 2016). However, only combinatorial ablation of two $G_1$ CDKs affected cortical progenitor proliferation *in vivo*, suggesting redundant or compensatory functions of CDKs during corticogenesis (Lim and Kaldis, 2013; Grison et al., 2018). Moreover, the effects of CDK2/4, CDK2/6, or CDK4/6 double-knockouts were progenitor type-dependent, indicating that discrete combinations of CDKs are used in distinct precursor populations (Lim and Kaldis, 2013; Grison et al., 2018).

**CDK2** kinase activity is high during *cerebral development* but drops rapidly thereafter (Carey et al., 2002). This suggests important functions of this enzyme in cortex development, which has been corroborated by several *in vitro* studies. For instance, cultured cortical precursors increased CDK2 activity upon FGF-2 stimulation (Lukaszewicz et al., 2002; Li and Dicicco-Bloom, 2004). Also, inhibition of CDK2 activity through adenoviral delivery of double-negative CDK2 mutants induced complete growth arrest in isolated NSC from embryonic forebrain (Ferguson et al., 2000). Nevertheless, no developmental deficits or impaired proliferation were observed in the SVZ of CDK2 mutant mice (Jablonska et al., 2007; Grison et al., 2018). Even the concomitant loss of CDK6 had no effect on the proliferation rate of cortical precursors, suggesting functional compensation by CDK1, CDK4 or other CDKs. Indeed, a subsequent study found altered CC parameters and increased neurogenic divisions in NSCs of CDK2/CDK4 double knockout embryos (Lim and Kaldis, 2012). This prevented the expansion of the basal IPC pool culminating in a striking thinning of the embryonic cortex. Nevertheless, isolated embryonic precursors of these mice retained their capacity to divide due to compensatory upregulation of CDK1 (Lim and Kaldis, 2012). CDK2 appears also dispensable in the neurogenic niches of the *adult brain*. Functional deletion of CDKs had neither an effect on proliferation, nor on differentiation or survival of hippocampal NPCs, both under basal and under seizure conditions (Vandenbosch et al., 2007). Others showed that CDK2 is expendable for proliferation of NSCs and neuroblasts in the adult SVZ (Jablonska et al., 2007; Caillava et al., 2011). Instead, these loss-of-function studies revealed an involvement of CDK2 in the proliferation, lineage commitment and differentiation of oligodendrocyte precursors in the adult SVZ (Jablonska et al., 2007; Caillava et al., 2011).

Two recent studies indicate that **CDK4** drives IPC expansion in the *embryonic* SVZ between E13.3 and E15.5 (Lim and Kaldis, 2013; Grison et al., 2018). This, however, became only apparent if CDK4 was depleted together with another $G_1$ CDK, i.e., CDK2 or CDK6. Interestingly, any of these combinatorial depletions left the population of apical NSCs in the VZ intact (Lim and Kaldis, 2013; Grison et al., 2018). Yet, adult single mutants are microcephalic (Beukelaers et al., 2011), pointing toward important non-redundant functions of CDK4 at developmental time points not investigated in the before mentioned studies. CDK4 is also expressed in dividing precursors of the *adult neurogenic niches* (Beukelaers et al., 2011), but like during development the picture is far from clear. Initial functional evidence came from loss- and gain-of-function experiments that described an involvement of cyclin D-CDK4 in fate specification of embryonic and adult NSCs (Lange et al., 2009; Artegiani et al., 2011). They observed that overexpression of cyclin D1-CDK4 reduces $G_1$ length and neurogenesis in the adult SGZ as well as in the embryonic cortex, while RNAi-mediated silencing of cyclin D1-CDK4 lengthens $G_1$ and increases neuronal differentiation during corticogenesis. However, Calegari and his group manipulated CDK4 together with D1, so no clear conclusions on the role of either of them can be drawn from these studies (which, by the way, was not the their aim). Rather, they provided persuasive evidence for a link between $G_1$ length and differentiation of NSC (Lange and Calegari, 2010; Salomoni and Calegari, 2010). More direct evidence was presented by subsequent *in vitro* studies (Roccio et al., 2013; Chirivella et al., 2017). Inhibition of CDK4 in precursors isolated from the adult SGZ or SVZ proportionally increased the number of cells in $G_1$ and forced neuronal differentiation. Recently, activation of CDK4 has been identified as mechanism of the proliferative effect of insulin in aNSC (Chirivella et al., 2017). Unexpectedly, this study also implies that CDK4 activity in response to insulin can promote terminal differentiation of NSCs. Yet, CDK4 appears to be dispensable *in vivo*. Although CDK4 is expressed by virtually all Ki67-positive cells in the adult SGZ, its loss had no effect on CC parameters, proliferation or neurogenesis (Beukelaers et al., 2011).

Available evidence for a role of **CDK6** in *brain development* is more or less indirect. A study in mice revealed that downregulation of CDK6 by Gli3 is required for the scheduled onset of cortical neurogenesis (Hasenpusch-Theil et al., 2018). In humans, mutations in the *cdk6* locus have been linked to autosomal recessive primary microcephaly (Naveed et al., 2018), suggesting important functions in the expansion of cortical precursor cells. However, brains of CDK6 knockout mice exhibit

no obvious phenotype despite smaller olfactory bulbs (Beukelaers et al., 2011), and even the concomitant deletion of CDK2 did not interfere with proliferation of cortical progenitors in mouse embryos (Grison et al., 2018).

In the *adult SGZ*, CDK6 displays essentially the same expression pattern as CDK4 (Beukelaers et al., 2011). Genetic inactivation of CDK6 reduced proliferation of SGZ precursors by >50%. Loss of CDK6 specifically prevents the expansion of neuronally committed precursors by lengthening $G_1$ phase duration and premature CC exit, resulting in decreased neurogenesis (Beukelaers et al., 2011). The group of Caron et al. (2018) then asked for the mechanism of CDK action in adult SGZ precursors. By generating mice bearing a kinase-dead allele of CDK6 they demonstrated that the function of CDK6 in hippocampal neurogenesis relies essentially on its kinase activity. They also observed that $p27^{cip1}$ is the main inhibitor of CDK6 activity in hippocampal progenitors.

Altogether, even if both early $G_1$ phase CDKs are widely expressed in the adult neurogenic niches, available data imply that they are not functionally redundant. Whereas CDK4 appears to be the prevailing CDK during corticogenesis, CDK6 exerts important functions in the adult DG. It needs to be determined whether the distinct outcomes of manipulating CDK4 and CDK6 are caused by intrinsically different functions of CDK4 and CDK6 (e.g., as transcription factor or through site-specific mono-phosphorylation of Rb), by selective interactions with regulatory D-cyclins, CAK and CKIs, or other mechanisms (Grossel and Hinds, 2006; Bryja et al., 2008; Bockstaele et al., 2009; Kollmann et al., 2013). In support of the first, it has been shown that CDK4 and CDK6 phosphorylate Rb with different residue selectivity (Takaki et al., 2005; Satyanarayana and Kaldis, 2009). In addition to its role in proliferation, findings suggest that CDK6 prevents terminal differentiation in a variety of cell types. This function, which involves phosphorylation of fate-determining transcription factors, is not shared with CDK4 (Grossel and Hinds, 2006). For instance, expression of CDK6, but not CDK4, in primary astrocytes resulted in expression of progenitor cell markers and dedifferentiation (Ericson et al., 2003). Moreover, CDKs can directly inhibit pro-neuronal transcription factors as demonstrated in frog eggs (Ali et al., 2011). There, rising CDK levels quantitatively phosphorylate Ngn2 on multiple sites which inhibits neuronal differentiation.

## CDK Inhibitors (CKIs)

**INK4 family members** are dynamically expressed during mouse development and adulthood (Zindy et al., 1997a,b; Canepa et al., 2007). $P15^{INK4b}$ and $p16^{INK4a}$ are first detected postnatally. Whereas $p16^{INK4a}$ becomes upregulated in all organs as mice age, $p15^{INK4b}$ is more restricted and lacking from brain (Zindy et al., 1997a,b). It is assumed that the ubiquitous increase in $p16^{INK4a}$ is important for preventing neoplastic transformation in later life (Serrano et al., 1996). On the contrary, $p18^{INK4c}$ and $p19^{INK4d}$ are steadily expressed during development and adulthood (Zindy et al., 1997a). Both are detectable also in embryonic and postnatal brains, albeit in disparate patterns (Zindy et al., 1997b).

In the developing cortex, **$p18^{INK4c}$** is restricted to proliferating neuroblasts around the time when they lengthen $G_1$ (Zindy et al., 1997b). Postnatally, $p18^{INK4c}$ is expressed by neuroblasts of the DG, but falls below detectable levels with reaching adulthood (Zindy et al., 1997b). Accordingly, loss of $p18^{INK4c}$ did neither impair proliferation nor neurogenesis in the adult SGZ (Caron et al., 2018).

**$P19^{INK4d}$**, on the other hand, is highly expressed in adult brain (Zindy et al., 1997a,b). However, $p19^{INK4d}$ is restricted to postmitotic neurons, independent of developmental age (Zindy et al., 1997a,b). Here, it actively represses CC re-entry together with the Cip/Kip family member $p27^{kip1}$ (Zindy et al., 1997b).

**$P16^{INK4a}$** is the only INK4 family member displaying an essential role in adult neurogenesis. Supporting the expression data from embryonic brain, deletion of $p16^{INK4a}$ had no obvious effect on brain development nor on proliferation and self-renewal capacity of NSCs from young adult mice, both, *in vivo* and *in vitro* (Bachoo et al., 2002; Molofsky et al., 2006). In the *aging SVZ*, however, studies show that accumulation of $p16^{INK4a}$ contributes to the progressive decline of NSC function (Molofsky et al., 2006; Nishino et al., 2008). Deletion of $p16^{INK4a}$ partially reverses this phenotype by increasing both, the self-renewal capacity of NSCs and precursor proliferation in the SVZ of aged mice (Molofsky et al., 2006). In support of these findings, overexpression of $p16^{INK4a}$ in NSCs from the embryonic SVZ strongly impairs their self-renewal capacity (Nagao et al., 2008). On the contrary, studies found no evidence for an involvement of $p16^{INK4a}$ in the aging-related decline in *adult hippocampal neurogenesis* (Molofsky et al., 2006). Rather, as demonstrated in a very recent report, $p16^{INK4a}$ appears to prevent aNSC's release from quiescence when neurogenic stimuli are present (Micheli et al., 2019). They showed that in the DG of middle-aged $p16^{INK4a}$ knockout mice, running highly increased aNSC numbers as well as IPCs through forcing their entry to CC, suggesting that $p16^{INK4a}$ plays a role in the maintenance of aNSCs after a neurogenic stimulus, to keep a reserve of their self-renewal capacity during aging (Micheli et al., 2019). Moreover, $p16^{INK4a}$ deletion counteracts the disruption of DG precursor proliferation after irradiation, demonstrating that $p16^{INK4a}$ expression is a mechanism mediating the radiation-induced loss of neurogenesis (Le et al., 2018). Another study showed that $p16^{INK4a}$ acts as a barrier to direct neuronal transdifferentiation and functions in the linage-restriction of astrocytes (Price et al., 2014). Altogether, in the light of INK4 family members being the main inhibitors of CDK4/6, their physiological significance in adult neurogenesis appears comparably low, with the exception of $p16^{INK4a}$ that emerged as important regulator of NSC self-renewal in the aging brain. However, whereas the aging-related rise in $p16^{INK4a}$ expression contributes to the progressive decline of NSC function and regenerative capacity in the SVZ, it helps to maintain the pool of quiescent NSCs in the DG through protecting them from excessive activation by neurogenic stimuli.

In accordance with their wider range of CDK inhibitory activity as compared to INK4 proteins, the **Cip/Kip family members** are vitally involved in a broad spectrum of cell fates, which is even extended through a range of CC-independent actions. The three Cip/Kip CKIs have distinct and overlapping functions, that either are surprisingly stable between

different niches (e.g., for p27) or vary depending on time and context (e.g., for p21).

Contrary to $p27^{kip1}$ and $p57^{kip2}$, **$p21^{cip1}$** expression in the developing cortex is weak, suggesting it plays no major role during development (Van Lookeren Campagne and Gill, 1998; Tury et al., 2011). $P21^{cip1}$ is expressed in the stem cell niches of the adult brain but, as seen for many other CC regulators, it has regionally distinct functions. In the *adult SVZ*, $p21^{cip1}$ is required to balance quiescence, proliferation and differentiation of NSCs (Kippin et al., 2005; Marques-Torrejon et al., 2013; Porlan et al., 2013). Loss of $p21^{cip1}$ results in CC shortening and cumulative hyperproliferation of aNSCs, leading to impaired long-term self-renewal and premature exhaustion of NSCs in aged mice (Kippin et al., 2005). The knockout had no effect on the proliferation of IPC, suggesting that the functions of $p21^{cip1}$ are highly specific for aNSCs. The mechanisms involved in $p21^{cip1}$-dependent regulation of NSCs were evaluated by two subsequent studies (Marques-Torrejon et al., 2013; Porlan et al., 2013). The first found that $p21^{cip1}$ acts as transcriptional repressor of *Sox2* in aNSCs, thereby preventing replicative stress and exhaustion of the NSC pool (Marques-Torrejon et al., 2013). Later, the same group reported that $p21^{cip1}$ maintains aNSC in an undifferentiated, multipotent state through transcriptional repression *Bmp2* (Porlan et al., 2013). Strikingly, $p21^{cip1}$-dependent transcriptional regulation of both genes emerged to be independent from its role as CDK inhibitor and CC regulator. Rather, $p21^{cip1}$ represses *Sox2* through direct interaction with the *Sox2* enhancer, whereas it modulates *Bmp2* expression through association with E2F (Porlan et al., 2013). These data demonstrate that $p21^{cip1}$ acts in distinct ways to link the relative quiescence of aNSCs to their longevity and potentiality (Porlan et al., 2013). $P21^{cip1}$ is also expressed at high levels in the *adult SGZ*. There, it is expressed by IPCs, neuroblasts and immature neurons, but absent from radial glia-like NSC and mature granule cells (Pechnick et al., 2008, 2011). Accordingly, several studies show that committed IPCs or neuroblasts divide more actively in the DG of $p21^{cip1}$ knockout mice, suggesting that $p21^{cip1}$ restrains their proliferation (Pechnick et al., 2008, 2011; Zonis et al., 2013; Li and Wong, 2018). Another study, despite not reproducing the effects of $p21^{cip1}$ deletion in native DG, identified $p21^{cip1}$ as intrinsic suppressor of precursor proliferation after ischemic brain injury (Qiu et al., 2004). Given its importance for restricting adult neurogenesis, it is not surprising that $p21^{cip1}$ is relevant also in neuropathological contexts. For instance, the studies of Pechnick et al. (2008, 2011) revealed that antidepressants exert their beneficial effects on hippocampal neurogenesis and behavior through down-regulation of $p21^{cip1}$. Another report suggests that an increase of $p21^{cip1}$ is responsible for the CC arrest of neuroblasts in response to acute systemic inflammation (Zonis et al., 2013). Together these studies again highlight the differences in the neurogenic niches of the adult brain, showing that $p21^{cip1}$ serves to maintain a population of undifferentiated NSCs in the SVZ, whereas it acts as brake for proliferation of later stages of neuron development in the SGZ.

Several studies have demonstrated the importance of the Cip/Kip family members **$p27^{kip1}$ and $p57^{kip2}$** as regulators of CC exit, differentiation and migration (Tury et al., 2012). Expression profiles and observations in mutant mice suggest that they regulate different sets of precursor cells in the *embryonic cortex* (Tury et al., 2012). Besides being co-expressed with $p57^{kip2}$ in the cortical plate, **$P27^{kip1}$** is exclusively found in IPCs of the SVZ (Van Lookeren Campagne and Gill, 1998; Mairet-Coello et al., 2012). In this way, genetic manipulation assays revealed that $p27^{kip1}$ promotes CC exit exclusively in IPCs (Goto et al., 2004; Tarui et al., 2005; Nguyen et al., 2006; Mairet-Coello et al., 2012; Clement et al., 2017). Moreover, they identified roles of $p27^{kip1}$ in precursor differentiation and migration of cortical neurons (Nguyen et al., 2006; Tury et al., 2011; Hasan et al., 2013; Clement et al., 2017). These effects are independent from its CC activity, as neuronal differentiation is driven through stabilization of Ngn2 and migration is controlled through inhibition of RhoA signaling (Vernon et al., 2003; Nguyen et al., 2006).

In the *adult SVZ*, $p27^{kip1}$ has very similar functions as during cortex development. As exemplified in $p27^{kip1}$-deficient mice, $p27^{kip1}$ specifically promotes CC exit of IPCs, while playing no role in NSCs (Doetsch et al., 2002; Gil-Perotin et al., 2011). Cells originating in the adult SVZ then migrate a long distance via the rostral migratory stream toward the olfactory bulb to replace resident granule neurons (Doetsch et al., 1997). $P27^{kip1}$ is ubiquitously expressed in these areas to prevent the proliferation of newborn neurons (Li et al., 2009). The size of the rostral migratory stream is increased and olfactory bulb development is delayed in $p27^{kip1}$ knockout mice, which may reflect a migration deficit (Li et al., 2009).

Two types of $p27^{kip1}$-positive cells exist in the *adult DG*, strongly positive cells in the SGZ and weakly positive postmitotic neurons in the GCL and hilus (Qiu et al., 2009; Beukelaers et al., 2011; Andreu et al., 2015). Detailed immunophenotyping revealed that $p27^{kip1}$ is expressed by virtually all type 1 NSCs, in the majority of IPCs, in neuroblasts and immature neurons (Qiu et al., 2009; Andreu et al., 2015). Deletion of $p27^{kip1}$ increases proliferation of radial aNSC *in vivo* and *in vitro*, and Ki67 can be detected only in those few aNSCs that are $p27^{kip1}$-negative, demonstrating a role of $p27^{kip1}$ in aNSC quiescence (Qiu et al., 2009; Andreu et al., 2015; Horster et al., 2017). Activation of $p27^{kip1}$ mutant NSCs has no effect on the total size of the aNSC pool, indicating that $p27^{kip1}$ has no role in the choice between symmetric and asymmetric aNSC divisions (Andreu et al., 2015). Mechanistically, $p27^{kip1}$ acts downstream of BMP4 to maintain aNSC quiescent (Andreu et al., 2015). Conversely, $p27^{kip1}$ is low in transit-amplifying type 2a cells that accordingly are resistant to deletion of $p27^{kip1}$ (Andreu et al., 2015). Later in the linage, $p27^{kip1}$ deletion delays the CC exit of neuroblasts, resulting in a net increase in newborn neurons, just as in the aSVZ (Qiu et al., 2009; Andreu et al., 2015). Summing up, these studies demonstrate that $p27^{kip1}$ acts as dual modulator of both aNSC quiescence and terminal CC exit of immature neurons. Recent studies also provided some insight into the role of $p27^{kip1}$ in differentiation. $P27^{kip1}$ increases upon neuronal differentiation *in vitro* and *in vivo* (Varodayan et al., 2009; Andreu et al., 2015; Horster et al., 2017). It becomes induced by proneural genes, such as NeuroD2 or Mash1, suggesting that $p27^{kip1}$ is employed by neural determination factors to force differentiation and CC exit (Farah et al., 2000). Others showed that selective

phosphorylation and stabilization of $p27^{Kip1}$ by CDK5 is crucial to neuronal differentiation of NSCs and promotes neurite outgrowth as neurons differentiate (Zheng et al., 2010). The role of $p27^{Kip1}$ in neuronal differentiation is further strengthened by observations in embryonic and pluripotent stem cells, in which $27^{Kip1}$ promotes neuronal differentiation through both stabilization of Ngn2 and repression of *Sox2* (Nguyen et al., 2006; Li et al., 2012).

**$P57^{kip2}$** is expressed in cycling precursors of the *embryonic* VZ and SVZ (Ye et al., 2009; Mairet-Coello et al., 2012). Studies in mutant mice revealed that $p57^{kip2}$ controls CC length and proliferation both in radial NSCs and IPCs (Tury et al., 2011; Mairet-Coello et al., 2012). Accordingly, it has been shown that $p57^{kip2}$ knockout mice display macrocephaly due to embryonically increased proliferation of radial glia NSCs (Mairet-Coello et al., 2012). In promoting neuronal differentiation $p57^{kip2}$ is even more effective than $p27^{kip1}$ (Tury et al., 2011). And similar to $p27^{kip1}$, $p57^{kip2}$ regulates migration in the developing cortex, albeit at later stages (Nguyen et al., 2006; Tury et al., 2011). A feature that clearly distinguishes the two CKIs is the suppressive action of $p57^{kip2}$ on astrogliogenic fate decisions (Tury et al., 2011). Noteworthy, all effects of $p57^{kip2}$ on neurogenesis mentioned so far require their interaction with cyclin/CDK complexes.

Information on the role of $p57^{kip2}$ in the *adult DG* is unfortunately sparse. Furutachi et al. (2013) show that conditional deletion of $p57^{kip2}$ in aNSCs activates their proliferation and increases neurogenesis both in young and aged mice. Prolonged deletion, however, led to depletion of aNSCs and reduced neurogenesis (Furutachi et al., 2013). They further observed that the running-induced increase in aNSC proliferation, which is accompanied by a decreased expression of $p57^{kip2}$ in wildtypes, is impaired in $p57^{kip2}$ mutants. Their results suggest that $p57^{kip2}$ maintains radial aNSCs in a quiescent state to preserve a pool of recruitable NSCs throughout life and that the reduction of $p57^{kip2}$ is required for activation of aNSCs by a neurogenic stimulus such as running. In addition, suppression of $p57^{kip2}$ directs NSCs isolated from the aSGZ toward the oligrodendrocyte linage while reducing astroglial characteristics, suggesting an involvement in linage commitment or maintenance of stem cell character (Jadasz et al., 2012).

## Rb Proteins and E2F

In agreement with research reporting Rb functions in diverse cellular pathways of various cell types, recent studies identified distinct requirements for **Rb/E2F** in the brain, including cell division, differentiation and migration of precursor cells. During brain development, telencephalon-specific or heterozygous Rb mutants displayed increased and ectopic division of neuroblasts, demonstrating a requirement of Rb for CC exit after commitment to a neuronal fate (Lee et al., 1994; Callaghan et al., 1999; Ferguson et al., 2002). Ectopic division may be supported through the concomitant increase in FGF-2 levels in the VZ and cortical plate of the mutant mice (McClellan et al., 2009). Others identified severe deficits in the proliferation, maturation and subsequent tangential migration of specific interneuron subtypes in these mice, suggesting essential involvement of Rb in differentiation and migration of cortical precursor cells (Ferguson et al., 2005; Ghanem et al., 2012). Rb overexpression in cultured NSCs from newborn rats increased their differentiation – according to the specification factor added – into neurons, astroglia or oligodendrocytes (Jori et al., 2007). This suggests a requirement of Rb in determination of NSC fate in response to extracellular linage specification signals. A recent study examined DG development and *adult hippocampal neurogenesis* in conditional Rb knockout mice, essentially describing similar defects as observed in the embryonic cortex (Vandenbosch et al., 2016). Both, in the developing and adult DG they observed delayed CC exit and increased ectopic proliferation of neuronally committed progenitors and neuroblasts. However, the increased neuron birth was counteracted by an increase in cell death, which resulted in a long-term reduction of neurogenesis in the adult DG. Consistent with these findings, Rb-deletion in the aSVZ and rostral migratory stream was associated with increased progenitor proliferation and neurogenesis but impaired long-term survival (Naser et al., 2016). Together, these studies suggest conserved requirements for Rb in embryonic and adult neurogenic niches, i.e., exit from CC, restriction of proliferation and survival of newborn neurons (Vandenbosch et al., 2016). Consistent with prior findings in hematopoietic stem cells, none of the studies noticed consequences of the Rb knockout in the NSC population, suggesting that other Rb proteins are responsible for the control of self-renewal and quiescence in NSCs (Vanderluit et al., 2004; Walkley and Orkin, 2006; Naser et al., 2016; Vandenbosch et al., 2016).

Current knowledge on the role of **p107** in neurogenesis relies only on studies conducted in the embryonic VZ and adult SVZ, in which it is highly expressed (Vanderluit et al., 2004, 2007). Contrary to Rb, p107 is expressed in uncommitted precursors and becomes downregulated as they commence to differentiate (Callaghan et al., 1999; Vanderluit et al., 2004). P107 knockout mice display increased proliferation of progenitors and slowly dividing NSCs *in vivo* that exhibit an increased self-renewal potential in *in vitro* (Vanderluit et al., 2004, 2007). Concomitantly, effectors of the Notch pathway including Notch1, Dll1, and Hes1 become upregulated in the mutated cells, suggesting that p107 controls the NPC population through suppression of Notch activity (Vanderluit et al., 2004, 2007). Indeed, p107 was shown to directly interact with regulatory sequences of Notch1 and Hes1, and accordingly the phenotype of p107 mutants could be rescued through deletion of Hes1 (Vanderluit et al., 2007). Nevertheless, p107 knockout mice ultimately display reduced neurogenesis due to decreased differentiation and increased apoptosis of uncommitted progenitor cells (Vanderluit et al., 2007). As reported by others, the p107/E2F pathway controls NPC division also through restricting the autocrine production of growth factors such as FGF-2 (McClellan et al., 2009). Together, these studies identified distinct mechanisms by which p107 regulates the expansion and neuronal commitment of stem and progenitor cells in the embryonic and adult brain.

Also, little is known about the role of **p130**, which is predominantly expressed in quiescent and terminally differentiated cells, during adult neurogenesis (Mulligan and Jacks, 1998). View lines of evidence suggest an involvement in NSC differentiation. For instance, p130 forms complexes with E2F4 and SIN3A to repress *Sox2* transcription during iPSC differentiation (Li et al., 2012). Additionally, p130 overexpression in differentiating NSCs isolated from newborn rats causes a shift toward the cell type induced by differentiation cues, similar to the effects of Rb overexpression. No such effect was observed under proliferating conditions, suggesting that p130 and Rb reinforce the cellular responses of NSCs to extracellular fate specification signals (Jori et al., 2007).

Together, these studies exemplified diverse functions of distinct Rb proteins in the regulation of neural stem and progenitor cells, not only by controlling genes required for CC progression but also by regulating genes relevant for fate control and linage specification.

## CONTROL OF FATE DECISIONS IN $G_1$

The commitment of cells to divide requires the presence of external mitogenic signals. It is during early $G_1$ that cells are highly susceptible to such extrinsic cues and make critical decisions about their fate, including the commitment to proceed, pause or exit the CC (Matsushime et al., 1994; Sherr, 1994a; Pauklin and Vallier, 2013). In the presence of mitogens, D-cyclins become upregulated and initiate the cascade driving the cell through the restriction point to commence a new round of division. If mitogens are withdrawn or anti-mitogenic cues are present (e.g., BMPs), D-cyclins are lost and Cip/Kip proteins accumulate, forcing the cell into a reversible quiescent state, a hallmark of aNSCs ($G_0$; Blomen and Boonstra, 2007; Andreu et al., 2015). Recent evidence suggests that aNSC quiescence is not merely an inactive state but rather is actively maintained (Wang et al., 2011; Morizur et al., 2018), at least partly by Cip/Kip proteins, whose deletion inevitably leads to excessive proliferation and premature depletion of aNSCs (Kippin et al., 2005; Cheung and Rando, 2013; Andreu et al., 2015). Exposure to differentiation cues is also accompanied by an increase in Cip/Kip CKIs, but this time forcing differentiation and terminal exit from CC (Lukaszewicz et al., 2002; Blomen and Boonstra, 2007; Horster et al., 2017). Of note, differentiation and proliferation are not mutually exclusive events, as is the case in neuronally committed progenitor cells. Cells rather continue to divide at slower pace and cease cycling toward the end of the differentiation process (Lukaszewicz et al., 2002; Coffman, 2003). Such a lengthening of the CC during neuronal differentiation has been initially described for the embryonic cortex (Takahashi et al., 1995). Subsequent studies applying sophisticated genetic tools to selectively manipulate important $G_1$ regulators suggest that the length of $G_1$ can directly influence proliferation/differentiation decisions in NPCs (**Figure 3**). For example, overexpression of cyclin D1-CDK4 complexes, or cyclin D1 and cyclin E alone were found to shorten $G_1$, which was accompanied by a deceleration of neurogenesis and enhanced expansion of IPCs, both in embryonic and adult neurogenic niches (Lange et al., 2009; Pilaz et al., 2009; Artegiani et al., 2011; Bragado Alonso et al., 2019). Conversely, silencing of cyclin D-CDK4, deletion

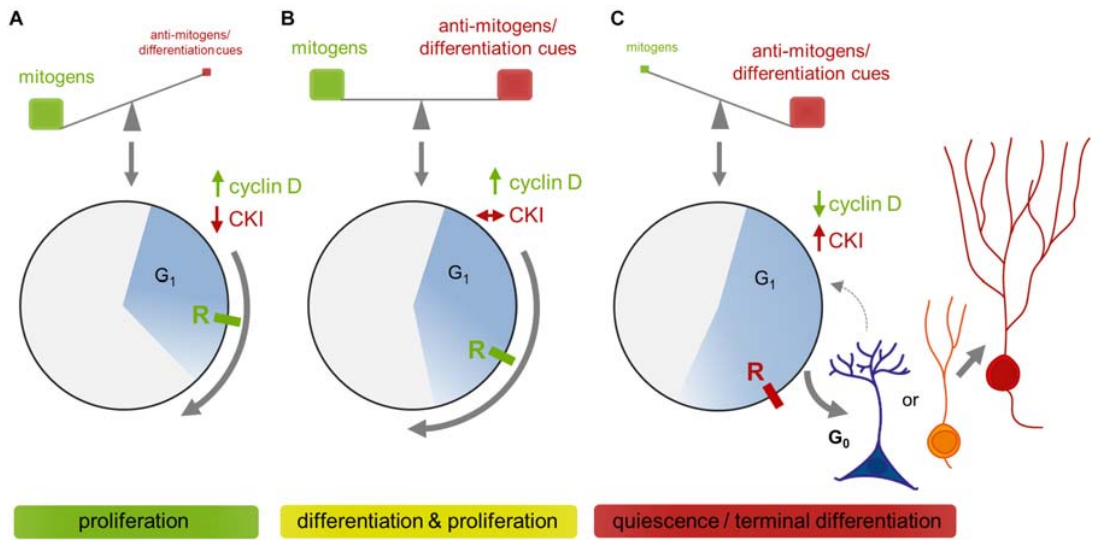


**FIGURE 3 |** Simplified model on the coordination of $G_1$ dynamics by extrinsic signals and their integration into fate decisions based on evidence from cyclin and CDK manipulation studies. **(A)** Fast proliferation is achieved in presence of high mitogen levels that stimulate the expression of D-cyclins, thus initiating the canonical CC cascade driving progression through $G_1$. If cells pass the R-point (point of no return), mitogen stimulation is no longer required for commencing cell division. D-cyclin levels stay high until mitogens are withdrawn. **(B)** If specification signals come on board, CKIs increase on top and restrain but don't block the activities of D-cyclins, resulting in a lengthening of $G_1$. This, as proposed by the "cell cycle length theory," provides sufficient time for specification signals to initiate the differentiation program before cells reach the R-point. **(C)** If mitogens decrease, D-cyclins become rapidly degraded and cells can not overcome the R-point. Adult NSCs then return into a reversible quiescent state ($G_0$), whereas cells that initiated their differentiation program terminally exit the cell cycle to become an immature neuron (IN).

of CDK6 or pharmacological inhibition of CDK2 or CDK4 lengthen $G_1$ and lead to premature neuronal differentiation (Calegari and Huttner, 2003; Lange et al., 2009; Beukelaers et al., 2011; Lim and Kaldis, 2012; Roccio et al., 2013). These observations have led to the hypothesis that CC lengthening may be a cause of differentiation, proposing that a longer $G_1$ will increase the propensity of fate-determining signals to accumulate and drive differentiation (Calegari and Huttner, 2003; Salomoni and Calegari, 2010). However, for the adult DG there is also evidence not completely in line with this hypothesis, coming from two studies which examine the correlation between voluntary exercise-induced proliferation and CC length (Farioli-Vecchioli et al., 2014; Fischer et al., 2014). Whereas the study of Fischer et al. (2014) despite finding a tendency toward $G_1$ shortening, reports no clear correlation between CC length and increased proliferation after a short period (5 days) of running, Farioli-Vecchioli et al. (2014) showed that the proliferative response after prolonged (12 days) running is accompanied by shortening of the CC, which was attributable to shortening of the S phase in neuronally committed progenitors. This is in line with others reporting that NPCs shorten S phase upon neuronal commitment both during development and in the adult DG (Arai et al., 2011; Brandt et al., 2012). Interestingly, Brandt and colleagues further observed that radial aNSCs, once activated, divide faster, also through shortening of their S phase, which is in agreement with the "disposable stem cell" model as put forward by Encinas et al. (2011), but in contrast to the "long-term repeated aNSC self-renewal" model proposed by Bonaguidi et al. (2011) and to the finding that expanding NPCs spend more time in S phase for DNA quality control (Arai et al., 2011). Taken together, the overall picture is more complex than previously assumed, and it still remains to be proven whether and how changes in CC dynamics translate into fate decisions. A possible explanation for the apparent discrepancies described before is that the $G_1$ proteins manipulated to induce changes in CC length may in addition to their CC regulatory function directly influence cell fates. Indeed, several studies have provided evidence for direct roles of $G_1$ proteins in differentiation, independent of their CC regulatory activity, through interaction with signaling pathways and transcription factors controlling fate decisions (Pauklin and Vallier, 2013; Pauklin et al., 2016). For cyclin D1, chromatin-wide location analyses identified hundreds of target genes, including *Notch1* and *Wnt3*, suggesting direct roles in the maintenance and differentiation of stem cells (Bienvenu et al., 2010; Pauklin et al., 2016). $P27^{Kip1}$ has been shown to promote differentiation through transcriptional repression of *Sox2* and stabilization of the proneural protein Ngn2 (Vernon et al., 2003; Nguyen et al., 2006; Li et al., 2012). Ngn2 is moreover targeted by CDKs through direct phosphorylation at multiple sites (Ali et al., 2011). This results in quantitative inhibition of Ngn2's ability to induce transcription of differentiation genes, thus providing a mechanism how CDK activity and perhaps also CC length can be sensed to coordinate neuronal differentiation.

## CONCLUDING REMARKS

Recently there has been a remarkable progress in our understanding about the control of adult hippocampal neurogenesis by the CC. However, despite accumulating evidence for multiple and diverse interactions between CC components and fate decisions of NPCs, we are far from drawing a comprehensive picture. Still, several CC proteins await investigation in adult NPCs or are subject to ongoing work. On the other hand, interpretation of existing data is frequently complicated because many $G_1$ regulators, including D-cyclins and CDKs, (i) emerge as pleiotropic molecules regulating multiple cellular functions, often independent from their CC regulatory role and localized to different protein domains, and (ii) display functional redundancy and counter-regulate if the expression of related proteins is experimentally manipulated. Additional detailed analyses of expression patterns in combination with spatiotemporally controlled manipulation of genes, as well as of individual functional domains of CC regulators, may help to answer outstanding questions. We believe that a detailed understanding of the regulatory mechanisms underlying adult neurogenesis may open new avenues for developing therapies of neurodegenerative diseases. The first attempts to exploit this potential have already been made and have produced promising results.

## AUTHOR CONTRIBUTIONS

AU conceptualized and wrote the manuscript and prepared the figures. OW conceptualized and edited the manuscript.

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# The Social Component of Environmental Enrichment Is a Pro-neurogenic Stimulus in Adult c57BL6 Female Mice

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In rodents, the hippocampal dentate gyrus gives rise to newly generated dentate granule cells (DGCs) throughout life. This process, named adult hippocampal neurogenesis (AHN), converges in the functional integration of mature DGCs into the trisynaptic hippocampal circuit. Environmental enrichment (EE) is one of the most potent positive regulators of AHN. This paradigm includes the combination of three major stimulatory components, namely increased physical activity, constant cognitive stimulation, and higher social interaction. In this regard, the pro-neurogenic effects of physical activity and cognitive stimulation have been widely addressed in adult rodents. However, the pro-neurogenic potential of the social aspect of EE has been less explored to date. Here we tackled this question by specifically focusing on the effects of a prolonged period of social enrichment (SE) in adult female C57BL6 mice. To this end, 7-week-old mice were housed in groups of 12 per cage for 8 weeks. These mice were compared with others housed under control housing (2–3 mice per cage) or EE (12 mice per cage plus running wheels and toys) conditions during the same period. We analyzed the number and morphology of Doublecortin-expressing ($DCX^+$) cells. Moreover, using RGB retroviruses that allowed the labeling of three populations of newborn DGCs of different ages in the same mouse, we performed morphometric, immunohistochemical, and behavioral determinations. Both SE and EE increased the number and maturation of $DCX^+$ cells, and caused an increase in dendritic maturation in certain populations of newborn DGCs. Moreover, both manipulations increased exploratory behavior in the Social Interaction test. Unexpectedly, our data revealed the potent neurogenesis-stimulating potential of SE in the absence of any further cognitive stimulation or increase in physical activity. Given that an increase in physical activity is strongly discouraged under certain circumstances, our findings may be relevant in the context of enhancing AHN via physical activity-independent mechanisms.

**Keywords: adult hippocampal neurogenesis, environmental enrichment, social enrichment, retrovirus, behavior**

## INTRODUCTION

Adult neurogenesis occurs in a limited number of brain regions in most mammalian species (Altman and Das, 1965; Kempermann et al., 2018; Moreno-Jiménez et al., 2019). Among these regions, the hippocampal dentate gyrus gives rise to newly generated dentate granule cells (DGCs) throughout life. This process, named adult hippocampal neurogenesis (AHN), converges in the functional integration of mature DGCs into the trisynaptic hippocampal circuit (Zhao et al., 2006). AHN is believed to participate in certain types of hippocampal-dependent learning, as well as in mood regulation (Sahay et al., 2011; Hill et al., 2015). In fact, this process is impaired in animal models of neurodegenerative and psychiatric diseases (Lazarov and Marr, 2010; Lazarov et al., 2010).

Adult hippocampal neurogenesis encompasses a series of sequential, tightly regulated stages (Zhao et al., 2006). Both intrinsic and extrinsic factors regulate the integration of newly generated neurons into the hippocampal circuitry. Among these factors, environmental enrichment (EE) and physical exercise are two of the most potent paradigms that stimulate AHN (Kempermann et al., 1997; van Praag et al., 1999). In fact, they exert neuroprotective actions both on healthy and diseased animals (van Praag et al., 2000, 2002; Teixeira et al., 2018). In contrast, social isolation causes stress for social rodents and markedly decreases the rate of AHN (Stranahan et al., 2006; Ibi et al., 2008).

Environmental enrichment generally consists of a combination of three major components, namely physical activity, cognitive stimulation, and social interaction. An increased level of physical activity is usually achieved by including voluntary running wheels in the enrichment cages. Cognitive stimulation is ensured by periodically changing the non-social components of the cage, such as toys, tubes or bedding material. Finally, the social component of EE, namely increased social interaction, occurs naturally as a consequence of the higher number of mice housed together in enriched cages. In this regard, the individual contribution of the cognitive and physical activity components of EE to the pro-neurogenic effects of this protocol during adulthood has been extensively addressed (van Praag et al., 1999; Steiner et al., 2008; Fabel et al., 2009). Previous studies demonstrate that physical activity and EE increase AHN via independent mechanisms (Olson et al., 2006; Garthe et al., 2016). However, the selective contribution of the social component of EE during adulthood has received less attention to date. In this regard, numerous studies have examined the long-term cellular and behavioral consequences of brief periods of EE applied early after weaning in comparison to social isolation (Branchi and Alleva, 2006; Branchi et al., 2006a,b, 2013; Curley et al., 2009; Gracceva et al., 2009; Cirulli et al., 2010; D'Andrea et al., 2010). However, to the best of our knowledge, the pro-neurogenic effects of a prolonged period of social enrichment (SE) during adulthood in comparison to EE and control housing conditions have not been examined to date. Here we addressed this question by determining the specific contribution of SE to the pro-neurogenic and behavioral effects of EE during adulthood in female C57BL6/J mice.

## MATERIALS AND METHODS

### Animals

Five-week-old female C57BL6/J Ola Hsd mice were purchased from EnVigo laboratories (Spain). Animals were housed at the *Centro de Biología Molecular "Severo Ochoa"* (CBMSO) in a specific pathogen-free colony facility in accordance with European Community Guidelines (directive 86/609/EEC) and handled following European and local animal care protocols. Given that the hierarchy/dominance relationships established between male mice have a negative impact on AHN (Kozorovitskiy and Gould, 2004; McQuaid et al., 2018), only female mice were used in this work in all the housing conditions tested. Animals were left undisturbed for 2 weeks before starting any experimental manipulation. During this period, they were housed in groups of four mice per cage. Experiments were approved by the CBMSO Ethics Committee (AEEC-CBMSO-23/172) and the National Ethics Committee (PROEX 205/15). In stereotaxic injection experiments, five mice were used for each experimental condition. In cell count and behavioral determination experiments, seven animals per experimental condition were used.

### Experimental Design

To label three cell subpopulations of newborn DGCs of different ages in the same mouse, we stereotaxically injected each one of the three so-called *RGB retroviruses* (Gomez-Nicola et al., 2014) at a different time point. The time schedule of stereotaxic injections is shown in **Figure 1A**. 1 week after the last injection, animals were assigned to one of three experimental conditions, namely Control Housing (CH), EE, or SE. Mice were housed under these conditions for the following 8 weeks. It should be noted that stereotaxically injected mice were housed with naïve age-matched counterparts under each experimental condition. Consequently, each experimental group comprised five stereotaxically injected mice +7 naïve mice. After completion of this 8-week period, naïve mice were subjected to the Open Field and Social Interaction behavioral tests. Finally, the animals were sacrificed and immunohistochemical determinations were performed. Animals in EE and SE conditions were housed in groups of 12 animals per cage, whereas four mice were housed together in CH conditions.

### Preparation of Viral Stocks

We used three retroviral stocks encoding for either mCherry (Red, R), Venus (Green, G), or Cerulean (Blue, B) fluorescent proteins on a RSF91 backbone (Schambach et al., 2006; Gomez-Nicola et al., 2014). The plasmids used to produce these viruses were kindly provided by Profs. Tsien (Howard Hughes Medical Institute Laboratory at the University of California, San Diego, CA, United States), Baum and Schambach (Hannover Medical School, Germany), Miyawaki (RIKEN Brain Science Institute, Saitama, Japan), Riecken (University Medical Center Hamburg-Eppendorf, Germany), and Gage (Salk Institute, CA, United States). Retroviral stocks were concentrated to working titers of $1 \times 10^7$–$2 \times 10^8$ pfu/ml by ultracentrifugation

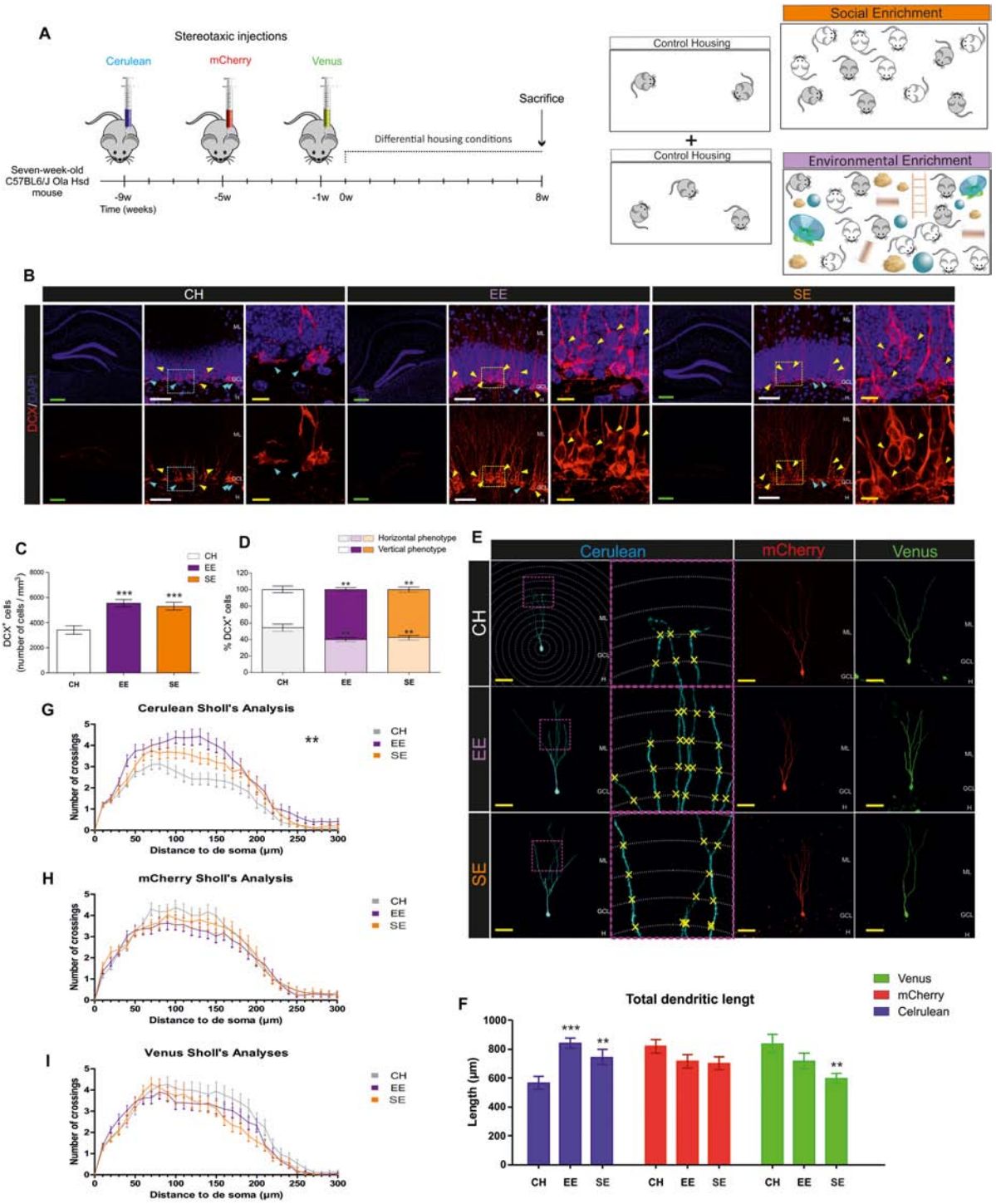


**FIGURE 1 |** Effects of Environmental enrichment (EE) and Social enrichment (SE) on the number and morphological maturation of newborn dentate granule cells (DGCs). **(A)** Experimental design. **(B)** Representative images of Doublecortin-expressing ($DCX^+$) cells in control housing (CH), EE, and SE animals. **(C)** Number of $DCX^+$ cells. **(D)** Percentage of "Horizontal type" and "Vertical type" $DCX^+$ cells in the different experimental conditions. **(E)** Representative images of newborn dentate granule cells (DGCs) transduced with either Cerulean-, mCherry-, or Venus-encoding retroviruses in the different experimental conditions. **(F)** Total dendritic length of newborn DGCs transduced with either Cerulean-, mCherry-, or Venus-encoding retroviruses in the different experimental conditions. **(G)** Sholl's analysis of dendritic branching in Cerulean-transduced newborn DGCs. **(H)** Sholl's analysis of dendritic branching in mCherry-transduced newborn DGCs. **(I)** Sholl's analysis of dendritic branching in Venus-transduced newborn DGCs. ML, molecular layer; GCL, granule cell layer; H, hilus. Green scale bar: 250 µm. White scale bar: 50 µm. Yellow scale bar: 10 µm. Blue triangles: "Horizontal type" $DCX^+$ cells. Yellow triangles: "Vertical type" $DCX^+$ cells. $^{**}0.01 > p \geq 0.001$; $^{***}p < 0.001$. Asterisks indicate changes with respect to CH animals.

(Zhao et al., 2006). Since the retroviruses used are engineered to be replication-incompetent, only cells dividing at the time of surgery are infected (Zhao et al., 2006). In the dentate gyrus (DG), these proliferative cells are almost totally restricted to newborn DGCs (Zhao et al., 2006).

## Stereotaxic Surgery

Seven-week-old mice were anesthetized with isoflourane and placed in a stereotaxic frame. Viruses were injected into the DG at the following coordinates (mm) relative to bregma [−2.0, ± 1.4, 2.2] in the anteroposterior, mediolateral, and dorsoventral axes. 2 μl of each retrovirus was injected with a glass micropipette at a rate of 0.2 μl/min. Micropipettes were kept in place at the site of injection for an additional 5 min to avoid any suction effect of the solution injected, before being slowly removed.

## Differential Housing Conditions

Housing under differential conditions started 1 week after the last stereotaxic injection was performed and lasted 8 weeks.

### Control Housing (CH)

Animals under CH conditions were housed in groups of 2–3 in standard polycarbonate cages.

### Environmental Enrichment (EE)

We used a previously described EE protocol (Llorens-Martin et al., 2010). All enriched cages were equipped with various types of running wheel. Animals under EE conditions were housed in groups of 12 (five stereotaxically injected +7 naïve animals) in large transparent polycarbonate cages (55 cm × 33 cm × 20 cm, Plexx Ref. 13005). They had free access to voluntary running wheels and toys of different shapes, sizes, materials, and surface textures. A set of 10 different toys and new bedding were placed in the cages every other day in order to alter the environment (Llorens-Martin et al., 2010).

### Social Enrichment (SE)

Animals under SE conditions were housed in groups of 12 (five stereotaxically injected +7 naïve animals) in large transparent polycarbonate cages (55 cm × 33 cm × 20 cm, Plexx Ref. 13005).

## Sacrifice

Mice were fully anesthetized by an intraperitoneal injection of pentobarbital (EutaLender, 60 mg/kg) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 N phosphate buffer (PB). Brains were immediately removed and post-fixed at 4°C overnight in the same fixative. They were then washed three times in 0.1 N PB.

## Immunohistochemistry (IHC)

We obtained 50-μm thick coronal brain sections on a Leica VT1200S vibratome (Vivar et al., 2012). Series of brain slices were randomly made up of one section from every eighth for immunohistochemical analyses. Slices were initially pre-incubated in phosphate buffer with 1% Triton X-100 and 1% bovine serum albumin for 10 min. Dual or triple immunohistochemistry was then performed as described previously (Llorens-Martin et al., 2013), using the following primary antibodies: goat anti-Doublecortin (DCX) (Santa Cruz Biotechnology Cat# sc-8066, **RRID:AB_2088494**; 1:500); guinea pig anti-PSD95 (Synaptic Systems Cat# 124 014, **RRID:AB_2619800**; 1:1000); and rabbit anti-Piccolo (Synaptic Systems Cat# 224 003, **RRID:AB_2263066**; 1:500). The binding of these primary antibodies was detected by incubation with the following secondary antibodies: Alexa-488 donkey anti-rabbit (Molecular Probes Cat# A-21206, **RRID:AB_141708**; 1:1000); Alexa-488 donkey anti-mouse (Molecular Probes Cat# A-21202; 1:1000); Alexa-555 donkey anti-rabbit (Molecular Probes Cat# A-31572, **RRID:AB_162543**; 1:1000); Alexa-555 goat anti-ginea pig (Molecular Probes Cat# A-21435; 1:1000); Alexa-555 donkey anti-goat (Molecular Probes Cat# A-21432, **RRID:AB_141788**; 1:1000); Alexa-555 donkey anti-rat (Molecular Probes Cat# A-21434, **RRID:AB_141733**; 1:1000); Alexa-647 donkey anti-goat (Molecular Probes Cat# A-21447; 1:1000); and Alexa-647 donkey anti-rabbit (Molecular Probes Cat# A-31573; 1:1000). To label cell nuclei, all the sections were counterstained for 10 min with DAPI (Merck, 1:5000).

## Cell Counts

The number of $DCX^+$ cells was determined using the physical dissector method coupled to confocal microscopy, as previously described (Llorens-Martin et al., 2014, 2016). Briefly, *z*-stacks of images were obtained under a LSM710 Zeiss confocal microscope (25× Oil immersion objective). Various parameters were used to accurately determine the number of $DCX^+$ cells (XY dimensions: 103.81 μm; *Z*-axis interval: 1.7 μm; *Z*-axis thickness: 20 μm). Five stacks of 20 images were analyzed per cell marker and animal. The number of cells counted was divided by the reference volume in order to calculate cell densities, as previously described (Llorens-Martin et al., 2013).

Moreover, to determine the effects of EE on the morphological maturation of $DCX^+$ cells, these cells were classified following previously published criteria (Plumpe et al., 2006). In this regard, "Horizontal type" referred to $DCX^+$ cells with an immature morphology, which included those with no neurites or those with several undifferentiated neurites oriented parallel to the hilar border of the granule cell layer. In contrast, "Vertical type" referred to more differentiated $DCX^+$ cells that exhibited a single primary apical neurite oriented perpendicular to the hilar border toward the molecular layer. We calculated the percentage of cells of each type and used averaged values per animal in the graphs (**Figure 1D**).

## Morphometric Analysis of Retrovirally Labeled Newborn Dentate Granule Cells

RGB retroviruses have traditionally been injected simultaneously as a cocktail (Gomez-Nicola et al., 2014). However, in order to label three cell populations of different ages in the same animal, each retrovirus was injected at a different time point. This experimental design allowed the labeling of newborn DGCs in three colors, namely red ($mCherry^+$), green ($Venus^+$), and blue ($Cerulean^+$) (Schambach et al., 2006; Gomez-Nicola et al., 2014). At least 50 randomly selected newborn DGCs per mouse

were reconstructed in a LSM710 Zeiss confocal microscope (25× oil immersion objective, XY dimensions: 103.81 μm). Confocal stacks of images were obtained (*Z*-axis interval: 2 μm), and *Z*-projections were analyzed. Total dendritic length was determined using the *NeuronJ* plugin in *Fiji*. Dendritic branching was analyzed using the *ShollAnalysis* plugin for *Fiji* (Llorens-Martin et al., 2013; Pallas-Bazarra et al., 2017).

## Measurement of PSD95$^+$ and Piccolo$^+$ Area

Five confocal images corresponding to each of the three sub-regions of the molecular layer (ML) [namely external (EML), medial (MML) and inner (IML) layer, or to the granule cell layer (GCL)] were obtained per animal in a LSM710 Zeiss confocal microscope (63× oil immersion objective; XY dimensions: 24.1 μm). Next, an invariant threshold for fluorescence intensity was established in *Fiji* software, and the area over the threshold was measured following previously described procedures (Pallas-Bazarra et al., 2016).

## Western Blotting

Extracts for western blot analysis were prepared by homogenizing the hippocampus in ice-cold extraction buffer consisting of 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM EDTA, a protease inhibitor cocktail (Roche), and 1 μM okadaic acid. Samples were homogenized and protein content was determined by the Pierce BCA Protein Assay (Thermo Fisher #23225) method. Twenty-five micrograms of total protein were electrophoresed on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, United States). Data were normalized with respect to CH mice. A mouse anti-PSD95 (UC Davis/NIH NeuroMab Facility Cat# 75-028, **RRID: AB_2292909**; 1:1000) primary antibody was used. Membranes were incubated with the antibody at 4°C overnight in 5% nonfat dried milk. Secondary anti-Mouse antibodies (1:1000; Invitrogen, San Diego, CA, United States) were incubated for 2 h at room temperature, and ECL detection reagents (Amersham Biosciences, Arlington Heights, IL, United States) were used for immunodetection. Quantification was performed by densitometric scanning. The densitometry values were obtained in the linear range of detection with these antibodies. Values were normalized with respect to anti-β-Actin (Sigma Cat#A5441, **RRID: AB_476744**; 1:5000) to correct for total protein content.

## Behavioral Tests

### Open Field

To analyze general ambulatory and anxiety-like behaviors, animals were exposed to a square (45 cm × 45 cm), constantly illuminated, open-field methacrylate arena for 10 min (See **Figures 2A,B** for schematic diagrams). The behavior of the animals was recorded with a zenithal video camera connected to AnyMaze (Stoelting Co.) software. The distance and speed traveled, the percentage of time the animals spent immobile, and the percentage spent in the center of the arena were calculated using the software (Llorens-Martin et al., 2014).

### Social Interaction Test

***Habituation phase***

24 h after being tested in the Open field, mice were placed in the same cage for 10 min. Cages were equipped with two empty identical compartments made of thin iron bars. The percentage of time the animals spent exploring these compartments was calculated.

***Testing phase***

24 h later, animals were exposed to the same environment explored during the training phase. In this case, one of the compartments held a non-familiar female mouse. The percentage of time the animals spent interacting with this mouse (either relative to the total exploratory or the total testing time) was calculated as a measurement of social interaction (McQuaid et al., 2018).

## Statistical Analysis

Statistical analysis was performed using SPSS 25 software (SPSS, 1989; Apache Software Foundation, Chicago, IL, United States). To test the normality of sample distribution, the Kolmogorov–Smirnov test was used. For comparisons between the three experimental groups, a One-way ANOVA test was used in case of normal sample distribution, whereas the nonparametric Kruskal-Wallis test was used in those cases in which normality could not be assumed. The DMS *post hoc* test was used to compare individual differences between groups when the aforementioned tests produced statistically significant differences. Dendritic arborization (Sholl's analysis) was analyzed using a repeated measurements ANOVA test. Simple main effects of genotype at each point of the dendritic tree were tested to determine the differences between the groups. Graphs represent mean values ±SEM. A 95% confidence interval was used for statistical comparisons.

# RESULTS

## Effects of Social Enrichment and Environmental Enrichment on the Number and Morphological Maturation of Newborn Dentate Granule Cells

Mice were housed under CH, SE, or EE conditions for 8 weeks. To label cellular populations of three distinct ages, animals received single stereotaxic injections of a retrovirus that encodes one of the following fluorescent proteins: Cerulean, mCherry, or Venus. Stereotaxic injections were performed at different time points following the experimental design shown in **Figure 1A**. In addition, seven additional mice per housing condition did not receive any stereotaxic injection but were housed under the same conditions. These animals were used to perform cellular counts and behavioral analyses.

First, we counted the number of Doublecortin-positive (DCX$^+$) cells in the different experimental conditions. Both EE ($p \leq 0.001$) and SE ($p = 0.008$) produced a similar increase in the number of these cells with respect to CH conditions

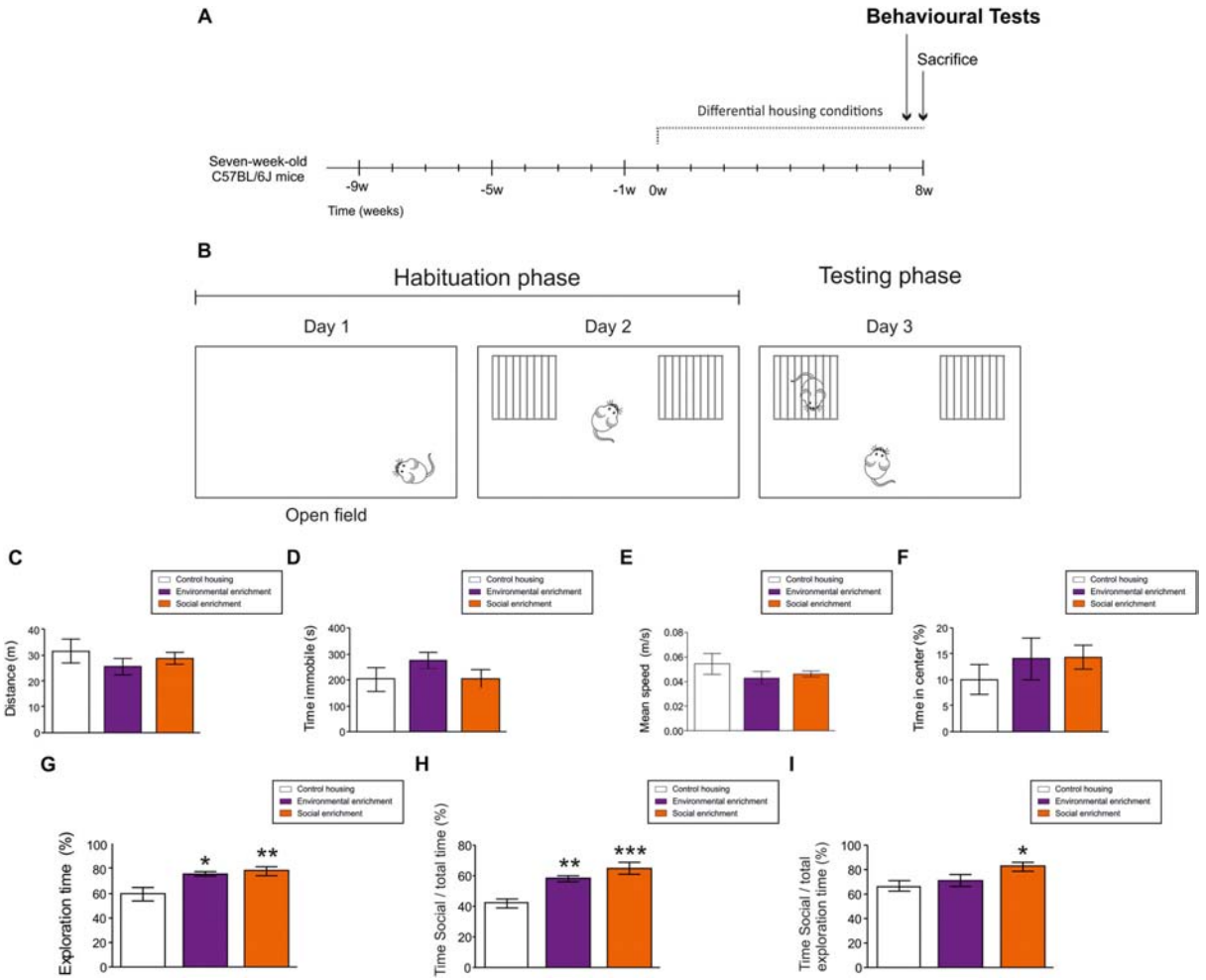


**FIGURE 2 |** Behavioral effects of Environmental enrichment (EE) and Social enrichment (SE). **(A)** Experimental design. **(B)** Schematic diagram of behavioral test performed. **(C)** Total distance moved in Open field test. **(D)** Total time immobile in Open field test. **(E)** Mean speed in Open field test. **(F)** Percentage of time spent in the central part of the arena in Open field test. **(G)** Total exploration time during day 2. **(H)** Percentage of time exploring the cage holding a mouse divided by the total testing time during day 3. **(I)** Percentage of time exploring the cage holding a mouse divided by the total exploratory time during day 3. *$0.05 > p \geq 0.01$; **$0.01 > p \geq 0.001$; ***$p < 0.001$. Asterisks indicate changes with respect to control housing animals.

($F_{2,4} = 10.419$; $p \leq 0.001$). No differences were observed in this parameter between SE and EE mice ($p = 0.993$). In **Figure 1B**, representative images of DCX$^+$ cells are shown. High-power magnification images show two populations of DCX$^+$ cells with remarkable morphological differences. As can be observed, some cells exhibited an immature phenotype (characterized by the presence of several neurites oriented parallel to the SGZ), whereas others had a more mature phenotype (characterized by the presence of a single primary apical dendrite perpendicular to the SGZ and oriented toward the ML) (**Figure 1C**). EE and SE conditions increased the morphological maturation of these cells, since both ($p = 0.002$ and $p = 0.009$, respectively) increased the percentage of the more differentiated DCX$^+$ cells, and reduced that of the immature type cells ($F_{2,4} = 5.755$; $p = 0.004$) (**Figure 1D**). No differences in the percentage of immature ($p = 0.637$) or mature ($p = 0.637$) DCX$^+$ cells were observed between SE and EE mice.

Next, we analyzed the effects of housing conditions on the morphology of previously generated newborn DGCs, which were 9- (Cerulean$^+$), 5- (mCherry$^+$), or 1- (Venus$^+$) week-old at the beginning of the differential housing conditions (**Figure 1E**). EE ($p \leq 0.001$) and SE ($p \leq 0.001$) increased the total dendritic length of 17-week-old Cerulean$^+$ newborn DGCs with respect to CH conditions ($F_{2,4} = 10.307$; $p \leq 0.001$) (**Figure 1F**). Accordingly, EE ($p \leq 0.001$) and SE ($p = 0.033$) increased the dendritic branching of these cells (Greenhouse-Geisser Interaction $F = 2.600$; $p = 0.008$) (**Figure 1G**). No differences in the total dendritic length ($p = 0.130$) of Cerulean$^+$ cells were observed between SE and EE mice, although differences in dendritic branching were detected ($p = 0.017$). In contrast, none of these conditions modified the total dendritic length ($F_{2,4} = 2.044$; $p = 0.136$) (**Figure 1F**) or dendritic branching [Greenhouse-Geisser Interaction $F = 0.903$; $p = 0.475$) (**Figure 1H**) of 13-week-old mCherry$^+$ cells. Finally,

SE ($p$ = 0.003 (with respect to WT mice), and $p$ = 0.126 (with respect to EE mice)] caused a slight decrease in the total dendritic length of 9-week-old Venus$^+$ newborn DGCs ($F_{2,4}$ = 4.743; $p$ = 0.011) (**Figure 1F**), although the branching pattern remained unaltered in these cells (Greenhouse-Geisser Interaction $F$ = 1.295; $p$ = 0.269) (**Figure 1I**).

These data support the notion that SE triggers effects similar to those induced by EE on the morphological maturation of newborn DGCs of different ages.

## Behavioral Effects of Social Enrichment and Environmental Enrichment

To test the potential effects of EE and SE on ambulatory, anxiety-like and exploratory behaviors, animals were tested in the Open field (OF) and the Social interaction (SI) tests, respectively (**Figures 2A,B**). During the OF test, housing conditions did not affect the total distance moved ($F_{2,4}$ = 0.711; $p$ = 0.505) (**Figure 2C**), the immobility time ($F_{2,4}$ = 1.225; $p$ = 0.317) (**Figure 2D**), the speed of displacement ($F_{2,4}$ = 0.206; $p$ = 0.816) (**Figure 2E**), or the percentage of time spent in the center of the arena ($F_{2,4}$ = 1.612; $p$ = 0.229) (**Figure 2F**). These data suggest that housing conditions did not affect the ambulatory or anxiety-like behavior of the mice.

In contrast, both EE ($p$ = 0.04) and SE ($p$ = 0.010) increased the percentage of time the animals spent exploring the empty compartments during the second day ($F_{2,4}$ = 6.533; $p$ = 0.007). No differences in this parameter were observed between SE and EE mice ($p$ = 0.670) (**Figure 2G**). Moreover, EE ($p \leq 0.001$) and SE ($p$ = 0.004) led to an increase in the percentage of time the mice spent exploring the compartment that held another mouse [with respect to the total testing time ($F_{2,4}$ = 12.901; $p \leq 0.001$) (**Figure 2H**)], although only SE ($p$ = 0.016 with respect to WT mice) increased the percentage of time the animals spent exploring the cage containing another mouse [with respect to the total exploratory time ($F_{2,4}$ = 3.686; $p$ = 0.046) (**Figure 2I**)].

These data suggest that housing conditions determine the social and exploratory behaviors of female C57/BL6 mice without affecting their locomotor activity.

## Effects of Housing Conditions on the Afferent Excitatory Connections of the Dentate Gyrus

To test the potential effect of housing conditions on the excitatory afferent connections to the DG, we analyzed the expression of a presynaptic (namely, Piccolo) and a postsynaptic (namely, PSD95) marker of glutamatergic synapses in several regions of the ML and in the GCL (**Supplementary Figures S1A–C**). To this end, the number and area occupied by PSD95$^+$ and Piccolo$^+$ particles were determined. The number of PSD95$^+$ puncta (**Supplementary Figure S1B**) was increased by EE ($p$ = 0.006) in the IML ($F_{2,4}$ = 5.208; $p$ = 0.008). Accordingly, the area occupied by these particles was also increased by EE in the same region ($F_{2,4}$ = 15.209; $p \leq 0.001$) (**Supplementary Figure S1D**). Regarding the expression of Piccolo, EE increased the number of these particles in the EML ($F_{2,4}$ = 7.874; $p$ = 0.001), MML ($F_{2,4}$ = 6.775; $p$ = 0.002), and IML ($F_{2,4}$ = 4.027; $p$ = 0.023) regions of the ML, and in the GCL ($F_{2,4}$ = 4.806; $p$ = 0.011) (**Supplementary Figure S1C**). Accordingly, the area occupied by these particles was also increased in the same regions (EML: $F_{2,4}$ = 23.740; $p \leq 0.001$; MML: $F_{2,4}$ = 24.268; $p \leq 0.001$; IML: $F_{2,4}$ = 31.642; $p \leq 0.001$; and GCL: $F_{2,4}$ = 5.555; $p$ = 0.006) (**Supplementary Figure S1E**). However, no changes in markers of excitatory synapses were observed in SE animals. These data suggest that EE and SE exert differential effects on the afferent connectivity of the DG.

Moreover, we measured the protein levels of PSD95 in the hippocampus of CH, EE, and SE mice (**Supplementary Figures S1F,G**). As shown, no differences in the levels of PSD95 ($F_{2,4}$ = 2.752; $p$ = 0.083) expression were observed between the different experimental groups. These data point to a selective increase in the expression of excitatory synapse markers in the ML caused by EE.

# DISCUSSION

The rodent DG continuously gives rise to new DGCs during life (Altman and Das, 1965). Newborn DGCs go through a tightly orchestrated sequence of maturative stages before becoming fully integrated into the pre-existing hippocampal trisynaptic circuit (Zhao et al., 2006). In this regard, EE and physical exercise are two of the most potent positive modulators of the rate of AHN (Kempermann et al., 1997; van Praag et al., 1999). The pro-neurogenic effects of EE are complex and multi-faceted since this manipulation differentially affects the sequential maturative stages that newborn DGCs go through. In this regard, EE increases the survival and synaptic integration of newly generated DGCs (van Praag et al., 2000) and exerts a profound rewiring of their afferent connectivity (Bergami et al., 2015). However, recent evidence points to remarkable differences in the maturation-promoting effects of EE on newborn DGCs of different ages. In fact, a recent concept, referred to as the *critical period*, reflects the limited period during which newborn DGCs exhibit the highest sensitivity to the stimulatory effects of EE and physical exercise (Bergami et al., 2015; Temprana et al., 2015). However, the duration of this period remains controversial. A study by Bergami et al. (2015) showed that a 4-week period of EE selectively increases the maturation and number of afferent synaptic partners of 2- to 6-week-old DGCs. In contrast, Alvarez et al. demonstrated that newborn DGCs exhibit the highest sensitivity to the stimulatory effects of a 2-day period of EE between the first and the second week of cell age (Alvarez et al., 2016). Moreover, studies by Sah et al. (2017) and Vivar et al. (2016) showed that running rewires the afferent connections of 1- and 4-week-old newborn DGCs respectively. Previous data from our laboratory indicate that a 6- to 8-week period of EE increases the number of postsynaptic densities and the size of mossy fiber terminals (MFTs) of 4- and 1-week-old newborn DGCs, respectively (Llorens-Martin et al., 2013; Pallas-Bazarra et al., 2016). Moreover, other authors demonstrate that EE increases the morphological complexity of fully mature DGCs (Faherty et al., 2003; Darmopil et al.,

2009), although it does not modify this parameter in 4-week-old newborn DGCs (Llorens-Martin et al., 2013). As shown, research conducted by various groups point to differential, or even contradictory, effects of EE on the maturation of newborn DGCs of distinct ages. In the present study, we aimed to address these differences by labeling three populations of newborn DGCs in the same mouse. Our data show that under a paradigm in which animals received three subsequent stereotaxic injections, only 9-week-old cells exhibited an increase in dendritic branching and length after EE, whereas 4- and 1-week-old newborn DGCs did not experience variations in these parameters in response to an 8-week period of EE. Despite the prolonged inter-stereotaxic injection interval, the possibility that the inflammation caused by previous injections conditioned the morphological development of mCherry$^+$ and Venus$^+$ cells in response to enriched conditions cannot be completely ruled out. However, the number and morphological maturation of DCX$^+$ cells born during the course of the EE period was markedly increased by enriched conditions, which strongly argues against the aforementioned possibility. Interestingly, similar effects on the population of DCX$^+$ cells have been reported after both short- (Beauquis et al., 2010) and long-term EE protocols (Llorens-Martin et al., 2007). Thus, our data confirms that newborn DGCs of different ages show a markedly different sensitivity to the stimulatory actions of EE.

Regarding the pro-neurogenic mechanisms triggered by EE, various components of this paradigm have been demonstrated to potentiate specific stages of the neurogenic process (Fabel et al., 2009). It has been largely acknowledged that the increased physical activity component of EE selectively increases precursor cell proliferation (van Praag et al., 1999; Holmes et al., 2004; Plumpe et al., 2006; Fabel and Kempermann, 2008; Steiner et al., 2008; Kobilo et al., 2011; Marlatt et al., 2012), whereas exercise is not required to enhance the survival of these cells (Brown et al., 2003; Olson et al., 2006; Fabel et al., 2009). Among the exercise-independent components of the EE paradigm, one critical aspect, namely the increased social interaction between enriched mice, has received little attention in the literature. Although long-lasting pro-neurogenic effects of an early post-weaning model of SE, referred to as communal nesting, have been reported (Branchi et al., 2006b), the putative existence of these effects when SE is applied to mice during adulthood have not been explored to date. To address this question, we compared the pro-neurogenic effects of an 8-week period of SE to those of a standard protocol of EE and to CH conditions. Surprisingly, we found that SE increased the number of DCX$^+$ cells and the morphological maturation of newborn DGCs of different ages, and these effects were comparable to those exerted by EE. It should be noted that our study included mice housed in small groups as in CH conditions, rather than socially isolated animals, the latter being a highly stressful situation for social rodents with detrimental effects on the rate of AHN (Stranahan et al., 2006; Ibi et al., 2008; Kulesskaya et al., 2011). Thus, our data reveal unexpected effects of SE on newborn DGCs of different ages. This manipulation increased the number and morphological maturation of DCX$^+$ cells and selectively increased the dendritic length and branching of 9-week-old retrovirally labeled newborn DGCs. Importantly, the magnitude of these effects was equal to that observed in EE mice. Hence, our findings shed light on a crucial component of EE, namely increased social interaction, as a potent stimulator of AHN in the absence of any further cognitive stimulation or increased physical activity.

Furthermore, EE exerts a plethora of neurogenesis-dependent and independent effects on hippocampal plasticity and behavior (Farmer et al., 2004; Meshi et al., 2006; Pena et al., 2006; Segovia et al., 2006; Schloesser et al., 2010; Bekinschtein et al., 2011; Kulesskaya et al., 2011; Lehmann and Herkenham, 2011; Doulames et al., 2014; Freund et al., 2015; Bechard et al., 2016; Garthe et al., 2016). Regarding the behavioral consequences of SE, they have been reported to be strongly dependent on species, gender and the developmental period at which the intervention is applied. In general terms, early post-weaning SE increases stress resilience (Cirulli et al., 2010; Branchi et al., 2013) and alters anxiety-like or depressive-like behaviors (Branchi and Alleva, 2006; Branchi et al., 2006b; Curley et al., 2009; D'Andrea et al., 2010), whereas when applied during adulthood it either increases (Branchi et al., 2006a) or decreases (Gracceva et al., 2009) social interaction. Here we observed no alteration of motor behavior in any of the experimental groups studied. However, we detected a selective increase in social interaction in response to SE and EE. In this regard, previous observations reported that EE increased the number of social interaction contacts between adult female mice (Freund et al., 2015). This finding, together with the observation that EE and SE regulated excitatory afferent innervation in the DG in distinct manners, points to differential mechanisms of action exerted by the components of EE.

In addition to the aforementioned effects of EE and SE under physiological conditions, both interventions have been demonstrated to have therapeutic effects under pathological conditions. In this regard, both EE and SE induce recovery in various models of central nervous system injury (Berrocal et al., 2007; Gajhede Gram et al., 2015; Lajud et al., 2018). Moreover, EE increases AHN in mouse models of Down syndrome (Chakrabarti et al., 2011; Pons-Espinal et al., 2013), Alzheimer's disease (Levi and Michaelson, 2007; Mirochnic et al., 2009; Rodriguez et al., 2011; Valero et al., 2011; Llorens-Martin et al., 2013; Marlatt et al., 2013), Huntington's disease (Lazic et al., 2006), diabetes (Pamidi and Nayak, 2014), ischemia (Rojas et al., 2013), and chronic pain (Zheng et al., 2017), after cranial irradiation (Garbugino et al., 2016), and during physiological aging (Kempermann et al., 1998, 2002; Kempermann, 2008, 2015; Speisman et al., 2013). In contrast, early SE reverses social deficits in animal models of autism (Garbugino et al., 2016; Campolongo et al., 2018), Parkinson's disease (Goldberg et al., 2012), and Fragile X syndrome (Oddi et al., 2015). Thus, our data take on greater relevance when examined from a therapeutic perspective. Given that SE alone is capable of inducing potent pro-neurogenic effects in the absence of any further cognitive stimulation or increase in physical activity and that these effects are of a similar magnitude to those exerted by EE, then SE emerges as an interesting alternative approach to increase AHN under certain pathological conditions. In this regard, it should be noted that numerous neurodegenerative conditions course with

motor coordination impairments and with a general compromise of motor abilities. Hence, the possibility of increasing AHN by means of SE gains further relevance in the context of these pathological conditions.

## ETHICS STATEMENT

Five-week-old female C57BL6/J Ola Hsd mice were purchased from EnVigo laboratories (Spain). Animals were housed at the Centro de Biología Molecular "Severo Ochoa" (CBMSO) in a specific pathogen-free colony facility in accordance with European Community Guidelines (directive 86/609/EEC) and handled following European and local animal care protocols. Given that the hierarchy/dominance relationships established between male mice have a negative impact on AHN (Gracceva et al., 2009; D'Andrea et al., 2010), only female mice were used in this work. Animals were left undisturbed for 2 weeks before starting any experimental manipulation. During this period, they were housed in groups of four mice per cage. Experiments were approved by the CBMSO Ethics Committee (AEEC-CBMSO-23/172) and the National Ethics Committee (PROEX 205/15). In stereotaxic injection experiments, five mice were used for each experimental condition. In cell count and behavioral determination experiments, seven animals per experimental condition were used.

## AUTHOR CONTRIBUTIONS

ML-M designed and conceived the experiments. EM-J, JJ-A, and ML-M performed the experiments. EM-J and ML-M acquired confocal images. EM-J analyzed the data. EM-J and ML-M wrote the manuscript. JA and ML-M obtained funding. All the authors critically discussed the data and revised the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00062/full#supplementary-material

**FIGURE S1 |** Non-neurogenic effects of Environmental enrichment (EE) and Social enrichment (SE). **(A)** Representative high-power magnification images showing staining with presynaptic (Piccolo) and postsynaptic (PSD95) markers of glutamatergic synapses in the Molecular layer (ML) of the DG. **(B)** Density of $PSD95^+$ particles in various sub-regions of the DG. **(C)** Density of $Piccolo^+$ particles in sub-regions of the DG. **(D)** Total $PSD95^+$ area in sub-regions of the DG. **(E)** Total $Piccolo^+$ area in different sub-regions of the DG. **(F)** Measurement of PSD95 protein expression level in the hippocampus by western Blot (WB). Anti-β-Actin was used as a loading control. Data are normalized to the expression levels of control housing (CH) mice. **(G)** WB membranes showing PSD95 expression in the different experimental groups. EML, external molecular layer; MML, medial molecular layer; IML, inner molecular layer; GCL, granule cell layer. Yellow scale bar: 5 μm. $^{**}0.01 > p \geq 0.001$; $^{***}p < 0.001$. Asterisks indicate changes with respect to control housing animals.

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BRIEF RESEARCH REPORT
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# Lack of Brain Serotonin Affects Feeding and Differentiation of Newborn Cells in the Adult Hypothalamus

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Serotonin (5-HT) is a crucial signal in the neurogenic niche microenvironment. Dysregulation of the 5-HT system leads to mood disorders but also to changes in appetite and metabolic rate. Tryptophan hydroxylase 2-deficient (*Tph2*$^{-/-}$) mice depleted of brain 5-HT display alterations in these parameters, e.g., increased food consumption, modest impairment of sleep and respiration accompanied by a less anxious phenotype. The newly discovered neural stem cell niche of the adult hypothalamus has potential implications of mediating stress responses and homeostatic functions. Using *Tph2*$^{-/-}$ mice, we explore stem cell behavior and cell genesis in the adult hypothalamus. Specifically, we examine precursor cell proliferation and survival in *Tph2*$^{-/-}$ mice at baseline and following Western-type diet (WD). Our results show a decline in BrdU numbers with aging in the absence of 5-HT. Furthermore, wild type mice under dietary challenge decrease cell proliferation and survival in the hypothalamic niche. In contrast, increased high-calorie food intake by *Tph2*$^{-/-}$ mice does not come along with alterations in cell numbers. However, lack of brain 5-HT results in a shift of cell phenotypes that was abolished under WD. We conclude that precursor cells in the hypothalamus retain fate plasticity and respond to environmental challenges. A novel link between 5-HT signaling and cell genesis in the hypothalamus could be exploited as therapeutic target in metabolic disease.

**Keywords: hypothalamus, BrdU, 5-HT, Tph2, NG2, Western-type diet**

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## INTRODUCTION

Besides the well-known neurogenic niches, the subventricular zone and the subgranular zone of the dentate gyrus, the hypothalamus has emerged as third region of postnatal neurogenesis and gliogenesis (Kokoeva et al., 2005; Lee et al., 2012; Robins et al., 2013a; Chaker et al., 2016). Positioned between the third ventricle and the median eminence, specialized radial glial tanycytes are thought to regulate the hypothalamic in- and output of hormones and nutrients to maintain body homeostasis (reviewed in Bolborea and Dale, 2013). Cell genesis within the adult hypothalamus may have an important role in feeding and reproduction control, in mediating stress

responses, and in energy metabolism. Recent studies indicate the stem cell niche is responsive to mitogens (Haan et al., 2013; Robins et al., 2013a), leptin (Kokoeva et al., 2005) and dietary challenges (Lee et al., 2012, 2014), and thereby regulates appetite and energy expenditure (Pierce and Xu, 2010). The widespread monoamine serotonin (5-HT) impacts the variety of these functions (reviewed in Donovan and Tecott, 2013). Dysregulation of the 5-HT system leads to age-related memory loss and depression (reviewed in Alenina and Klempin, 2015), but also to changes in appetite and energy metabolism (Nigro et al., 2013; D'Agostino et al., 2018). At the same time, the precise role of 5-HT in the regulation of hypothalamic functions remains unknown. Here, we address this using tryptophan hydroxylase 2-deficient ($Tph2^{-/-}$) mice selectively depleted of brain 5-HT. $Tph2^{-/-}$ mice exhibit transient early postnatal growth retardation, modest impairment of sleep and respiration, accompanied by a less anxious and highly aggressive phenotype (Alenina et al., 2009; Mosienko et al., 2012). Given the role of 5-HT in stress response and energy balance, we studied cell proliferation and fate plasticity in the hypothalamic nuclei of $Tph2^{-/-}$ mice, and the effect of high fat high cholesterol diet Western-type diet (WD) on cell survival. Our data reveal an age-dependent decline in BrdU numbers and alterations in food intake and cell phenotypes in the lack of brain 5-HT.

# MATERIALS AND METHODS

## Animals and Treatment

$Tph2^{-/-}$ mice (Alenina et al., 2009) were bred onto the C57BL/6N background for more than 10 generations. Mice were housed under standard laboratory conditions with a light/dark cycle of 12 h each. Brain slices of young-adult (6 weeks of age, postnatal day (P) 42; $n$ = 10), adult (3 months of age, P80; $n$ = 8), and 1-year-old (P1y; $n$ = 8) female mice and their littermates (CTR) (Klempin et al., 2013) were analyzed for baseline cell proliferation in the hypothalamus. Animals received three intraperitoneal injections (i.p.) of BrdU (5-bromo–2′-deoxyuridine, 50 mg/kg; Sigma-Aldrich) at 8:00 (2 h after lights went on), 14:00, and 20:00, and were killed 24 h after the first injection. Estrous cycle was not determined. Another cohort of three-month-old female $Tph2^{-/-}$ mice and their littermates ($n$ = 20) were randomly assigned to two groups for standard diet (SD, 5.0% fat, 17.8% protein, 11.0% sugar) or WD (21.2% fat, 17.5% protein, 33.2% sugar, 2.071 mg/kg Cholesterol; TD-88137 ssniff, Germany). At day 6, 7, and 8 of the diet, BrdU (50 mg/kg) was injected twice per day. Animals were sacrificed after 7 weeks (49 days) of either diet to determine proliferation and survival of newly generated cells in the hypothalamus.

## Tissue Preparation and Immunohistochemistry

Mice were deeply anesthetized and perfused transcardially with 0.9% sodium chloride followed by 4% paraformaldehyde (PFA). Brains were removed and placed into PFA overnight, and transferred into 30% sucrose the following day. BrdU immunohistochemistry followed the peroxidase method in accordance with an established protocol (Klempin et al., 2013). Briefly, one in-six series of sequential 40 μm coronal sections were stained free floating, and all immunoreactive cells detected throughout the hypothalamus were counted. The mean of both hemispheres was taken, and the total number of BrdU-positive (BrdU+) cells was estimated by multiplying cell counts by six. For the first experiment, BrdU numbers were determined per hypothalamic nuclei (arcuate nucleus, median eminence, third ventricle, and ventromedial nucleus). For Ki67 and phenotypic analysis, one-in-twelve series of sections were labeled for multiple immunofluorescence staining. BrdU+ cells were evaluated using Keyence (BioRevo BZ-9000) or Leica TCS SP5 confocal microscope (Leica, Germany) for three-dimensional colocalization. Primary antibodies were applied in the following concentrations: anti-BrdU (rat, 1:500; Biozol/AbD serotec), anti-Glial Fibrillary Acidic Protein (GFAP; rabbit, 1:1000; Abcam), anti-Iba1 (rabbit, 1:500; Wako), anti-Ki67 (rat/mouse, 1:500; eBios), anti-NG2 (rabbit, 1:200; Abcam), anti-NeuN (rabbit, 1:500; Millipore), anti-RIP (rabbit, 1:1000; Merck), anti-S100β (mouse, 1:1000; SIGMA) and anti-Sox2 (goat, 1:1000; Santa Cruz Biotechnology); Alexa488-, Cy3-, or Cy5-conjugated secondary antibodies were used (1:250; Invitrogen, United States).

## Statistical Analysis

Statistical tests to detect differences between group means were performed by two-way ANOVA (experiment 1: genotype × age; experiment 2: genotype × diet), followed by Tukey's *post hoc* tests, in cases where a significant $F$ statistic was obtained (GraphPad PRISM 6.01 software). Student's $t$-test was used for pair-wise comparison of BrdU and Ki67 analysis, and of hypothalamic subnuclei. All values are expressed as mean ± SEM. $P$-values of 0.05 were considered statistically significant.

# RESULTS

## Cell Proliferation in the Adult Hypothalamus of $Tph2^{-/-}$ Mice Declines With Aging

We first quantified baseline cell proliferation (**Figure 1A**) in the hypothalamus of $Tph2^{-/-}$ and CTR mice at different ages. In $Tph2^{-/-}$ mice, a dramatic decline in BrdU+ cells was observed with aging [$F(2,20) = 9.786$, $p_{\text{genotype} \times \text{age}} = 0.0011$; **Figure 1B**]. At P42, the number of BrdU+ cells was significantly higher relative to CTR ($p = 0.0027$; **Figure 1B**) while it was lower at P1y ($p = 0.0431$; **Figure 1B**). At P80, the amount of BrdU+ cells in the hypothalamus of CTR and $Tph2^{-/-}$ mice was similar ($p = 0.5353$; **Figure 1B**). When we looked at the distribution of BrdU+ cells within the various hypothalamic areas and nuclei (**Figure 1C**), the increase at P42 in $Tph2^{-/-}$ mice was reflected by increased cell numbers in the median eminence (Student's $t$-test $p = 0.0339$) and arcuate nucleus ($p = 0.0166$), a tendency was observed for the ventromedial nucleus ($p = 0.0668$), while at P1y, $Tph2^{-/-}$ mice

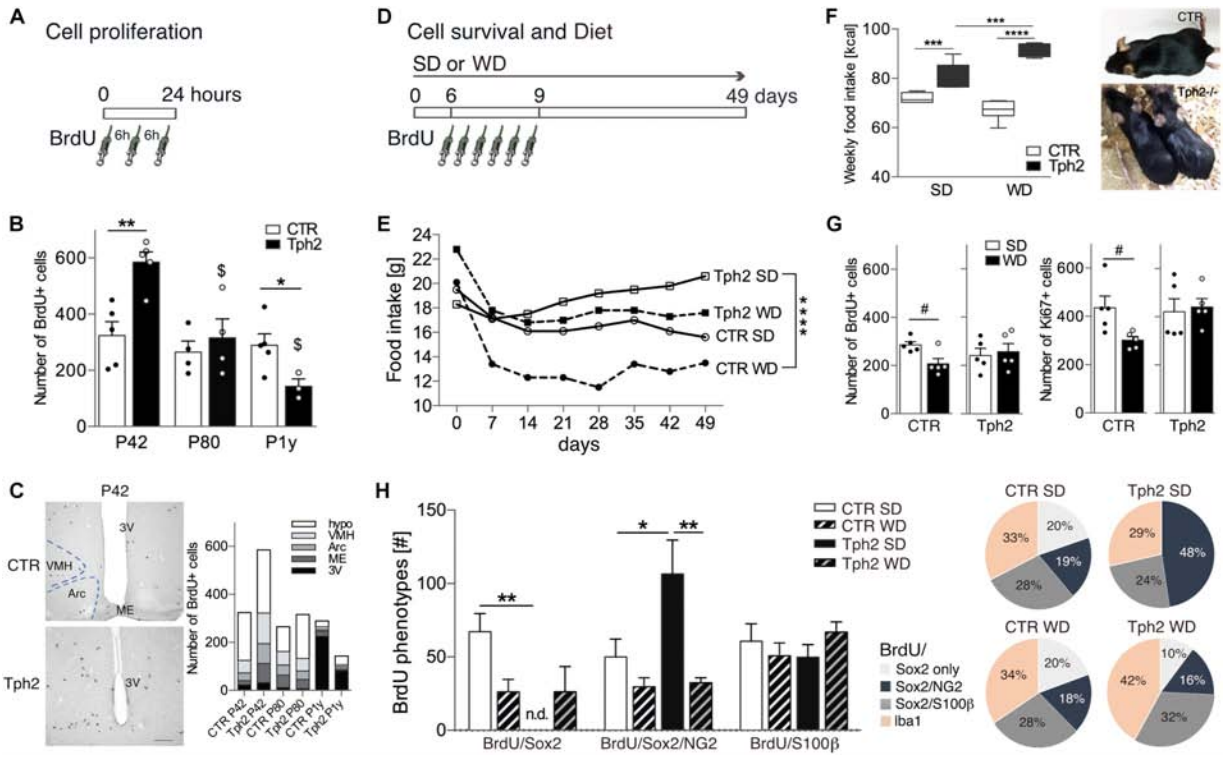


**FIGURE 1 |** Cell proliferation, food intake and cell survival at baseline and after Western-type diet (WD). **(A)** The number of BrdU-positive (BrdU+) cells in the hypothalamic nuclei was quantified at 24 h after 3 injections of BrdU 6 h apart. **(B)** Cell proliferation (entire hypothalamus) was significantly higher in young-adult (P42) Tph2$^{-/-}$ mice (Tph2), declined with aging and was significantly lower at 1 year of age (P1y) relative to littermates (CTR). **(C)** Representative images of BrdU peroxidase staining at P42, and cell numbers per hypothalamus (hypo; outside subnuclei) and per Arc, arcuate nucleus, VMH, ventromedial nucleus, ME, median eminence and 3V, third ventricle; Scale bar 150 μm. **(D)** Three-month-old Tph2 and littermates (CTR) were subjected to either standard diet (SD) or WD and BrdU was injected on days 6, 7, 8, twice per day, to determine cell proliferation and cell survival 6 weeks later. **(E,F)** Average daily food intake was least in CTR and largest in Tph2 (**E**, in g per cage of n = 5; **F**, in Kcal). Tph2 displayed oily and sticky fur upon WD. **(G,H)** At 49 days, WD resulted in a significant decrease in BrdU+ and Ki67+ cells in the hypothalamus of CTR, while it had no effect in Tph2 (**G**, Student's *t*-test #p < 0.05). Decreased cell survival in CTR was reflected by a reduction in BrdU/Sox2+ cells, however, phenotype distribution was unaffected. In Tph2 under SD, half of cells co-express BrdU/Sox2/NG2, abolishing the number (graph) and percentage (pie diagram) of BrdU/Sox2-only cells **(H)**; notably, this was not observed after WD. Two-way ANOVA followed by Tukey's *post hoc* test *p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001 between genotypes, $p < 0.05 to P42 of the same genotype.

revealed significant fewer cells lining the third ventricle relative to CTR ($p = 0.0057$).

## Cell Survival Is Not Affected by Increased High-Calorie Food Intake in *Tph2*$^{-/-}$ Mice

Given the role in energy metabolism, we elucidated the effect of WD for 7 weeks on the number of newly generated cells in the hypothalamus of three-month-old *Tph2*$^{-/-}$ mice and littermates (**Figure 1D**). During the first week of WD, food consumption [g] declined but then remained constant for each group over time (**Figure 1E**). Average food intake [g] was least in CTR fed WD and largest in *Tph2*$^{-/-}$ mice fed SD [$F(3,21) = 21.06$, $p < 0.0001$; **Figure 1E**]. Translated to calorie intake (Kcal), *Tph2*$^{-/-}$ mice consumed more of both diets, most of the WD [$F(1,23) = 27.99$, $p_{\text{genotype} \times \text{diet}} < 0.0001$; **Figure 1F**]. *Tph2*$^{-/-}$ mice displayed oily and sticky fur starting at day 3 of WD that was absent in CTR (**Figure 1F**). Seven weeks of WD resulted in significantly decreased survival of BrdU+ cells in the hypothalamus of CTR (SD vs. WD, Student's *t*-test $p = 0.0154$; **Figure 1G**). In contrast, increased high-calorie consumption by *Tph2*$^{-/-}$ mice did not affect cell numbers (SD vs. WD, Student's *t*-test $p = 0.7351$; **Figure 1G**). Cell proliferation at 49 days was assessed by Ki67-labeling; here too, WD significantly decreased the number of newly generated cells in CTR (Student's *t*-test $p = 0.0284$) while no effect was observed in *Tph2*$^{-/-}$ mice (Student's *t*-test $p = 0.7665$; **Figure 1G**).

## Lack of Brain 5-HT Affects Phenotype Distribution

We next assessed phenotypes of BrdU+ cells (**Figure 1H**) to determine the cell type affected in the absence of 5-HT and upon WD. BrdU+ cells were co-stained for the neural stem/progenitor markers GFAP and Sox2 (SRY-related HMG-box gene 2), the proteoglycan NG2, the oligodendrocyte lineage marker RIP, the astrocytes marker S100β, and the microglia marker Iba1. Sox2+ cells were highly proliferative; lining the third ventricle, they were considered as tanycytes

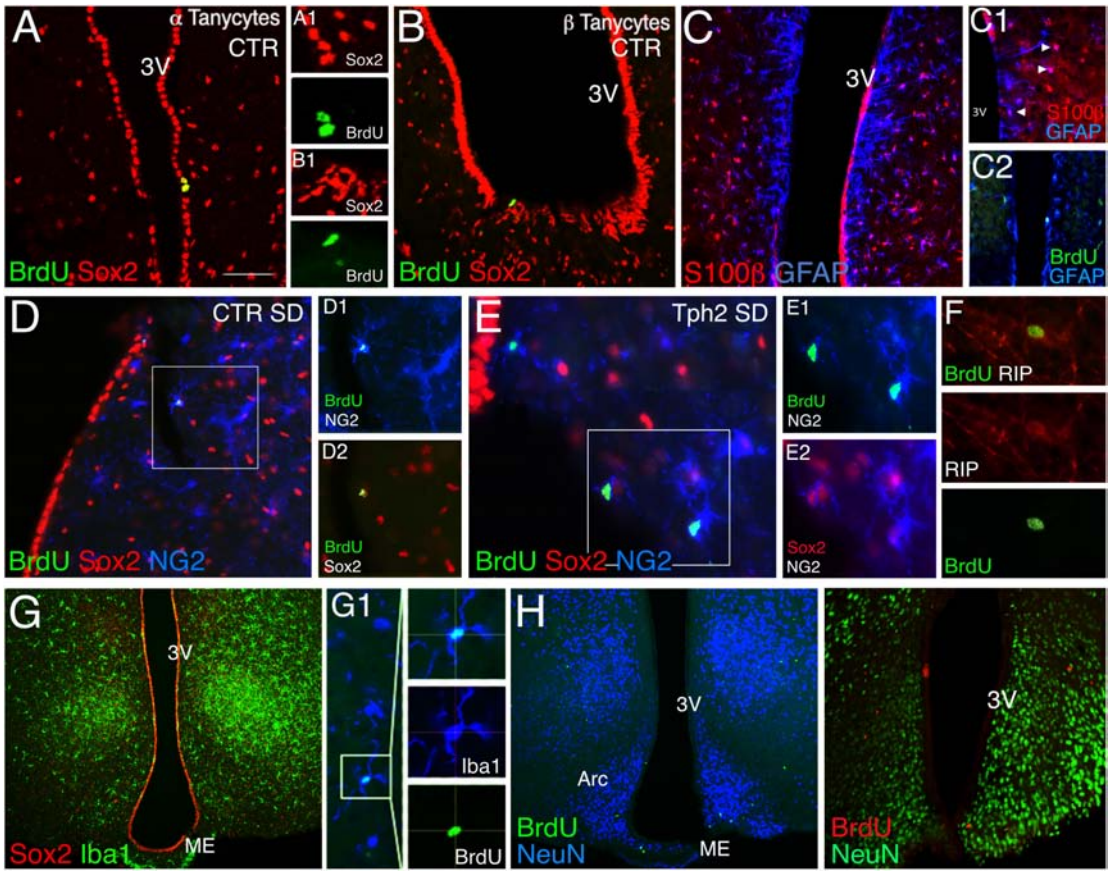


**FIGURE 2 |** Immunohistochemistry of cell phenotypes. **(A,B)** In CTR mice, a sparse number of BrdU/Sox2-tanycytes was found lining the third ventricle and was absent in Tph2$^{-/-}$ mice (Tph2; α tanycyte, **A1**, β tanycyte, **B1**) Scale bar 150 μm. **(C)** GFAP+ cells lining the dorsal part of the ventricle often co-express S100β, arrowheads **(C1)**, and were rarely dividing (BrdU/GFAP+, **C2**). **(D–F)** BrdU/Sox2/NG2+ cells account to ∼20% in CTR **(D)** and ∼50% in Tph2 **(E)** at standard diet, SD. Approx. 30% of these cells followed the oligodendrocyte fate expressing the lineage marker RIP **(F)**. **(G)** One third of BrdU+ cells adopted a microglia fate in all groups (Iba1+/Sox2−). **(H)** No BrdU/NeuN co-expressing cell was found in all groups at 49 days of the experiment. Arc, arcuate nucleus, ME, median eminence, and 3V, third ventricle.

(**Figures 2A,B**). Our results show a remarkably sparse number of BrdU-expressing tanycytes in CTR independent of the diet (<2.0% of total BrdU). Notably, no BrdU/Sox2-tanycyte was observed in *Tph2*$^{-/-}$ mice. GFAP+ cells located along the dorsal part of the ventricle (α2 tanycytes, (Robins et al., 2013a); **Figure 2C**) often co-expressed S100β (**Figure 2C1**), and were rarely dividing (**Figure 2C2**). BrdU/GFAP+ cells were only seen at SD for both genotypes accounting to 4% in CTR, and 5.8% of total BrdU in *Tph2*$^{-/-}$ mice (**Figure 2C2**). Analysis of glial phenotype distribution revealed two-thirds of BrdU+ cells expressed Sox2 (**Figure 1H**). In CTR, cells were predominantly triple labeled for BrdU/Sox2/S100β (∼30%) and BrdU/Sox2/NG2 (∼20%; **Figures 1H**, **2D**). Notably, all BrdU/S100β+ cells co-expressed Sox2. In *Tph2*$^{-/-}$ mice at SD conditions, half of the cells co-expressed BrdU/Sox2/NG2 [$F(2,46) = 7.764$, $p_{\text{genotype}} = 0.0012$; **Figures 1H**, **2E**], lowering the amount of BrdU/Sox2-only cells. After WD, the decrease in BrdU+ cell numbers in CTR was reflected by decreased double and triple labeling of glial markers with a significant reduction in BrdU/Sox2+ cells (SD vs. WD, $p = 0.024$; **Figure 1H**). However, phenotype distribution was unaffected by WD in CTR. In *Tph2*$^{-/-}$ mice upon dietary challenge, the large population of BrdU/Sox2/NG2+ precursor cells was diminished to 16%, and fate distribution among glial cells was similar to CTR (**Figure 1H**, pie diagram). Approximately 30% of cells in all groups adopted an oligodendrocyte fate co-expressing BrdU/RIP (**Figure 2F**). Another third of BrdU+ cells (Sox2−) adopted a microglia fate characterized by co-expression of Iba1 (**Figure 2G**). We did not observe BrdU/NeuN+ cells in any group (**Figure 2H**). Congruent to NeuN staining, no BrdU+ cell expressing the neuronal marker β-III-Tubulin was found (Tuj1; not shown).

## DISCUSSION

Our data demonstrate an age-dependent decline in cell proliferation in the hypothalamus of *Tph2*$^{-/-}$ mice, but not in CTR, and a large population of proliferating Sox2/NG2 cells. In the lack of brain 5-HT, no proliferating Sox2-tanycytes were detected and the high-calorie-induced decrease in cell

proliferation and survival was absent. Furthermore, we did not find newly generated neurons at 6 weeks after BrdU but a diet-induced altered phenotype distribution in *Tph2*$^{-/-}$ mice.

Neural stem/progenitor cell populations of α and β tanycytes in the adult hypothalamus, and postnatal born neurons, contribute to brain plasticity and potentially respond to hormones and nutrition to maintain metabolic rate (Lee et al., 2012; Bolborea and Dale, 2013; Haan et al., 2013; Robins et al., 2013a). Serotonin is involved in the regulation of feeding and metabolism, e.g., food intake enhances hypothalamic 5-HT indicating satiety (Leibowitz and Alexander, 1998; Schwartz et al., 2015; Nectow et al., 2017). On cellular level, 5-HT2C receptors on POMC neurons might particularly mediate the 5-HT effects (Lam et al., 2008; D'Agostino et al., 2018). Our data show that in the absence of brain 5-HT, *Tph2*$^{-/-}$ mice consumed significantly more suggesting they might lack satiety. This might correspond to no detectable proliferating Sox2-tanycytes; in a next step, POMC neurons in *Tph2*$^{-/-}$ mice should be studied (Klein et al., 2019). Furthermore, in the lack of brain 5-HT, a different subpopulation of precursor cells, Sox2/NG2, is favored and affected by WD; indicating 5-HT plays an important role at the stem/progenitor cell stage. Our earlier findings on neurogenesis in the hippocampus of *Tph2*$^{-/-}$ mice also revealed changes at Sox2 cell stage, suggesting physiological adaptations to changes in 5-HT supply to maintain homeostasis (Klempin et al., 2013). Hypothalamic Sox2/NG2 or NG2-only cells are highly proliferative with glial and some neuronal potential (Robins et al., 2013b). Fate plasticity makes them favorably affected to changes in the environment and in turn might act as compensatory mechanism. Increased proliferation of Sox2/NG2+ cells in *Tph2*$^{-/-}$ mice might compensate for the lack of BrdU/Sox2-tanycytes. Although a small number of newborn neurons was reported in few lineage tracing studies (Lee et al., 2012; Robins et al., 2013a) we have not detected any using our treatment protocol–based on BrdU and available markers of lineage progression–and it remains speculative whether the high number of NG2 cells eventually become neurons (Robins et al., 2013b).

In CTR mice, BrdU/Sox2+ cells were affected upon high-calorie consumption, reducing this precursor subset. In aged CTR mice, no difference in cell proliferation was observed; and a decline in only Sox2-tanycytes, e.g., starting at 11 months of age, was reported earlier (Zhang et al., 2017). In *Tph2*$^{-/-}$ mice, lack of brain 5-HT *per se* might not be responsible for the age-dependent decline in cell proliferation but overall changes in tanycyte and precursor numbers which in turn lead to reduced BrdU detected at P1y.

In this study, we explored the novel neural stem cell niche of the adult hypothalamus and analyzed the role of brain 5-HT in food intake and cell genesis. We show that precursor cells in the hypothalamus retain fate plasticity and respond to both intrinsic changes and environmental stimuli.

## ETHICS STATEMENT

All procedures were approved by LAGeSo (Berlin, Germany) and carried out in accordance with the European Communities Council Directive 2010-63 UE.

## AUTHOR CONTRIBUTIONS

MvL and FK designed the research, analyzed data and wrote the manuscript. MvL, MS, and FK performed experiments. NA bred and provided *Tph2*$^{-/-}$ animals.

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frontiers in Cell and Developmental Biology

MINI REVIEW
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# Adult Neural Stem Cells: Born to Last

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The generation of new neurons is a lifelong process in many vertebrate species that provides an extra level of plasticity to several brain circuits. Frequently, neurogenesis in the adult brain is considered a continuation of earlier developmental processes as it relies in the persistence of neural stem cells, similar to radial glia, known as radial glia-like cells (RGLs). However, adult RGLs are not just leftovers of progenitors that remain in hidden niches in the brain after development has finished. Rather, they seem to be specified and set aside at specific times and places during embryonic and postnatal development. The adult RGLs present several cellular and molecular properties that differ from those observed in developmental radial glial cells such as an extended cell cycle length, acquisition of a quiescence state, a more restricted multipotency and distinct transcriptomic programs underlying those cellular processes. In this minireview, we will discuss the recent attempts to determine how, when and where are the adult RGLs specified.

**Keywords: neurogenesis, quiescence, hippocampus, neural stem cell, transcriptional profile**

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## INTRODUCTION

During the formation of the central nervous system, RGCs proliferate and differentiate to first generate neurons in a process known as neurogenesis and later, in a second wave, glial cells. While the latter process of gliogenesis continues at postnatal stages and it is widespread throughout the adult vertebrate brain (Rowitch and Kriegstein, 2010; Gallo and Deneen, 2014), neurogenesis ceases soon after birth in most mammalian brain regions. In rodents, two exceptions are the SGZ of the DG in the hippocampus and the V-SVZ of the lateral ventricles, in which, respectively, GN and progenitors of olfactory bulb interneurons are generated throughout life (Altman and Das, 1965; Doetsch et al., 1999; Fuentealba et al., 2012; Gonçalves et al., 2016). Adult neurogenesis depends on the persistence of neural stem cells that share properties with developmental RGCs, to which we will refer throughout the review as radial glia-like cells (RGLs). In the adult human brain, RGLs from the V-SVZ are thought to contribute new interneurons to the striatum (Ernst et al., 2014) while the SGZ contributes cells to the DG (Eriksson et al., 1998; Spalding et al., 2013; Boldrini et al., 2018; Moreno-Jiménez et al., 2019). However, adult neurogenesis in humans is still a matter of controversy (Cipriani et al., 2018; Sorrells et al., 2018) and although several technical issues have been considerably improved (Moreno-Jiménez et al., 2019), additional approaches should be

**Abbreviations:** BLBP, brain lipid binding protein; BMP, bone morfogenetic protein; DG, dentate gyrus; DGN, dentate gyrus neuroepithelium; EGFR, epidermal growth factor receptor; FAO, fatty acid oxidation; FGF, fibroblast growth factor; GABA, gamma-aminobutyric acid; GFAP, glial fibrillary acidic protein; GN, granule neuron; IPC, intermediate progenitor cell; LPA1, lysophosphatidic acid receptor 1; RGC, radial glial cell; RGL, radial glia-like cell; SGZ, subgranular zone; VCAM1, vascular cell adhesion molecule 1; V-SVZ, ventricular-subventricular zone.

undertaken before the whole scientific community accepts its existence (discussed in Lee and Thuret, 2018; Kempermann et al., 2018; Paredes et al., 2018; Snyder, 2018).

The question then arises as to why, at least in most mammals, are the adult neurogenic niches so restricted? And, why is the neurogenic process extended in time and reduced in number in the adult brain? Is it related to specific properties of adult RGLs such as quiescence? When and how is the quiescent pool of RGLs established? This minireview will revisit these questions with a focus on the DG niche of the rodent brain. Nevertheless, some aspects related to V-SVZ neurogenesis will be mentioned.

## THE DEVELOPMENT OF THE DENTATE GYRUS

One of the approaches to start understanding the uniqueness of the adult neurogenic process is to look at its origin during brain development. The development of the DG is quite distinct, first because it is more protracted in time than that of other cortical regions and also because, in comparison with the neocortex and the rest of the hippocampus, it involves the migration of a separate group of neural progenitors from the neuroepithelium, away from the VZ and close to the pial surface.

The DG progenitors originate at around embryonic day (E) 13.5 in mice from a restricted area of the medial pallium neuroepithelium, the DG neuroepithelium (DGN) or primary ($1^{ry}$) matrix (Altman and Bayer, 1990) that receives patterning signals from the adjacent cortical hem (a hippocampal organizer; Mangale et al., 2008; **Figure 1**). DG progenitors migrate through the secondary ($2^{ry}$) matrix, next to the fimbria border and toward the pial side of the cortex, forming the dentate migratory stream, composed of a mix of IPCs and postmitotic immature GNs, the principal neuron of the DG. At the end of their migration, GNs accumulate in the DG anlage or hilus and a new germinative pool, called the tertiary ($3^{ry}$) matrix, is established (**Figure 1**). While DG morphogenesis starts early in embryonic development, the vast majority of GNs are generated within the first two postnatal weeks and originate from the $3^{ry}$ matrix (Muramatsu et al., 2007). Significantly, between postnatal day (P) 20 and P30, proliferating cells become gradually confined to the SGZ, which serves as source of newly born neurons in the adult DG (Altman and Das, 1965; Altman and Bayer, 1990; Urban and Guillemot, 2014). Thus, the DGN only generates one type of neuron, the GN, and even DG astrocytes will be generated from a different region, the fimbria neuroepithelium, that is a derivative of the cortical hem (Altman and Bayer, 1990).

The majority of adult RGLs emerge in the DG during the first postnatal week. Ablation of proliferating Nestin-creERT$^{+}$ stem cells in this period leads to a lasting depletion of the adult RGL pool in the DG with a corresponding inhibition of adult neurogenesis and a RGL fate bias toward astrocytic progeny (Youssef et al., 2018). However, the same experiment performed from P14-P21 does not alter the pool of adult RGLs and only leads to reduced adult neurogenesis. In the same direction, using a reporter line (Hopx-creERT2) that labels mostly DG neural progenitors during development (Li et al., 2015), it has been genetically determined that a common neural precursor population with a restricted cell lineage continuously and exclusively contributes GNs to the DG formation from the $1^{ry}$ matrix up to adulthood (Berg et al., 2019). These experiments also confirm that the first progenitors with a typical RGL morphology appear around P7-P8 (**Figure 1**).

Interestingly, it has been also proposed that a subpopulation of RGLs and neural progenitors along the hippocampal longitudinal axis (septal/dorsal to temporal/ventral axis) is generated in the ventral part of the hippocampus and migrate perinatally from temporal to septal poles before settling (Li et al., 2013). This migrating population could be the origin of around 69% of the RGLs in the SGZ of the young P15 DG, although their contribution at adult stages has not been estimated. Recently it has been shown that ventral and dorsal populations respond differentially to Shh signaling as *Sufu* deletion (acting in this context as a Shh signaling inhibitor) only impairs the proliferation of RGLs in the dorsal DG, but not in the ventral DG (Noguchi et al., 2019). This difference could be due to underlying molecular differences between RGLs and the surrounding cells residing in these regions. Nevertheless, it is still unclear how the caudal temporal population, or even the rostral septal RGL population, acquire the molecular and functional characteristics of adult RGLs.

## DEVELOPMENTAL ORIGIN OF QUIESCENT RGLs

Even from early stages (E14.5), there are differences between the DG progenitors and those that will give rise to the hippocampus proper or the cortex. A subpopulation of GFAP expressing cells can be detected in the DGN, whereas in the adjacent dorsolateral neuroepithelium (cortical and hippocampal) RGCs do not express GFAP but Pax6 and BLBP. BLBP expression is acquired progressively in the GFAP expressing DG stem/progenitor cells from P1 to P14 (from 30 to 75% of total GFAP$^{+}$ cells; Seki et al., 2014; Matsue et al., 2018). These results suggest that the properties of hippocampal granule stem/progenitor cells are rapidly altered from an embryonic to adult type soon after birth. But, what are those properties that define the adult RGLs? Is there a distinct population of specified RGLs or are the developmental RGCs that start behaving differently?

Perhaps one of the characteristics that distinguish adult RGLs most clearly from their embryonic counterparts is the acquisition of quiescence by which the adult RGLs remain for long periods out of the cell cycle, in G0. The state of G0 quiescence is shared with many somatic stem cells in other mature vertebrate tissues and is crucial to maintain tissue homeostasis and avoid stem cell exhaustion (Simons and Clevers, 2011; van Velthoven and Rando, 2019). In invertebrates such as *Drosophila*, quiescent neural stem cells can be arrested in either G0 or G2 (Otsuki and Brand, 2018).

Taking a candidate gene approach, several groups have examined the role of cell cycle related genes in the regulation of RGL quiescence. There are some indications of cell cycle genes differentially involved in embryonic versus adult neurogenesis.

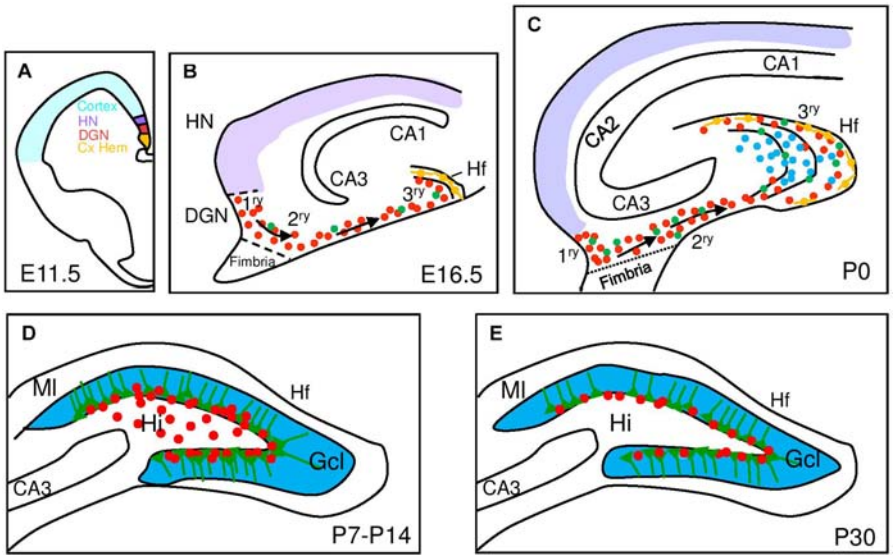


**FIGURE 1 |** Development of the mouse dentate gyrus. Schematic representation of hippocampal development from embryonic (E) to postnatal stages (P) with a focus on dentate gyrus development. **(A)** At E11.5 signals from the cortical hem (Cx HEM, orange) specify the adjacent dentate gyrus neuroepithelium (DGN, red) and the hippocampus neuroepithelium (HN, purple). **(B)** Late embryonic DG development (E16.5) showing dentate progenitor cells delaminating from the DGN forming the primary matrix ($1^{ry}$) and the migratory stream of proliferating migratory RGCs (green) and progenitor cells (red) forming the secondary matrix ($2^{ry}$) and then the tertiary matrix ($3^{ry}$), formed around the hippocampal fissure (Hf). Cajal-Retzius cells (orange) are derived from the Cx HEM and follow the Hf. **(C)** After birth, the upper and lower blades of the DG are observed. Primary and secondary matrices soon disappear but progenitors in the tertiary matrix continue actively dividing and producing granule neurons with continued insertion of radial glial fibers to organize the addition of new DG neurons. **(D)** At P7 mitotic cells gather at the border between the hilus (Hi) and the granular cell layer (Gcl) and establish the future subgranular zone (SGZ) at P14. **(E)** At P30, mitotically active RGL and IPCs are restricted to the SGZ. CA, Cornu Ammonis; Ml, molecular layer.

Among them, we encounter the *CyclinD* genes, which are necessary for the mid-G1 cell cycle checkpoint. The three *CyclinDs* are differentially expressed in the brain regions during embryonic and postnatal stages (Glickstein et al., 2007). Surprisingly, *CyclinD2* but not *D1* mutation severely reduced proliferation of RGLs and progenitors in the SGZ from P7 onward causing almost a 10-folds less proliferation at P30 (Kowalczyk et al., 2004; Ansorg et al., 2012). These data indicate that postnatal neurogenesis is controlled by CyclinD2 together with at least one other D-type cyclin, and that the age at which DG neurogenesis becomes exclusively dependent on the expression of functional CyclinD2 lies between P14 and P28 (Ansorg et al., 2012; **Figure 2**). However, it is not clear if the importance of CyclinD2 is because it is enriched in adult RGLs or because CyclinD2 confers differences in cell cycle dynamics with respect to CyclinD1. In that sense, CyclinD1 can be only incompletely compensated for by knock in of *CyclinD2* into the *CyclinD1* locus, indicating non-redundant functions of these proteins (Carthon et al., 2005). Moreover, as *CyclinD2* mutants have also embryonic defects resulting in a reduced DG postnatally, conditionally removing *CyclinD2* from the adult niche is still required to establish if the defects in adult neurogenesis are due to a defect in the specification or maintenance of the adult RGL cell population during development.

Other cell cycle regulators that could be important in the establishment of the RGL pool are the cyclin-dependent kinase inhibitors. Among them, the Cip/Kip family which includes $p21^{Waf/Cip1}$ (referred to as p21), $p27^{Kip1}$ (referred to as p27), and $p57^{Kip2}$ (referred to as p57). *p21* and *p27* deletion in mice during development results in increased progenitor proliferation in the hippocampus (Pechnick et al., 2008; Qiu et al., 2009). p27 is expressed in RGL cells in the adult DG and in the full *p27* mutant mice or in animals carrying a disruption in the cyclin-CDK interaction domain of p27 there is an increase in the proliferation of adult RGLs (Andreu et al., 2015). It is currently unclear if this leads to the loss of RGLs with time and/or if RGC proliferation is affected during DG development. Similarly, p57 is expressed in quiescent RGLs and its specific deletion in adult RGLs abrogates their quiescence (reduction in the number of RGLs that retain BrdU for long periods of time or BrdU-LRCs) and activates their proliferation (Furutachi et al., 2013). That leads to an increase in the number of new neurons, but subsequently at long-term (in 2 years old animals) it leads to excessive reduction of both RGLs and neurogenesis in the aged brain. It is not clear for how long the RGLs maintain high levels of proliferation in the p57 adult mutant before losing prematurely that potential in old animals. Furthermore, it has not been described what happens to hippocampal neurogenesis when p57 is removed during developmental stages.

Some clues about the possible relevance of p57 in establishing the pool of adult quiescent RGLs have emerged from the studies of the V-SVZ neurogenic niche in Nestin-cre/p57 mice (Furutachi et al., 2015). During embryonic ganglionic eminences development, the cell cycle of a subset of neural progenitor cells of the V-SVZ slows down, between E13.5 and E15.5, while other neural progenitors continue to divide rapidly

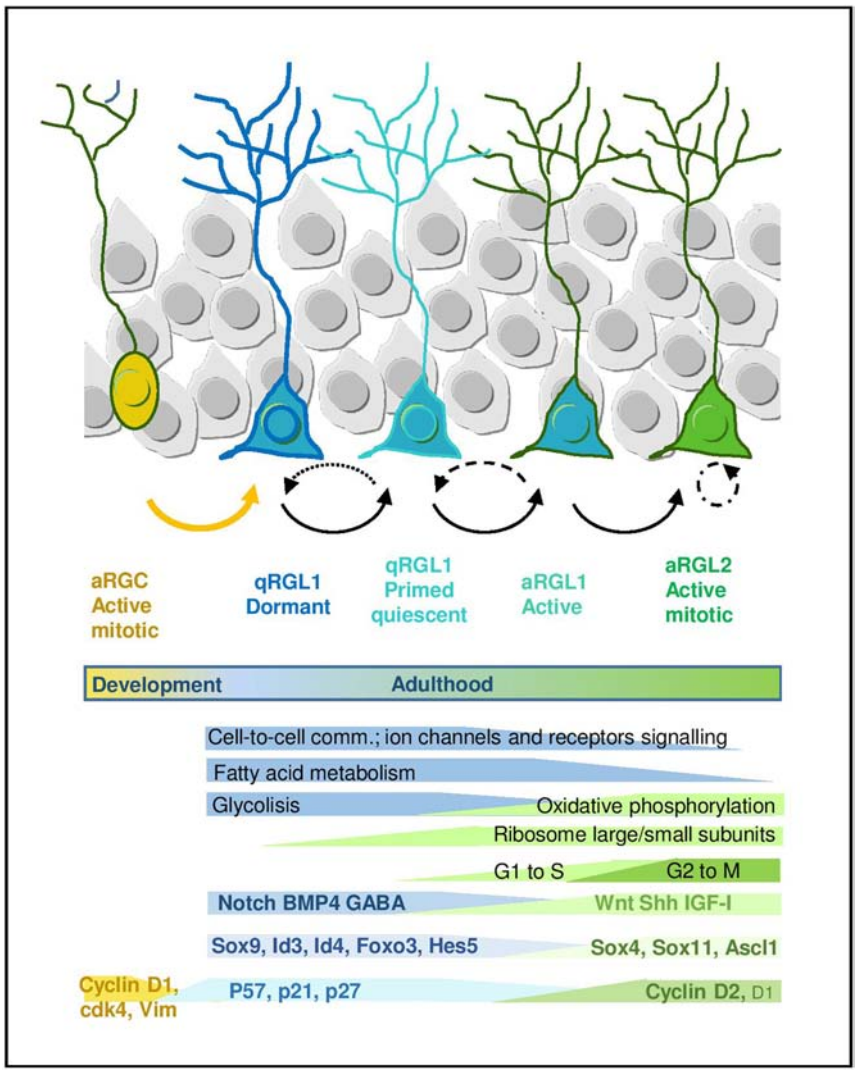


**FIGURE 2 |** Quiescence and activation in RGL cells in the hippocampal neurogenic niche. Scheme representing the RGL cell stages during early lineage progression. Main molecular programs are highlighted (adapted from Llorens-Bobadilla et al., 2015 and Shin et al., 2015). It also includes molecular signatures discussed in the main text.

(Fuentealba et al., 2015; Furutachi et al., 2015). A majority of RGLs in the young adult V-SVZ originate from these slowly dividing embryonic progenitors that are "set aside" and express high levels of p57. The conditional deletion of *p57* reduces the pool and proliferation of adult V-SVZ RGLs from E17.5 till at least P24 and also decreases the generation of new neurons. However, these studies do not exclude the possibility that some adult RGLs could derive from rapidly dividing embryonic progenitors, as adult RGLs are heterogeneous.

Unlike precursors for adult RGLs in the V-SVZ, recent results suggests that precursors for adult DG RGLs are not clearly "set-aside" dormant in quiescence during embryonic development, but instead seem to transition predominantly to a quiescent state postnatally. $Hopx^{+}$ cells give rise to RGLs with quiescence properties (RGLs that retain BrdU after a 30 day pulse) from midgestation but the production clearly peaks during the first postnatal week (Berg et al., 2019). In parallel, another group has demonstrated that *Sufu* conditional deletion (which causes a reduction in Shh signaling) impairs the ability of RGLs to expand in the first postnatal week that results in the premature entry of RGLs into the quiescent state (Noguchi et al., 2019). These recent data suggest that Shh signaling activity must be continuously maintained to promote RGL expansion postnatally.

Apart from cell cycle inhibition, the quiescent state involves changes in cell adhesion molecules as well. A similar function to that of p57 in the establishment of the adult RGL pool has been suggested for vascular cell adhesion molecule-1 (VCAM) in the V-SVZ (Hu et al., 2017). Blocking VCAM function in adult SVZ, it was shown that VCAM1 is required for keeping adult RGLs in a quiescent state (Kokovay et al., 2012). Moreover, conditional deletion of VCAM1 in the embryonic brain caused RGCs premature differentiation during mid-gestation and reduced

quiescence in slowly dividing RGCs with a reduction in the pool of postnatal and adult BrdU-LRCs (Hu et al., 2017). Thus, VCAM1 is required for the preservation of the embryonic RGCs into the adult stage. However, its possible function in the hippocampal niche has not been reported.

Altering the control of quiescence during development could provoke the exhaustion of the pool of active RGLs in mutant mice. For instance, loss of the phagocytosis factor Mfge8 during development promotes an increase in RGC proliferation at P15 and leads to the exhaustion of the neurogenic pool causing a decrease in RGL cell proliferation and neurogenesis by P30 (Zhou et al., 2018). Nevertheless, it is not clear if the depletion of RGLs in the adult brain is due to the premature overproduction or also to the fact that deletion of Mfge8 in the adult brain causes a change in cell fate specification and RGLs adopt an astrocytic fate (Zhou et al., 2018). It is also striking that in only 15 days (from P15 to P30) the pool of proliferating RGLs could be depleted. Moreover, the possible alterations in DG development and SGZ specification in Mfge8 mutants at earlier stages (before P15) have not been analyzed.

In summary, there are differences between the main adult neurogenic niches in the way and time the adult RGLs are generated. Thus, in the neuroepithelium of ganglionic eminences, neural precursors that give rise to adult RGLs (source of olfactory bulb interneurons) will be set aside at E13.5/15.5 from other precursors that will continue generating late born cortical interneurons and glial cells pre- and perinatally. In contrast, in the DG neuroepithelium (where development is protracted and cell lineage is very restricted), at around E15.5 subsets of precursors will continuously generate adult RGLs (source of only one type of neuron, the GN) with a clear peak in the first postnatal week, while the rest of DG precursors will generate also GN pre- and perinatally. These differences between adult niches could be probably related to early lineage specification and restriction during the development of their respective neuroepithelial progenitors, but the specific signals and mechanism involved in those early processes need to be revisited in the light of the generation of adult RGLs.

Nevertheless, the question of what exactly drives the acquisition of the adult RGL identity in the DG during early postnatal development remains open. In that sense, an in-depth analysis of the molecular program that controls the state of quiescence may eventually shed light on how quiescent RGLs become established. In addition, it is unclear if embryonic/postnatal quiescent RGLs are the same as adult quiescent RGLs.

## THE QUIESCENCE-SPECIFIC GENE EXPRESSION PROGRAM OF ADULT RGLs

Many efforts have been recently devoted to the identification of the molecular signature that defines the quiescent state of adult RGLs. The development of fluorescence-activated cell sorting protocols based on combinations of markers has allowed to prospectively isolate populations of quiescent and proliferating RGLs from the brain, facilitating the analysis of their transcriptomic fingerprint. The current cell sorting approaches rely on the use of: (1) a variety of cell surface epitopes to enrich in stem/progenitor cells, (2) transgenic animals expressing fluorescent proteins under the regulation of GFAP, Nestin and more recently LPA1 and Hopx for the hippocampus, and (3) fluorescent EGFR ligands to distinguish between active ($EGFR^+$) vs. quiescent ($EGFR^-$) RGLs (Capela and Temple, 2002; Beckervordersandforth et al., 2010; Daynac et al., 2013, 2015; Codega et al., 2014; Mich et al., 2014; Llorens-Bobadilla et al., 2015; Shin et al., 2015; Walker et al., 2016; Dulken et al., 2017; Morizur et al., 2018; Berg et al., 2019). Based on these isolation approaches, both genome-wide microarray, RNAseq and single-cell RNAseq transcriptomic datasets have been generated and some laboratories have even developed on-line tools that allow to explore the single-cell RNAseq data interactively[1,2]. In general, datasets obtained using different combinations of markers and strategies partly overlap and there is wide consonance in the functional interpretation of this valuable transcriptomic information (**Figure 2**).

A common theme uncovered in the multiple transcriptomic datasets through gene ontology and pathway analyses is the enrichment in genes related to lipid metabolism, glycolysis, cell signaling/communication and cell adhesion in the RGL quiescent state, both in the V-SVZ and DG. This profile is in sharp contrast to the enrichment in genes linked to the cell cycle, DNA/RNA metabolism, transcription and protein translation that characterizes the proliferative state (Llorens-Bobadilla et al., 2015; Shin et al., 2015; Morizur et al., 2018; Berg et al., 2019). In single-cell RNAseq studies, in which expression dynamics are reconstructed along a pseudo-time, the first mark for the exit from quiescence is the increase in the expression of genes that code for ribosomal proteins and that therefore participate in protein biosynthesis. Indeed, lentiviral overexpression of some ribosomal proteins is sufficient to induce proliferation of adult DG RGLs (Mukherjee et al., 2016). The upregulation of ribosomal genes is followed by a shift in energy metabolism genes from glycolysis to mitochondrial oxidative phosphorylation (Llorens-Bobadilla et al., 2015; Shin et al., 2015). Fatty acid metabolism has also emerged as a crucial regulator of RGL activity in the DG. *De novo* lipogenesis is key for proliferation (Knobloch et al., 2013) while lipid breakdown through FAO in the mitochondria is most prominently required in the quiescent state (although FAO is also used by proliferating cells). Whole proteome analysis of active vs. quiescent stem/progenitor cell cultures corroborates the transcriptomic data, evidencing an increase in FAO in quiescence, and metabolic experiments show that FAO is required during quiescence for energy production and as an alternative carbon source (Knobloch et al., 2017). Recent data also suggest similar shifts in lipid metabolism, cell cycle and translation in DG progenitors coinciding with the transition from postnatal RGCs to adult quiescent RGLs (Berg et al., 2019).

Transcriptomic data comparing quiescent and activated V-SVZ RGLs have also uncovered differences in the protein homeostasis network (Leeman et al., 2018). Activated RGLs

[1] https://martin-villalba-lab.shinyapps.io/scRNAseq_CSC2015/
[2] https://linnarssonlab.org/dentate/

mostly rely on proteasomal degradation for protein quality control while quiescent RGLs accumulate protein aggregates in lysosomes, a phenomenon that is exacerbated during aging. *In vitro*, stimulating the lysosome-autophagy pathway clears the protein aggregates and enhances RGL activation in response to growth factors. Generally speaking, metabolic changes are required to meet the increased biosynthetic demands of actively proliferating cells and are thus observed during the exit from quiescence of many cell types, including adult somatic stem and non-stem cells (García-Prat et al., 2017; Chapman and Chi, 2018). It is currently unclear if the aforementioned metabolic pathways (FAO, glycolysis, protein homeostasis) are merely adaptive responses to cell cycle withdrawal of quiescent cells or perhaps also drivers of RGL transformation *in vivo* during postnatal development.

In addition to the metabolic genes, transcription factors (TFs) are also differentially regulated in the quiescence-to-proliferation transition of RGLs, both in the DG (Shin et al., 2015) and the V-SVZ (Llorens-Bobadilla et al., 2015; Morizur et al., 2018). Some of the TFs (or their paralogs) are involved in embryonic neurogenesis and/or in the regulation of somatic stem cells in other non-neural adult niches. This suggests the existence of stage-specific genetic programs that would be controlled in a coordinated manner by those TF codes. The systematic analysis of the Shin et al. (2015) dataset, for instance, revealed around 80 TFs up and downregulated during the initial stages of adult hippocampal neurogenesis. As expected, TFs and transcription regulators such as *Sox9*, *Id4*, or *Id3* appear associated with the quiescent state in the hippocampus, while SoxC TFs (*Sox4* and *Sox11*) raise during activation, yet more than half of the regulated TFs identified in the dataset are still largely unexplored in the context of RGL quiescence (Shin et al., 2015). Similarly, in the V-SVZ *Sox9*, *Id2*, and *Id3* are associated with the quiescent state while *Ascl1*, *Sox4*, and *Sox11* are enriched in the active sate (Llorens-Bobadilla et al., 2015). Despite the indisputable relevance of these mRNA profiles, we should keep in mind that some of the TFs involved in the activation of RGLs are finely regulated post-translationally, as it is the case for Ascl1, a major player in shaping the transcriptomal landscape of active RGL and IPCs (Andersen et al., 2014; Llorens-Bobadilla et al., 2015). The E3-ubiquitin ligase Huwe1 destabilizes the Ascl1 protein in proliferating DG RGLs, preventing the accumulation of cyclinDs and returning the cells to the quiescent state (Urban et al., 2016). Supporting Ascl1 pivotal role of, it has been shown that Ascl1 oscillations, which in turn depend on Hes1 oscillations, regulate RGL activation, while high Hes1 expression and the resulting Ascl1 suppression promote DG RGL quiescence (Sueda et al., 2019).

Another characteristic of the quiescent state of RGLs is the upregulation of genes that code for membrane proteins involved in intercellular communication, cell adhesion and transport. In the DG for instance, among the top 1,000 quiescence-enriched genes downregulated during activation, 51% encode proteins associated with the membrane (Shin et al., 2015). Quiescent RGLs overexpress at the mRNA level both receptors of multiple relevant pathways in the SGZ niche (such as Dll/Notch, BMP, Insulin, FGF, or neurotrophins) and receptors of the neurotransmitters GABA, glutamate and calcium channels, suggesting that quiescent RGLs are probably more sensitive to extrinsic stimuli than their committed progeny (Shin et al., 2015). Cell membrane genes are also markedly increased in DG progenitors over development, pointing to a switch from an intrinsic mode of regulation in RGCs to a niche-dependent mode in adult RGLs (Berg et al., 2019). However, we should not forget that this scheme is based on the extrapolation of the transcriptomic information to the membrane proteome and to its potential signaling activity, not on direct signaling measurements. Given the enormous post-transcriptional regulation and recycling by internalization suffered by membrane receptors, functional correlations are not guaranteed. An example of the mRNA/membrane protein discrepancy has already been reported for members of the transmembrane syndecan family heparan sulfate proteoglycans in quiescent RGLs of the V-SVZ (Morizur et al., 2018).

In addition to the membrane receptors, several cell adhesion proteins, including neural cell adhesion molecules and cadherins/protocadherins are overexpressed in quiescent RGLs (Morizur et al., 2018). This further highlights the key role of cell adhesion and niche interaction in the modulation of RGL activity, as described previously for VCAM1 (Kokovay et al., 2012) or N-cadherin in V-SVZ RGLs (Porlan et al., 2014).

In summary, the transcriptomic data have allowed to study the molecular signature of quiescent and active RGLs, and based on the available information, new concepts are already emerging. The transcriptomic data have been partly endorsed by functional assays employing previously established *in vitro* quiescence protocols (Mira et al., 2010; Martynoga et al., 2013) and to a lesser extent, employing prospectively isolated cells or transgenic mouse models. Together, the transcriptomic, proteomic and functional data point to profound bioenergetic and cell signaling differences between quiescent RGLs and their active counterparts and highlight the potential of metabolic pathways as direct regulators of RGL transitions, as shown in other adult stem cell populations (García-Prat et al., 2017). Future mechanistic studies will be fundamental to gain insight on their putative role on the acquisition of the RGL identity and quiescence during development.

## INSIGHTS INTO THE DEVELOPMENTAL ORIGIN OF HIPPOCAMPAL RGL CELLS THROUGH SINGLE-CELL TRANSCRIPTOMICS

This question has been only directly addressed by the Linnarsson lab (Hochgerner et al., 2018). They performed a large-scale single-cell RNA-seq analysis of DG cell types throughout development and into adulthood, including RGCs and adult RGLs, but also neuroblasts, GN, astrocytes and other cell types (none of the latter analyzed in Shin et al., 2015). The power of the study relies on the vast number of cells analyzed (>24,185 cells), the unbiased sampling, the inclusion of perinatal, juvenile, and adult DG tissue, and the use of two complementary platforms to minimize batch effects. The data strongly support that adult

neurogenesis from development to adulthood, proceeds through a set of defined cellular states and transitions. Thus, while IPCs, neuroblasts and immature neurons express sequentially a conserved neurogenic TF cascade (Sugiyama et al., 2013) and are nearly indistinguishable across time, RGCs/RGLs, by contrast, display great shifts in their molecular profile as development proceeds (see below). Moreover, RGLs do not express cell-cycle genes such as *Top2a* or *Cdk1*, basically because they mostly identify the adult quiescent RGLs. The small population of actively proliferating RGLs probably cluster with IPCs, but can be distinguished because dividing RGLs have not entered the neurogenic program, while quiescent RGLs appear as a defined population disconnected from the main neurogenic trajectory that starts from the IPCs (yet this could be an artifact of visualizing the cells using t-distributed stochastic neighbor embedding (t-SNE) plot, see Kalamakis et al., 2019). RGLs are clearly distinct from astrocytes, expressing for instance TFs such as *Sox4* and *Ascl1*, or *Thrsp* (SPOT14), a key lipid metabolism regulator that when knocked down leads to a shift in quiescent RGLs toward more proliferative progenitors (Knobloch et al., 2013). According to the Linnarsson study, the key event in DG neurogenesis would be the cellular decisions occurring at the time of RGL activation, after which cells proceed to the IPC state, upregulating neurogenic TFs and engaging irreversibly in a conserved neuronal fate program, at least under non-pathological conditions.

But perhaps the most interesting finding of the Linnarsson lab is that related to the developmental origin of the adult RGL population (Hochgerner et al., 2018). The comparison of cells across time, from E16.5 to adulthood (P132) allowed them to suggest that the transcriptome of RGCs is profoundly affected as they transition to RGLs. Perinatal RGCs (E16.5, P0, and P5) are distinguishable from juvenile and adult RGLs, in that they express *Vim*, display higher expression of *Sox4* and *Sox11* and lower of *Notch2* and *Padi2*. Moreover, in relation to cell cycle changes, embryonic RGCs show comparatively higher levels of *Cdk4* than juvenile RGL, whereas RGLs express higher levels of *Cdk1* than RGCs. It remains to be analyzed if those changes in Cyclin-dependent kinase could be related to the temporal differences in Cyclins that we discussed above. Thus, they find that although postnatal and adult neurogenesis in the hippocampus is fundamentally similar, there is an early postnatal transformation of RG from embryonic progenitors (RGCs) to adult quiescent stem cells (qRGLs, **Figure 2**).

In this same line, the Song lab compared, by RNAseq, pools of mixed neural progenitors from the Hopx-creERT mouse hippocampus at different stages (E15.5 and P3) and from the adult DG at P45. However, a caveat of the study is that it relies on a gene, Hopx, that is dynamically expressed at early stages in progenitors for both the DG and CA and at adult stages in quiescent RGLs but is absent from adult IPCs. They identified a shared signature of 1,306 genes among all Hopx-creERT labeled progenitors, supporting their developmental relationship. GO analysis revealed consistent changes over time in several gene sets, an observation interpreted by the authors as a gradual and continuous transformation of progenitor cells over the course of DG development. Nevertheless, as in the Linnarsson's model, the most abrupt progenitor DG transition at the molecular level occurs postnatally. Marked gene expression differences are detected between the early postnatal and adult progenitors, including for instance the downregulation of the cell cycle genes encoding CyclinD1 and D2 and the upregulation of the cell cycle inhibitor p21. However, these data could reflect just the difference between the pool of embryonic Hopx-creERT neural precursor (the majority of which will be cycling) with respect to the exclusively quiescent RGL population labeled in the adult Hopx-creERT line.

In summary, all these transcriptomic data are in accordance with previous histological observations describing the temporal heterogeneity of RGCs and RGLs in the DG at the level of marker expression, and already showing that, structurally, the "adult" configuration of SGZ niche gets established between the first and second postnatal weeks, before individuals reach "adulthood" (Nicola et al., 2015).

Linnarsson and co-workers also described a fast maturation of GNs and mossy cells around the third postnatal week. It has been previously suggested that the establishment of the commissural fiber tract of the DG around P15 (Fricke and Cowan, 1977; Ribak et al., 1985) might have an impact on DG development (Nicola et al., 2015). RGL quiescence is also regulated by the input from contra- and ipsi-lateral mossy cells and by long-range GABAergic projections from the medial septum (Bao et al., 2017; Yeh et al., 2018). For that reason, it is conceivable that postnatal changes in connectivity contribute to the establishment of the quiescent RGL reservoir and the formation of the adult DG niche, as previously proposed (Nicola et al., 2015). The single-cell RNAseq resource generated by the Linnarsson lab may allow to further explore at the molecular level which genes are key players in the postnatal transition from RGCs to RGLs[3].

## CONCLUSION

We have witnessed a breakthrough in transcriptomic and proteomic analyses of the quiescence state of adult RGLs and in the signals and TFs involved in the transition from quiescence to activation in the adult brain. Moreover, recent data have uncovered a common origin from Hopx-expressing progenitors for embryonic RGCs, postnatal and adult RGLs in the hippocampal niche, although additional embryonic origins cannot be ruled out. Further mechanistic studies are indeed still warranted to define how exactly are quiescent RGLs specified during the early postnatal period and what is the precise role, if any, exerted by the players we discussed above in the process. Another important question is how the differences in gene expression found between embryonic RGCs and adult RGLs determine their cycling, lineage and cell-to-cell communication behavior. With all the available datasets, these fundamental aims are within reach. Overall, the understanding of the generation of new neurons in the adult brain through the control of the establishment and regulation of the quiescent RGL reservoir is of paramount importance in order to harness quiescent RGLs into neurogenic production in pathological and aging situations.

[3]https://linnarssonlab.org/dentate/

## DATA AVAILABILITY

No datasets were generated or analyzed for this study.

## AUTHOR CONTRIBUTIONS

AM and HM conceived the structure and content and wrote the manuscript. AM produced the figures.

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# Rewiring the Regenerated Zebrafish Retina: Reemergence of Bipolar Neurons and Cone-Bipolar Circuitry Following an Inner Retinal Lesion

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We previously reported strikingly normal morphologies and functional connectivities of regenerated retinal bipolar neurons (BPs) in zebrafish retinas sampled 60 days after a ouabain-mediated lesion of inner retinal neurons (60 DPI) (McGinn et al., 2018). Here we report early steps in the birth of BPs and formation of their dendritic trees and axonal arbors during regeneration. Adult zebrafish were subjected to ouabain-mediated lesion that destroys inner retinal neurons but spares photoreceptors and Müller glia, and were sampled at 13, 17, and 21 DPI, a timeframe over which plexiform layers reemerge. We show that this timeframe corresponds to reemergence of two populations of BPs (PKCα+ and *nyx::mYFP*+). Sequential BrdU, EdU incorporation reveals that similar fractions of PKCα+ BPs and HuC/D+ amacrine/ganglion cells are regenerated concurrently, suggesting that the sequence of neuronal production during retinal regeneration does not strictly match that observed during embryonic development. Further, accumulation of regenerated BPs appears protracted, at least through 21 DPI. The existence of isolated, *nyx::mYFP*+ BPs allowed examination of cytological detail through confocal microscopy, image tracing, morphometric analyses, identification of cone synaptic contacts, and rendering/visualization. Apically-projecting neurites (=dendrites) of regenerated BPs sampled at 13, 17, and 21 DPI are either truncated, or display smaller dendritic trees when compared to controls. In cases where BP dendrites reach the outer plexiform layer (OPL), numbers of dendritic tips are similar to those of controls at all sampling times. Further, by 13–17 DPI, BPs with dendritic tips reaching the outer nuclear layer (ONL) show patterns of photoreceptor connections that are statistically indistinguishable from controls, while those sampled at 21 DPI slightly favor contacts with double cone synaptic terminals over those of blue-sensitive cones. These findings suggest that once regenerated BP dendrites reach the OPL, normal photoreceptor connectomes are established, albeit with some plasticity. Through 17 DPI, some basally-projecting neurites (=axons) of regenerated *nyx::mYFP*+ BPs traverse

long distances, branch into inappropriate layers, or appear to abruptly terminate. These findings suggest that, after a tissue-disrupting lesion, regeneration of inner retinal neurons is a dynamic process that includes ongoing genesis of new neurons and changes in BP morphology.

**Keywords: retina, regeneration, zebrafish, circuitry, connectome, photoreceptor, retinal bipolar cell, birthdating**

## INTRODUCTION

A major challenge in the treatment of human neurodegenerative diseases and trauma affecting the central nervous system is that mammals do not regenerate neurons lost to such conditions, but instead launch a gliotic response that results in tissue scarring (Pekny et al., 2014; Okada et al., 2018), and nervous tissue function is not restored. In contrast, teleost fish respond to neuronal injury in a manner that not only replaces the lost neurons, but also restores tissue function (Mensinger and Powers, 1999, 2007; Madelaine and Mourrain, 2017; Ma et al., 2017; McGinn et al., 2018). The eye's neural retina, a component of the central nervous system, provides an accessible system for studying these differences in mammals vs. fish (Wan and Goldman, 2016), for identifying factors and conditions that favor regeneration, and for potentially delivering future clinical applications in the treatment of retinal diseases and injuries that would otherwise result in loss of vision.

Vertebrate retinas are highly conserved, with light-sensing rod and cone photoreceptors organized in an outer nuclear layer (ONL), and which transmit information to bipolar cells in the inner nuclear layer (INL), which in turn transmit signals to the output neurons of the retina, the retinal ganglion cells, located in the ganglion cell layer (GCL). Horizontal cells (also in the INL) and amacrine cells (in the INL and GCL) modulate this information flow. The zebrafish animal model has been particularly enlightening toward our understanding of retinal regeneration (Stenkamp, 2015; Wan and Goldman, 2016; Ail and Perron, 2017). In zebrafish, injuries that target all neurons (Sherpa et al., 2008), only photoreceptors (Kassen et al., 2007; Nagashima et al., 2013; Ranski et al., 2018), only inner retinal neurons (INL and GCL; Fimbel et al., 2007; Sherpa et al., 2014), are regionally specific (Fausett and Goldman, 2006), or target selected specific cell types (Fraser et al., 2013; D'Orazi et al., 2016; White et al., 2017), all result in a regenerative response that replaces these missing neurons. Studies making use of these injuries have revealed that the major glial cell type of the retina, the Müller glia, is the stem cell source of regenerated retinal neurons in the zebrafish (Fausett and Goldman, 2006; Bernardos et al., 2007; Nagashima et al., 2013). Several regulatory factors have been identified that promote or are necessary for Müller glia to re-enter the cell cycle and generate neural progenitors (Fausett et al., 2008; Qin et al., 2011; Ramachandran et al., 2011a,b; Lenkowski et al., 2013; Nagashima et al., 2013; Nelson et al., 2013), which then proliferate and produce the regenerated retinal neurons (Nagashima et al., 2013; Gorsuch and Hyde, 2014; Powell et al., 2016). Some of this knowledge has already been applied in strategies for coaxing the Müller glia of mouse retina to generate retinal neurons (Ahmad et al., 2011; Hyde and Reh, 2014; Jorstad et al., 2017; Yao et al., 2018).

A widely anticipated obstacle for the future application of endogenous regeneration strategies, as well as for the use of transplantation approaches to treat retinal disorders, is ensuring that the replacement neurons establish synaptic communication with the appropriate pre- and/or post-synaptic partners (Angueyra and Kindt, 2018). Indeed, teleost fish not only regenerate lost neurons, these regenerated neurons restore visual functions indicating successful rewiring (Mensinger and Powers, 1999, 2007; Sherpa et al., 2008, 2014; McGinn et al., 2018). Further, this restoration of visual function is possible even when pre- and post-synaptic partners are simultaneously destroyed (Mensinger and Powers, 1999, 2007; Sherpa et al., 2008, 2014; McGinn et al., 2018). Recently, Yao et al. (2018) showed that such successful rewiring is possible in a mouse model for rod photoreceptor degeneration, in that new rods regenerated by genetically altered Müller glia can transmit signals that are propagated through the visual system. However, this promising and exciting finding does not negate the obstacle of rewiring in mammalian retinal disorders, because in these disorders the retinal environment is altered by an inflammatory response (Arroba et al., 2018), neuronal remodeling takes place (Genove et al., 2014), gliotic scarring may block access to synaptic partners (Genove et al., 2014), and multiple neuronal cell types may be destroyed, damaged, or their function altered (Narayan et al., 2016).

The zebrafish retina again appears to represent a system that demonstrates the ability to overcome these obstacles. The damaged zebrafish retina also displays features that could be considered obstacles similar to those in damaged mammalian retina. The damaged zebrafish retina experiences an infiltration of microglia and macrophages (Mitchell et al., 2018), retains the potential for a gliotic response (Morris et al., 2008; Thomas et al., 2016), undergoes retinal remodeling (Saade et al., 2013), and the regenerating/regenerated zebrafish retina shows abnormal patterns of lamination of the retinal layers (Sherpa et al., 2014), and of the two-dimensional organization of neuronal classes within each layer (Vihtelic and Hyde, 2000; Stenkamp and Cameron, 2002). Regenerating zebrafish retinas also produce supernumerary neurons, and produce other neuronal types in addition to those lost to the initial injury (Sherpa et al., 2008, 2014; Powell et al., 2016). Despite these apparent obstacles, however, regenerated zebrafish retina is functional, as measured by behavioral assays for vision (Sherpa et al., 2008, 2014), and a physiological assay, the electroretinogram (ERG) (McGinn et al., 2018), suggesting at least some degree of accurate rewiring. Consistent with this idea, a recent report by D'Orazi

et al. (2016) noted minimal errors in connectivity patterns of regenerated retinal bipolar (BP) neurons after cell-selective lesion in larval zebrafish. Further, we recently reported that regenerated BPs of adult zebrafish also establish essentially normal cone connections, dendritic and axonal arbor morphologies, and axon terminal stratification patterns following a tissue-disrupting lesion that destroys inner retinal neurons (McGinn et al., 2018). These studies strongly suggest that the process of rewiring regenerated retinal neurons is successful in the zebrafish, regardless of the environmental conditions created by damage.

Do newly-regenerated BPs immediately display normal morphologies and connectivities, or are these features differentiated later? When do regenerated BPs emerge relative to their synaptic partners, when partners are also destroyed and regenerated? Does this match the sequence of synaptic partner generation seen during embryonic development? When do regenerated BPs show mature morphologies and accurate connectivities? This information will be of immense utility for therapeutic applications in which transplanted or regenerated retinal neurons must accomplish the same in an injured human retina (Angueyra and Kindt, 2018). As the next step toward answering these questions and achieving this goal, in the present study we describe the early stages of the BP regenerative process following a tissue-disrupting injury that selectively destroys inner retinal neurons. We find that regenerated BPs appear from 13 to 21 days post-injury (DPI), coincident with the reemerging plexiform layers (Fimbel et al., 2007), and birthdating indicates that, in contrast to the developmental sequence, these BPs are regenerated concurrently with their postsynaptic partners (amacrine and ganglion cells). The dendritic trees and axonal arbors of some newly regenerated BPs remain immature or highly abnormal at 13 and 17 DPI, but all sampled regenerated BPs show normal dendritic tree morphologies by 21 DPI. The generation and morphological differentiation of regenerated BPs appears distinctive in many ways in comparison with these processes during embryonic development, underscoring the need to further investigate this process.

# MATERIALS AND METHODS

## Animals and Retinal Lesioning

### Animals

Zebrafish (both sexes) used in this study were either of a wild-type strain originally obtained from Scientific Hatcheries (SciH, now Aquatica Tropicals), or were transgenic for one or both of the following transgenes: *nyx::mYFP, sws2:mCherry*. The *nyx::mYFP* transgenics are a line in which the *nyctalopin* (*nyx*) promoter drives Gal4, likely co-integrated with a construct in which the UAS enhancer drives expression of membrane-associated yellow fluorescence protein (mYFP) (Schroeter et al., 2006). In the adult *nyx::mYFP* zebrafish, a subpopulation of ON and Mixed (ON/OFF) retinal bipolar neurons (BPs) expresses YFP (McGinn et al., 2018). The *sws2:mCherry* transgene results in mCherry expression in blue-sensitive (SWS1) cone photoreceptors (Sifuentes et al., 2016). Zebrafish were maintained according to Westerfield (Westerfield, 2007) in monitored, recirculating system water, on a 14:10 h light:dark cycle. All procedures using animals were approved by the University of Idaho Institutional Animal Care and Use Committee.

### Retinal Lesioning

Retinas of adult fish were chemically lesioned as previously described (Fimbel et al., 2007; Nagashima et al., 2013; Sherpa et al., 2014; McGinn et al., 2018; Mitchell et al., 2018, 2019). Briefly, corneas of anesthetized fish were perforated with a sapphire blade to introduce a Hamilton syringe containing 40–70 μM ouabain (inhibitor of the $Na^+/K^+$ ATPase) in sterile saline. The injected volume (∼0.5 μL) resulted in an estimated intraocular concentration of 2 μM. Uninjected contralateral eyes were used as controls. This lesioning strategy has been demonstrated to result in death of inner retinal neurons, but spares photoreceptors, Müller glia, and microglia (Fimbel et al., 2007; Sherpa et al., 2014; Mitchell et al., 2018). Müller glia respond to this damage by re-entering the cell cycle and generating progenitors that in turn proliferate and generate the neuronal cell types lost to the lesion (Fimbel et al., 2007; Nagashima et al., 2013), ultimately restoring function (Sherpa et al., 2014; McGinn et al., 2018). The loss of BPs, survival of cones, and regeneration of BPs was monitored by observation of retinas of live, anesthetized fish using a Nikon SMZ 1500 epifluorescence stereomicroscope.

## Comparative Cell Birthdating During Retinal Regeneration

Sequential systemic exposure to bromodeoxyuridine (BrdU) and ethynyldeoxyuridine (EdU) was carried out according to Nagashima et al. (2013). For these experiments, wild-type zebrafish ($n = 3$ per condition) were transferred to 250 mL beakers containing 1.0 mM BrdU, and after a defined exposure period, were transferred to 125 μM EdU. The exposure protocols used in this study were: 4–6 DPI BrdU followed by 6–8 DPI EdU, and 6–9 DPI BrdU followed by 9–13 DPI EdU. Solutions were refreshed once daily during these exposures, and fish were fed after each refreshing.

## Tissue Processing and Immunofluorescence

Procedures were similar to those of McGinn et al. (2018). Briefly, dark-adapted fish were humanely sacrificed with MS-222, and eyes enucleated with forceps. For retinal cryosections, eyes were fixed with 4% paraformaldehyde containing 5% sucrose in phosphate buffer (pH = 7.4) for 1 h. Eyes were cryoprotected, embedded, and frozen in a 1:2 combination of Tissue-Tek OCT (Sakura Finetek):phosphate-buffered 20% sucrose, and then cryosectioned at 5 μm on a Leica CM3050 cryostat. For whole retinal flat mounts, corneas were perforated with dissecting scissors to gain access for removal of the lens. The sclera and RPE were removed with forceps and the freed retinas were rinsed in cold phosphate-buffered (pH=7.4) saline (PBS) or HEPES. Four radial incisions helped to flatten each retina, and they were then fixed in phosphate-buffered 4% paraformaldehyde containing 5% sucrose for 1 h. Retinas were then washed three times in PBS for 30 min each.

For immunofluorescence detection of antigens, 5 μm sectioned retinas were rinsed with PBS with 0.5% triton (PBST) and then blocked for 1 h with 20% normal goat serum and 0.1% sodium azide, diluted in PBST, at room temperature. Primary antibodies were diluted in antibody dilution buffer (PBST, 1% normal goat serum, and 0.1% sodium azide), and applied to sections, which were then incubated overnight at 4°C. Sections were washed with PBS three times, for 20 min each, incubated with secondary antibodies diluted in antibody dilution buffer (PBST with 1% normal goat serum and 0.1% sodium azide) and 4.25 μM DAPI overnight at 4°C, washed with PBST for 30 min and mounted in Vectashield (Vector Laboratories) or Fluoromount-G (SouthernBiotech). For detection of BrdU and EdU, an antigen retrieval step was incorporated (1:1 solution of 4N HCl:PBST) prior to the blocking step. Whole retinas were stained with primary antibodies in antibody dilution buffer for 1–2 weeks at 4°C with constant gentle agitation, washed with PBS three times (20 min each), and then incubated with secondary antibodies diluted in antibody dilution buffer and 4.28 μM DAPI at 4°C for another week. After staining, retinas were washed with PBS three times for 20 min each.

Primary antibodies that were used in this study, and their sources and dilution are as follows. ZPR1 is a mouse monoclonal that labels cone arrestin3a, staining both the red- and green-wavelength sensitive members of the double cone pair (Renninger et al., 2011) (ZIRC; 1:200). Anti-protein kinase Cα (PKCα) is a rabbit polyclonal antibody originally produced to target the C-terminus of human PKCα, and labels a subpopulation of BP neurons (Suzuki and Kaneko, 1990) (Santa Cruz Biotechnology; SC-10800 1:200). Anti-HuC/D is a rabbit polyclonal antibody that stains inner retinal neurons, primarily ganglion cells and amacrine cells (abcam, Eugene, OR; 1:100). Anti-BrdU is a mouse monoclonal antibody (Invitrogen, Carlsbad, CA; 1:200). EdU was detected using the "Click-It" reaction kit (Invitrogen). Secondary antibodies (Jackson ImmunoResearch, West Grove, PA, all 1:200) used in this study were donkey anti-mouse AlexaFluor 647, donkey anti-rabbit Cy3, donkey anti-rabbit TRITC, and donkey anti-rabbit FITC.

## Imaging and Quantification of Neurons in Cryosections

Sections were imaged using an Andor Zyla 5.5 sCMOS camera connected to a Nikon Ti inverted microscope with a Yokogawa spinning disk using a 20x 0.75NA air objective, a 40x 1.3NA oil immersion, a 60x 1.40NA oil, or a 100x 1.45NA oil immersion objective. BP neurons were counted in PKCα-stained retinal cryosections of *nyx::mYFP* fish (n=3 per condition, with 9 contralateral controls), by identifying PKCα+, DAPI+ cell bodies, *nyx::mYFP*+, and DAPI+ cell bodies, within 5 μm-thick radial sections imaged at 20x. To quantify BPs in cryosections, only cell bodies showing both DAPI and a BP-specific marker were counted. To quantify inner retinal neurons following cell birthdating by BRDU/EdU immersion (described above), BP neurons (PKCα+) and HuC/D+ neurons were counted in retinal cryosections following staining for PKCα, BrdU, and EdU, or for HuC/D, BrdU, and EdU. BPs and HuC/D+ neurons that incorporated BrdU or EdU were counted by identifying cell-specific marker+, nucleotide+ cell bodies. For both analyses, sections were sampled over the regions between the larval remnant (Allison et al., 2010) and the extreme periphery. We counted every 5th section to avoid double-counting. We note that, if the nuclei of newly regenerated BPs were smaller than those of control BPs, this counting method may under-sample neurons in the regenerated retinas (Coggeshall and Lekan, 1996). However, to our knowledge (and see **Figure 1**), nuclei of regenerated retinal neurons do not appear substantially different in size in comparison with those of their control counterparts.

## Imaging and Morphometric Analyses of Bipolar Neurons in Whole Retinas

Whole retinas were mounted in Fluoromount-G or Vectashield Hardset, with the GCL facing the coverslip, and using electrical tape as a spacer between the slide and coverslip. Retinas were imaged with the Andor/Nikon system described above using a 60x 1.2NA water immersion objective, with Immersol W 2010 (Zeiss). Images were collected from multiple locations throughout the retina when possible, but for some retinas only a very few *nyx::mYFP*+ neurons were found. Multiple image stacks were collected using 0.3 μm z-steps through the entire thickness of the retina. As in McGinn et al. (2018), each stack was 114.62 μm wide by 164.88 μm high, or 1028x1522 pixels, resulting in a scale of 9.23 pixels/μm.

Image stacks were analyzed in Fiji software (ver 1.51d) (Fiji, RRID:SCR_002285) (Schindelin et al., 2012). Selected, *nyx::mYFP*+ cells were traced with the Simple Neurite Tracer (SNT) plugin (Longair et al., 2011). Each BP was visually assessed for meeting the following set of criteria defining a "stereotypical appearance" of a BP: Cell body within the INL, having a single apical process with multiple branches within the outer plexiform layer (OPL), and having a single basal process with terminals in the IPL, and in general resembling one of the type specimens identified by Li et al. (2012). Only cells showing a clear soma, dendrite, and axon (or other projections) within the field of view were traced (numbers of traced neurons are provided in **Supplementary Table 1**).

Traced neurons were filled using the fill option in SNT. Because SNT was not designed to show larger 3D objects such as a cell body, only portions of cell bodies were traced and filled, while the axons and terminals, and dendritic trees were traced and filled in their entirety. The axon, soma, and dendrite of each BP were each converted in an image stack and imported into ImageJ's 3D viewer as surfaces. Each neuron was saved as a separate file in the OBJ format. OBJ files were imported into 3ds Max 2016, placed together by their respective time point and then rendered. Numbers of neurons subjected to surface rendering are provided in **Supplementary Table 1**.

Sholl analysis was performed on traced BPs to determine the characteristics of individual dendritic trees and axonal branching patterns. For BP dendrites, Sholl analysis was done for traced neurons that displayed a clear, apically-projecting primary dendrite, showed branching, and had dendritic spreads sufficient for the analysis to return meaningful values (e.g., dendrites with

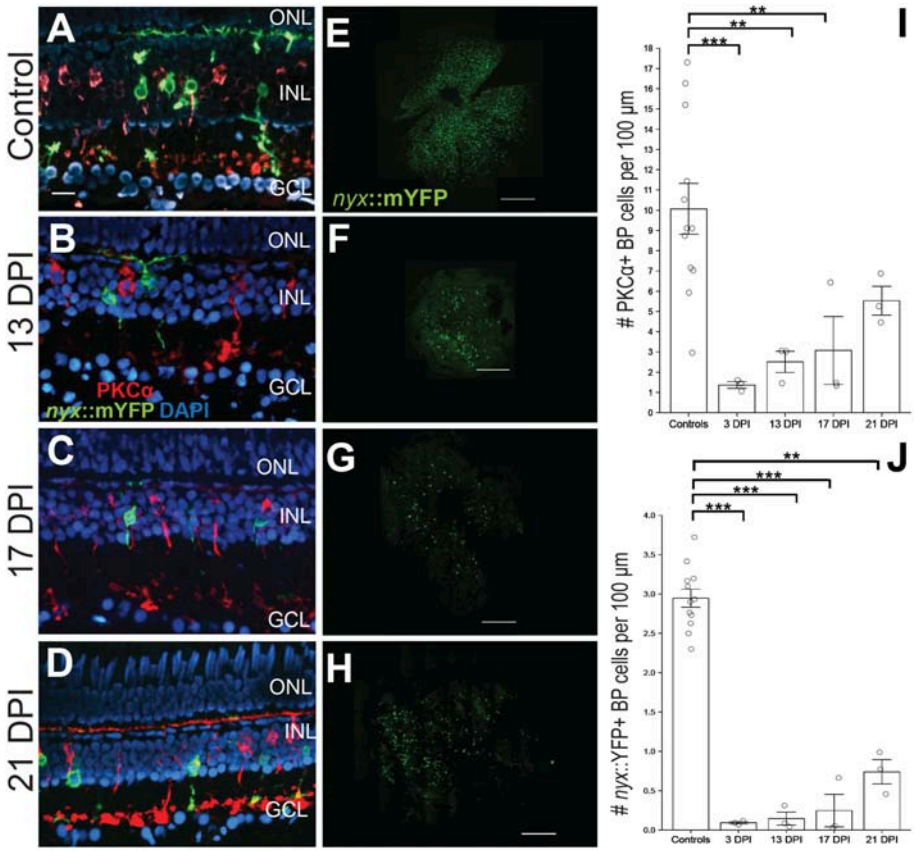


**FIGURE 1 |** Emergence of identifiable retinal bipolar (BP) neurons following a chemical lesion selective to the inner retina. **(A)** PKCα+ and *nyx::mYFP*+ BP cell bodies occupy the inner nuclear layer (INL), with dendritic trees in the OPL and axon terminals in the inner plexiform layer of control retinas. **(B–D)** New PKCα+ and *nyx::mYFP*+ BPs reappear over the time frame of 13 days post-injury (DPI) **(B)**, 17 DPI **(C)**, and 21 DPI **(D)**, and have recognizable apical processes (dendrites) and basal processes (axons). **(E–H)** Retinal flat mounts of control **(E)**, and regenerated, *nyx::mYFP* retinas at 13 DPI **(F)**, 17 DPI **(G)**, and 21 DPI **(H)** showing distributions of regenerated *nyx::mYFP*+ BP neurons ($n = 11$) controls, six 13 DPIs, five 17 DPIs, and five 21 DPIs prepared as whole mounts (**Supplementary Table 1**) and visually inspected for overall distribution of *nyx::mYFP* BPs. Three controls, two 13 DPIs, three 17 DPIs, and three 21 DPIs were imaged as in **(E-H)**. High resolution images of these retinas are provided in **Supplementary Figure 1**. **(I)** Numbers of PKCα+ BPs at 3 (McGinn et al., 2018), 13, and 17 DPI were significantly different from controls (**$p < 0.01$; ***$p < 0.001$), while there was no statistically significant difference between controls and 21 DPI ($p = 0.246$), or for any other *post-hoc* pairwise analysis (Kruskall-Wallis, Conover's *post-hoc* analysis; graph shows means ± SEM). **(J)** Numbers of *nyx::mYFP*+ BPs remained significantly reduced at 3, 13, 17, and 21 DPI (**$p < 0.01$; ***$p < 0.001$), but there was no statistically significant difference for any other *post-hoc* pairwise analysis (Kruskall-Wallis, Conover's *post-hoc* analysis; graph shows means ± SEM). Scale bar in **A** (applies to **A–D**) = 20 µm. Scale bars in **E-H** each = 200 µm.

only one very small branch would be excluded). For BP axons, Sholl analysis was done for traced neurons that displayed a clear, basally-projecting axon that reached the IPL, showed branching within the IPL, and could be traced for its entirety. The Sholl Fiji plugin utilized several different variants of the original Sholl Analysis (Sholl, 1953) in order to test for differences in neuronal architecture as the retina regenerates. A series of concentric shells (spheres in this case) were created around a centerpoint. For dendrites used in this study the point at which the primary dendrite branches was considered the center. For axons the point where the primary axon had the largest number of branches was considered the centerpoint. The software then counted how many branches crossed or intersected at each particular enclosing shell. Numbers of neurons subjected to Sholl analysis of dendritic trees and axonal arbors are provided in **Supplementary Table 1**.

Cone-*nyx::mYFP*+ BP contacts were identified using the strategy described by McGinn et al. (2018). In brief, raw.ND2 images were imported into Fiji, and YFP+ dendrites were traced with SNT, and filled in using the "fill" option. Independent stacks were generated for each dendrite, and re-merged with ZPR1+ fluorescence (double cones) and *sws2:mCherry* fluorescence (blue-sensitive cones) as separate channels. We next generated a partial Z-projection containing only cone synaptic pedicles and the untraced dendritic tree of the *nyx::mYFP*+ BP under analysis. BPs with highly truncated dendritic trees were excluded from this analysis. ZPR1+ vs. mCherry+ cone synaptic pedicles were outlined as separate colors using the region of interest tool. The total number of dendritic tips for each analyzed BP was counted, and each dendritic tip showing contact with an outlined cone pedicle was recorded. Numbers of primary apical (dendritic) and basal (axonal) processes were counted for each traced, *nyx::mYFP*+ BP.

Each BP was visually assessed for meeting the following set of criteria defining a "stereotypical appearance" of a BP: Cell

body within the INL, having a single apical process with multiple branches within the OPL, and having a single basal process with terminals in the IPL, and in general resembling one of the type specimens identified by Li et al. (2012).

Some selected neurons were traced using Filament Tracer, in Imaris version 8.2.0 (Bitplane), an alternative tracing strategy that allows visualization of traced neurons with a background grid and from multiple views. Neurons selected for this analysis were those that were physically isolated from other *nyx::mYFP*+ neurons and had morphologies that could not be as easily appreciated using the viewpoints available following use of SNT. Multiple views were collected in 3D View in order to demonstrate how the neurons were positioned within the retinal region represented by the confocal stack. Images showing the top and side views were collected in the Slice View in order to show partially projected top down and side views of neurons. In order to visualize the traced neurons in this module, a new color channel was created by selecting the "create channel from filament" option from the traced axon, and pseudocolored red. Another color channel was created for the traced dendritic tree and pseudocolored green.

## Statistics

Numbers of PKCα+ and *nyx::mYFP*+ cells/100 μm of retinal section were compared across samples, including the data from 3 DPI samples reported in McGinn et al. (2018), using a Kruskal-Wallis test, followed by a Conover's test for multiple comparisons of independent samples. For statistical analysis of morphometric data of individual BP neurons, data were imported into R Studio (ver 0.99.903) (R Project for Statistical Computing) using R (ver 3.3.1) for statistical analysis. ANOVAs and Kruskal-Wallis tests were used for parametric and most non-parametric data, respectively. Since none of the parametric data showed statistical significance ($p < 0.05$) as measured by a one-way ANOVA, no *post-hoc* tests were conducted. For any Kruskal-Wallis test outcome that had a $p$-value $< 0.05$, a *post-hoc* test was done using a Wilcoxon–Mann–Whitney test with a false discovery rate $p$-value adjustment. A generalized linear model with a Poisson distribution was applied for analysis of connectivity patterns, and any comparisons having $p$-values $< 0.05$ were considered significant. Sample sizes were $n = 3$ for quantifications in cryosections (with 12 contralateral controls for PKCα+ and *nyx::mYFP*+ BP counting); **Supplementary Table 1** contains the numbers of neurons subjected to morphometric analyses.

# RESULTS

## Emergence of Regenerated BPs Following Ouabain Lesion

We previously documented strikingly normal morphologies of regenerated BPs in retinas sampled at 60 days after a ouabain-mediated lesion of inner retinal neurons (McGinn et al., 2018). This lesioning strategy has been verified to destroy neurons of the inner retina by 3 days post-injury (DPI), sparing Müller glia, photoreceptors, and very few horizontal cells (Fimbel et al., 2007; Nagashima et al., 2013; Sherpa et al., 2014; McGinn et al., 2018; Mitchell et al., 2018). At 60-80 DPI after this lesion, functional recovery of vision is evident by behavioral and physiological analyses (Sherpa et al., 2014; McGinn et al., 2018). To gain insights into the process of the formation of BP dendritic trees and axonal branching patterns during regeneration, zebrafish subjected to such a lesion were sampled at 13, 17, and 21 DPI. This timeframe was selected in part because neurons of the layers that were destroyed (INL and GCL) are regenerated by this time, and plexiform layers re-emerge (Fimbel et al., 2007; Mitchell et al., 2018). Furthermore, microglia remain active over this timeframe (Mitchell et al., 2018), and their involvement in synaptic remodeling has been documented in other contexts (Schafer et al., 2012). We therefore reasoned that 13–17 DPI may represent a period of BP reemergence as well as synaptogenesis and refinement of neuronal processes during retinal regeneration. The loss of BPs and their regeneration was confirmed in lesioned, *nyx::mYFP*; *sws2:mCherry* transgenic zebrafish by observation of eyes of live, anesthetized fish ($n = 10$) at 3, 5, 10, 11, 12, and 13 DPI, by epifluorescence stereomicroscopy. These observations confirmed loss of mYFP+ signal (corresponding to BPs), and the continued presence of distinctive rows of mCherry+ cells (blue-sensitive, *sws2*-expressing cones) within retinas at 3–5 DPI (data not shown). Uninjected, contralateral eyes retained expression of both transgenic reporters. In lesioned fish, new YFP+ fluorescence was again visible for the first time at 13 DPI, and so we focused our detailed analyses beginning at this timepoint.

We first documented the recovery of *nyx::mYFP*+ BPs [a population including ON BPs and mixed ON/OFF BPs (Schroeter et al., 2006; McGinn et al., 2018)], and PKCα+ BPs [ON BPs (Suzuki and Kaneko, 1990; Schroeter et al., 2006; McGinn et al., 2018)] in regenerated retinas over 13–21 DPI (**Figure 1**). We previously demonstrated that these are separate populations of BPs in adult zebrafish retinas, with minimal overlap (McGinn et al., 2018) (**Figure 1A**). Consistent with our observations in live, anesthetized fish, small numbers of PKCα+ and of *nyx::mYFP*+ BPS were observed in retinal cryosections of fish sampled at 13 DPI (**Figure 1B**) and 17 DPI (**Figure 1C**), and these BP cell types appeared more abundant in cryosections of fish sampled at 21 DPI (**Figure 1D**). The restoration of these cell types was quantified in the cryosections and numbers were compared among groups, including previously-reported PCKα+ BP numbers at 3 DPI (McGinn et al., 2018), when considerable damage has occurred, and with contralateral controls. PKCα+ BPs were present, but were reduced in numbers at 13 and 17 DPI in comparison with controls, while there was no statistical significance regarding a difference between PKCα+ BP numbers at 21 DPI in comparison with controls (**Figure 1I**; Kruskal-Wallis with Conover's *post-hoc*), suggesting restoration of these BP neurons at this timepoint. However, *post-hoc* analyses of PKCα+ BP numbers for all other pairwise comparisons, including in comparison to numbers at 3 DPI, did not indicate statistically significant differences (**Figure 1I**). We note that the small sample number at each timepoint likely limits conclusions from this analysis. The *nyx::mYFP*+ BPs also appear over this time frame, in a similar temporal pattern, but at lower densities compared to PKCα+ BPs. The

*nyx::mYFP*+ BPs remained reduced in numbers at 13, 17, and 21 DPI in comparison with controls (**Figure 1J**; Kruskal-Wallis with Conover's *post-hoc*). Similar to the findings for PKCα+ BPs, *post-hoc* analyses of all other pairwise comparisons, including comparison to numbers at 3 DPI, did not indicate statistically significant differences of *nyx::mYFP*+ BPs (**Figure 1J**). Again, small sample sizes at all timepoints likely limit conclusions from this analysis. Findings for *nyx::mYFP* BPs should be interpreted with even further caution, because the low numbers of newly regenerated *nyx::mYFP*+ BPs may be in part related to transgene silencing (Goll et al., 2009) or delay in expression of the fluorescent reporter. The *nyx::mYFP*+ retinas were also sampled as whole mounts at 13, 17, and 21 DPI by confocal microscopy (**Figures 1E–H**; **Supplementary Figure 1**). BPs appeared in central and peripheral retina, and were visible in all quadrants of flat mounted retinas, suggesting no biases favoring a particular location of regenerated *nyx::mYFP* BPs (**Figures 1E–H**).

## Relative Birthdating of Regenerated BPs and Other Retinal Neurons

The quantification of regenerated BPs in cryosections, together with our previous studies showing that some regenerated BPs were labeled by BrdU exposures at either 4–7 or 6–12 DPI (McGinn et al., 2018), suggested that the regeneration of BPs is a protracted and likely asynchronous process, distinct from the rapid wave of BP generation that takes place during zebrafish embryonic development (Hu and Easter, 1999). Alternatively, or in addition, regenerating BPs may exhibit delayed and/or asynchronous expression of the markers used to identify them. To distinguish among these possibilities, we used two different, sequential BrdU-EdU exposures, and quantified PKCα+ BPs expressing either or both nuclear markers upon tissue collection at 21 DPI. Early exposures (4–6 DPI BrdU, 6–8 DPI EdU) revealed very little incorporation and retention of the BrdU label (data not shown), suggesting onset of BP regeneration begins after 6 DPI. Later exposures (6–9 DPI BrdU, 9–13 DPI EdU; **Figure 2A**) revealed sufficient incorporation and retention of label for quantification (**Figures 2B,C**). At 21 DPI, an average of ∼20% of the PKCα+ BPs were BrdU+ (born 6–9 DPI), and another ∼20% were EdU+ (born 9–13 DPI) (**Figure 2D**), consistent with steady, protracted production of this cell population. The highly limited numbers of *nyx::mYFP*+ BPs (**Figure 1J**) in regenerated retinas at the sampled timepoints, along with challenges related to the experimental procedure (YFP fluorescence not reliably surviving the antigen retrieval process for BrdU staining), and interpretation (outcomes related to possible transgene silencing), precluded any parallel birthdating analysis of this BP population.

We next compared the PKCα BP birthdating results with those for regenerated retinal neurons that are HuC/D+. These neurons correspond to retinal ganglion cells and amacrine cells (Kay et al., 2001), the postsynaptic partners of BPs, which are also destroyed and subsequently regenerated following ouabain injection (Fimbel et al., 2007; Sherpa et al., 2014). In zebrafish embryonic retinal development, cells of the GCL are generated prior to those of the INL (Hu and Easter, 1999), yet whether generation of these inner retinal neurons (and specifically BPs vs. their post-synaptic partners) follows similar relative timing is not yet known for regeneration. At 21 DPI, HuC/D+ cells were detected that incorporated BrdU (**Figure 2E**) or EdU (**Figure 2F**), consistent with accumulation of HuC/D+ neurons from 7 to 21 DPI (Fimbel et al., 2007). Interestingly, an average of ∼20% of the HuC/D+ cells were BrdU+, and another ∼20% were EdU+ (**Figure 2G**), very similar to the findings for the PKCα+ BPs. The HuC/D+ neuronal population that arises between 6 and 13 DPI is therefore also regenerated steadily over this timeframe, and not likely in a temporal pattern that is earlier or later than the PKCα+ BP neuronal population. The sequence of cell birth during retinal regeneration through at least 13 DPI therefore does not appear to match that observed during embryonic retinal neurogenesis, and also further supports that regeneration of inner retinal neurons following this damage paradigm is a protracted process resulting in accumulation of regenerated neurons over time (Fimbel et al., 2007; Sherpa et al., 2014). Further, and of additional importance to the present study, regenerated postsynaptic partners of the BPs are born over the same timeframe and at a similar rate as the BPs themselves.

## Morphologies of Newly Regenerated *nyx::mYFP* BPs: Qualitative Findings

The sparse cellular densities of the regenerated *nyx::mYFP*+ BP neurons made them amenable to detailed morphometric analyses, because many were physically isolated from any surrounding *nyx::mYFP*+ BPs, allowing complete tracing of individual cell dendritic trees, somas, and axonal processes after imaging. Such analyses were not possible for the PKCα+ BPs, most notably within undamaged retinas, where PKCα+ BPs are very densely distributed (**Figure 1I**), and because the PKCα antibody did not adequately penetrate whole mounted retina tissue. Several differentiating *nyx::mYFP*+ BPs identified in whole retinas sampled at 13, 17, and 21 DPI were therefore imaged and traced (**Supplementary Table 1**), visualized as resliced Z-projections (**Figure 3**), and also visualized following surface rendering (**Figure 4**). For purposes of comparison, a series of control, *nyx::mYFP*+ BPs sampled from undamaged, control retinas, is also shown in each Figure (**Figures 3**, **4**); some of these control neurons were also shown in McGinn et al. (2018). **Supplementary Videos 1–5** provide rotating perspective animations of the galleries shown in **Figure 4** (Videos 1-4), and of a gallery of regenerated *nyx::mYFP*+ BPs sampled at 60 DPI [Video 5; (McGinn et al., 2018)]. Note that the cell bodies of some of these BPs remained incompletely traced/filled due to limitations of the SNT plugin. The majority of sampled, regenerated *nyx::mYFP* BPs displayed apically-projecting neurites, as well as basally-projecting neurites (**Figures 3**, **4**). For simplicity, apically-projecting neurites with terminals within or approaching the OPL are referred to as dendrites (green processes in **Figures 3**, **4**), and basally-projecting neurites with terminals within or approaching the inner plexiform layer (IPL) are referred to as axons (red processes in **Figures 3**, **4**). At 13 and 17 DPI, some dendritic trees did not

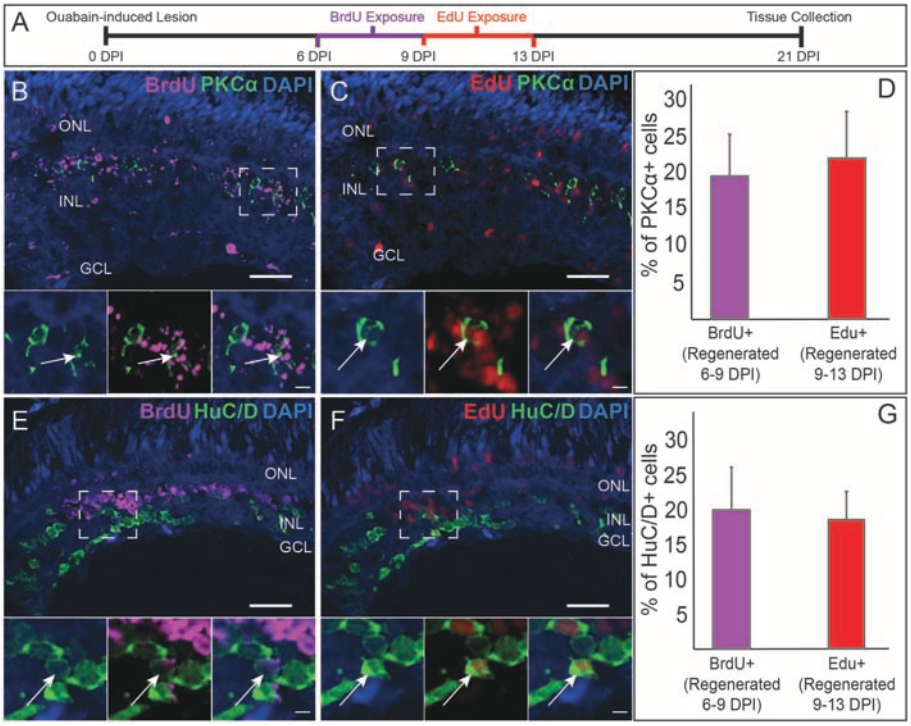


**FIGURE 2 |** Comparative birthdating of regenerated PKCα+ bipolar (BP) neurons and HuC/D+ amacrine and ganglion cells following a chemical lesion selective to the inner retina. **(A)** Timeline and experimental design; DPI, days post-injury. **(B)** Some regenerated PKCα+ (green) BPs incorporated BrdU (magenta) (6–9 DPI). Higher magnification insets of boxed region show PKCα and DAPI, PKCα, and BrdU, and all channels merged. Arrows indicate BrdU+ PKCα+ BP cell. **(C)** Some regenerated PKCα+ BPs incorporated EdU (red) (9–13 DPI). Higher magnification insets corresponding to boxed region show PKCα and DAPI, PKCα and EdU, and all channels merged. Arrow indicates EdU+ PKCα+ BP cell. **(D)** Percentage of PKCα+ cells that incorporate BrdU and EdU at the indicated timeframes (means ± s.d.; $n = 3$). **(E)** Some regenerated HuC/D+ (green) neurons incorporated BrdU (magenta) (6–9 DPI). Higher magnification insets corresponding to boxed region show HuC/D and DAPI, HuC/D and BrdU, and all channels merged. Arrow indicates BrdU+ HuC/D+ cell. **(F)** Some regenerated HuC/D+ BPs incorporated EdU (red) (9–13 DPI). Higher magnification insets corresponding to boxed region show HuC/D and DAPI, HuC/D and EdU, and all channels merged. Arrow indicates EdU+ HuC/D+ cell. **(G)** Percentage of HuC/D+ neurons that incorporate BrdU and EdU at the indicated timeframes (means ± s.d.) are similar to the percentages of PKCα+ BPs that incorporated these labels. Scale bar in **B** (applies to **B,C,E,F**) = 50 μm. Scale bar in insets (applies to all insets) = 10 μm.

projected fully into the OPL (one of 15 at 13 DPI, three of 13 at 17 DPI), while all of the BPs sampled at 21 DPI had dendritic trees that projected into the OPL (**Figure 3**; **Supplementary Table 2**). Some axons of BPs in 13 DPI retinas were apparently longer and meandered more than those of 17 DPI, 21 DPI, or undamaged retina, and axons of 21 DPI BPs appeared to have more complex morphologies than those sampled at earlier timepoints or from control retinas (**Figures 3**, **4**; **Supplementary Table 2**). The IPL could not be readily identified in some cases, particularly at 13 DPI, as there were many nuclei present within the presumed corresponding region (**Figure 3**).

In several cases, the morphologies of newly regenerated BPs in 13 DPI and 17 DPI retinas were highly atypical (arrows in **Figures 3**, **5**; **Supplementary Table 2**; **Supplementary Figure 2**). For example, one BP displayed a single major neurite that then branched into both apical and basal projections, sometimes with extensive branching (**Figures 5A,A'**), while others (three of 15 BPs at 13 DPI and two of 13 BPs at 17 DPI) displayed numerous neurites projecting from the cell body (**Figures 5B,B',C,C'**; **Supplementary Table 2**). Some of the BPs displayed neurites that were intertwined and seemingly lacked polarity with respect to the surrounding retinal tissue (**Figures 5B,B'**; **Supplementary Table 2**). Two of 15 BPs at 13 DPI and two of 13 BPs at 17 DPI showed highly truncated dendritic trees, visible only as short primary dendrites (**Figures 5D,D'**; **Supplementary Table 2**; **Supplementary Figure 2C**). Furthermore, some of the BPs displayed dendrites having secondary branches, even though the primary dendrite did not reach the OPL (one of 15 at 13 DPI and two of 13 at 17 DPI; **Figure 5B**; **Supplementary Figure 2B**). This type of feature has not been reported in regenerated retinas at 60 DPI (McGinn et al., 2018), in adult undamaged retina (Li et al., 2012; McGinn et al., 2018), or in developing zebrafish retina (Schroeter et al., 2006), and so may be distinct to newly regenerated BPs. Two of the 15 axons of newly regenerated BPs studied at 13 DPI displayed long, wandering axons (**Figures 3**, **4**). Only seven of 15 BPs at 13 DPI displayed a single axon with one to three branching points (**Supplementary Table 2**) and could be considered stereotypical BP neurons (Li et al., 2012). A summary of BP numbers at each time point displaying characteristics not meeting criteria defining a "stereotypical appearance" of a BP (see Methods), is provided in **Supplementary Table 2**. These

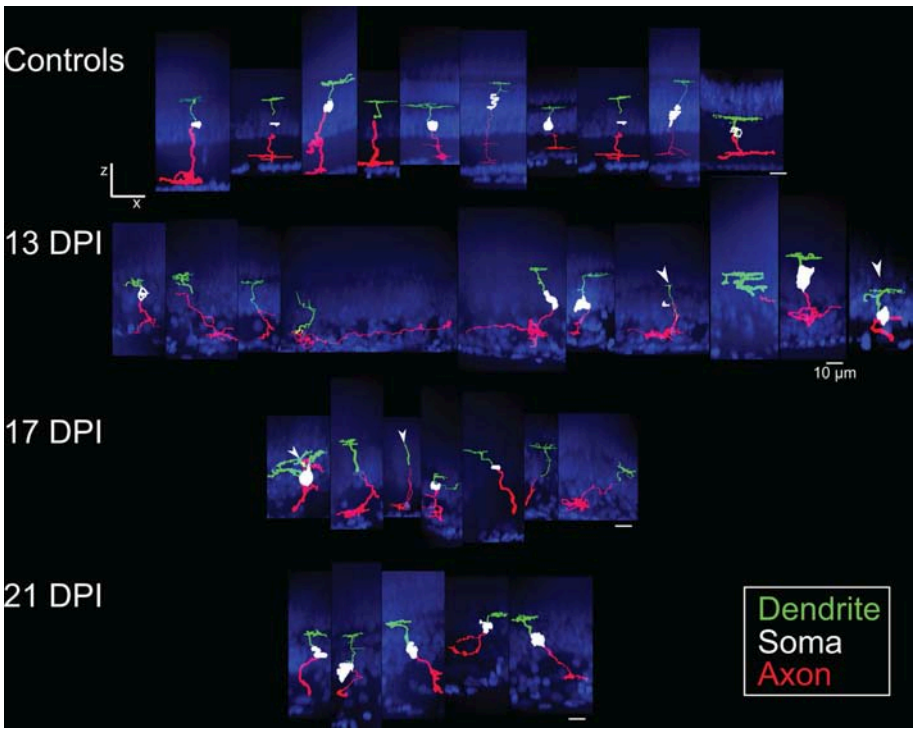


**FIGURE 3 |** Galleries of traced *nyx::mYFP+* retinal bipolar (BP) neurons. Neurons were traced in Simple Neurite Tracer (SNT), dendrites (green), cell bodies (white), axons (red) were pseudocolored, and then merged with images of the DAPI-stained (blue) nuclei to visualize retinal layers; merged images were then resliced to show the orthogonal views, with preservation of alignment of the traced cells and DAPI stained nuclei. For some BPs the cell body was not traced or only minimally traced due to limitations of SNT. Top row: BPs sampled from control retinas included some that were also shown in McGinn et al. (2018). Second row: BPs sampled at 13 days post-injury (13 DPI) vary in appearance, with some displaying long, wandering axons, and others displaying simple apical processes but no dendritic trees, and others presenting an apparently normal morphology. Third row: BPs sampled at 17 DPI also vary in appearance, with unusual dendritic tree structures or only simple apical processes, and others appearing normal. Bottom row: BPs sampled at 21 DPI show morphologies that more closely resembled those of control retinas. Scale bar = 10 µm.

atypical morphologies, found far more frequently at 13 and 17 DPI than at 21 DPI, may represent immature stages of BP differentiation, steps in the process of axon and dendritic tree pathfinding, and/or abnormal regenerated neurons that may later be eliminated by cell death or clearance. It is also important to note that the relative numbers of morphologically abnormal-appearing BPs may be the consequence of uncontrollable variables such as transgene silencing, and/or by unavoidable potential biases in the selection of isolated, *nyx::mYFP+* neurons for tracing. Because many of these morphologically abnormal neurons were difficult to unambiguously trace, and/or did not display the process branching characteristics required for the further morphometric analyses, they were not included in the following more detailed quantitative studies of dendritic trees and axonal branching patterns.

## Morphometric Analyses of Dendritic Trees of Newly Regenerated *nyx::mYFP* BPs

### Dendritic Spread

Dendritic spreads of several (**Supplementary Table 1**) traced, regenerated nyx::YFP+ BPs were measured using an ellipse or polygonal method (see Methods). Interestingly, the dendritic spreads of regenerated BPs at 13 DPI were not different than those of control BPs ($p = 0.216$, measured by a convex polygon; $p = 0.072$, measured by an ellipse) (**Figure 6**). However, the dendritic spreads of BPs in the 17 DPI and 21 DPI retinas were smaller than those in control retinas ($p = 0.002$ and $p = 0.038$, complex polygon; $p = 0.004$ and $p = 0.016$, ellipse) (Wilcoxon–Mann–Whitney) (**Figure 6**).

### Dendritic Tree Characteristics

Using the Sholl Analysis plugin in ImageJ, the extent of dendritic branching was calculated separately for several individual neurons (see Methods; **Supplementary Table 1**). A "mean of intersections" was returned by the plugin, a measure which is calculated by dividing the sum of the number of dendrite crossings of each concentric sphere by the number of concentric spheres (Ferreira et al., 2014). A higher value for mean of intersections indicates more extensive branching. This measure of dendritic branching was lower for BPs in 13 DPI retinas than for those in control retinas ($p = 0.006$) (**Figure 7A**). However, the extent of dendritic branching reached control levels for regenerated BPs sampled at 17 DPI and 21 DPI ($p = 0.054$ and $p = 0.883$) (**Figure 7A**). Therefore, while the sizes of the dendritic trees of 13 DPI BPs were similar to those of controls

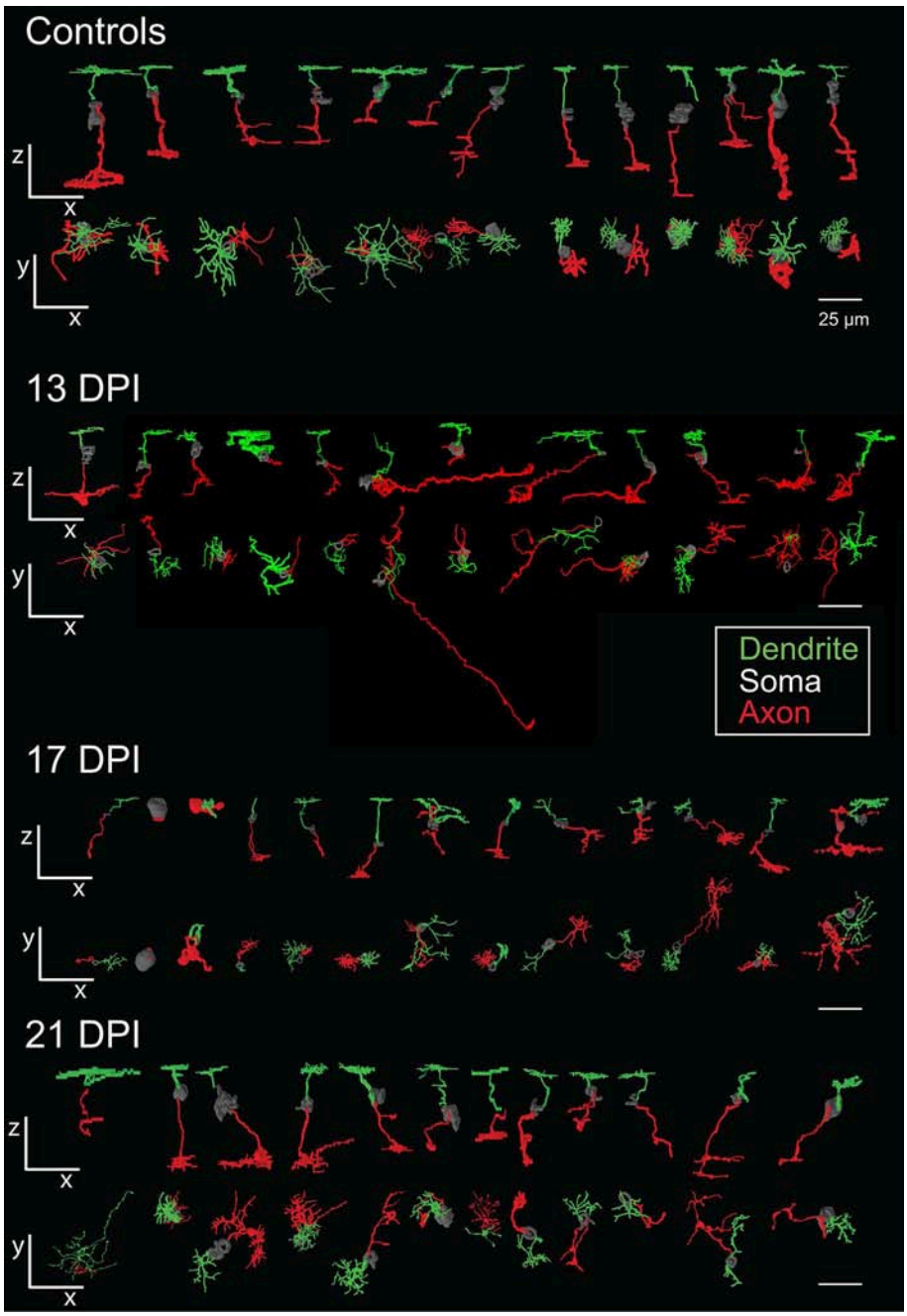


**FIGURE 4 |** Galleries of surface rendered *nyx::mYFP+* retinal bipolar (BP) neurons. Neurons were traced in Simple Neurite Tracer (SNT), colorized, filled, created into three-dimensional surface models, imported into 3ds Max 2016, and then entered into a single image to display overall similarities and differences of BPs. For some BPs the cell body was not traced or only minimally traced due to limitations of SNT. Each timepoint shows the rendered neurons in the x-z radial "side" views (top row) and the x-y tangential "top-down" views (bottom row) relative to the imaging plane. BPs shown from control retinas included some that were also shown in McGinn et al. (2018). Dendritic tree alignment of the seventh 13 DPI neurons is offset vertically in order to fit all into the display without overlap. Scale bars = 25 µm. DPI, days post-injury.

(**Figure 6**), these trees were less extensively branched, a finding that could be considered consistent with dendrite restructuring toward presynaptic partners of regenerated BPs. Results from Sholl analysis also revealed significant reductions in the Sholl critical value for 13 DPI and 17 DPI BPs as compared with controls ($p = 0.0088$ and $p = 0.0237$), but dendrites of 21 DPI BPs had Sholl critical values matching those of controls ($p = 0.921$) (**Figure 7B**). The Sholl critical value is the distance at which the maximum number of dendritic crossings is found, and so these results indicate that the BPs sampled at earlier regeneration times

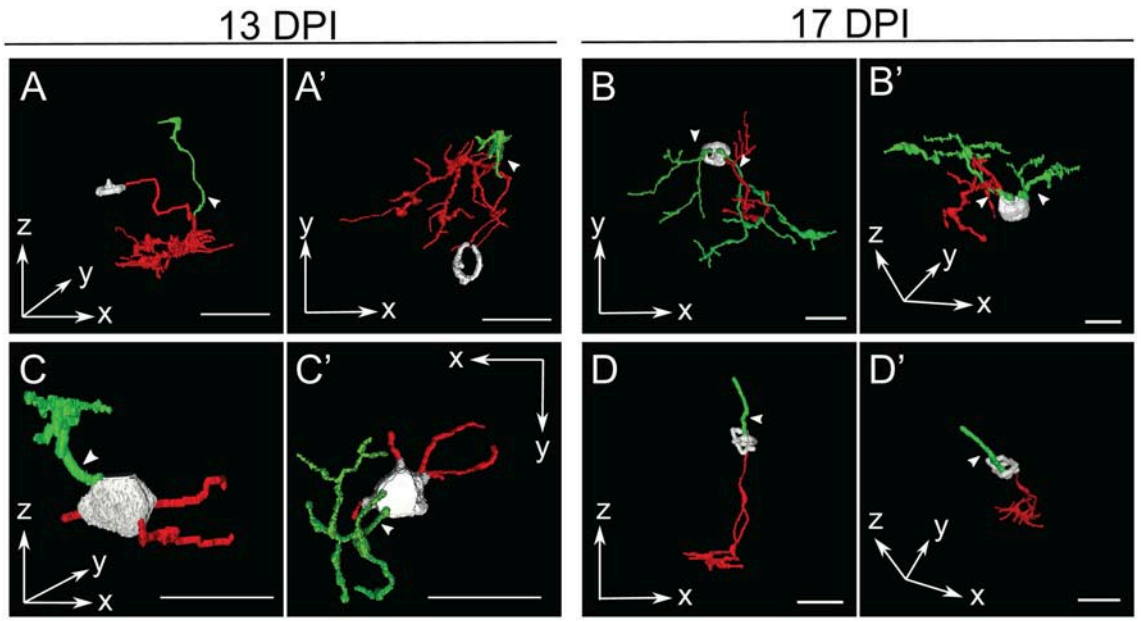


**FIGURE 5 |** Selected retinal bipolar (BP) neurons that demonstrate the unusual range of morphologies observed at 13 and 17 days post-injury (DPI). Neurons were traced in Simple Neurite Tracer and then rendered in ImageJ's 3D viewer. **(A,A')** Neuron showing an apparently displaced soma and multiple neurites, shown from two different orientations (**A** = radial "side" view, **A'** = tangential "top-down" view). The cell body was only minimally traced due to limitations of SNT. **(B,B')** Neuron that has two distinct apparent dendritic trees connected to the same cell body. **(C,C',D,D')** Neurons with apparently simple morphologies sampled at 13 DPI and 17 DPI, respectively, each shown from two different orientations. In contrast to A and B, these BPs have a bipolar shape, but have smaller dendritic trees with fewer branches than most other neurons sampled at 13 and 17 DPI. The neuron in **(C,C')** appears to have more than one basal outgrowth (possibly multiple axons), all of them truncated, while the neuron in D has an axon with a normal appearance, but a single apical process rather than a dendritic tree. The cell body in **(D,D')** was only minimally traced due to limitations of SNT. Arrows designate investigator assignment of primary dendrites (green). Axons, red; incompletely traced cell bodies, grayscale. Scale bar(s) in all panels = 10 μm.

likely had fewer dendritic branches at the greater distances from the primary dendrite.

As a further measure of overall dendritic morphology, the Sholl regression coefficient was calculated, using the semi-log method; the regression coefficient can be used to determine if neurons fall into the same broad morphological category (Ristanovic et al., 2006). A one-way ANOVA across all sampling times revealed no significant differences ($p = 0.444$) (**Figure 7C**), indicating that the dendritic trees of newly regenerated BPs were not sufficiently distinct from those of control BPs as to constitute a separate morphological neuronal type.

## Analyses of Dendritic Tips and Cone Contacts of Newly Regenerated *nyx::mYFP* BPs

### Dendritic Tips

Endpoints (dendritic tips, which project apically toward their presynaptic photoreceptor partners) of *nyx::mYFP* BPs were counted in regenerated retinas at 13, 17, and 21 DPI and compared to controls (McGinn et al., 2018) (**Supplementary Tables 1, 3**). The average total numbers of dendritic tips per BP showed no statistically significant differences across all conditions ($p = 0.194$, one-way ANOVA.) (**Figure 7D**). It is important to again note, however, that neurons with highly truncated dendritic trees (e.g., **Figure 5D**), were excluded from this analysis because they had no endpoints to count, and so these numbers of dendritic tips do not represent the entire BP population sampled.

### Cone Contacts

Traced, *nyx::mYFP* BP dendritic tips were evaluated for the presence of presumptive synaptic connections with cone photoreceptors. To this end, we simultaneously visualized mCherry+ synaptic terminals of blue-sensitive cones, and ZPR1-antibody labeled synaptic terminals of double cones (a.k.a. red- and green-sensitive cones) (**Figures 8A–E**; **Supplementary Table 3**) in whole *nyx::YFP* transgenic retinas. For this analysis we again excluded BPs that did not reach the OPL with their apical dendrites. The patterns of connections, measured as the proportion of endpoints contacting blue cones vs. double cones vs. unassigned endpoints (which could represent UV cones, rods, or unconnected tips), were quantified (**Figure 8F**; **Supplementary Table 3**). In our previous study, ZPR1+ cone pedicles could readily be identified as belonging to green-sensitive or red-sensitive cones based upon their location with respect to surrounding cone terminals of the organized mosaic (McGinn et al., 2018). Unfortunately, we were unable to unambiguously make these specific assignments as the mosaic of cone terminals at 13, 17, and 21 DPI was difficult to appreciate, perhaps due to tissue fragility (**Figures 8B–D**). Instead, each

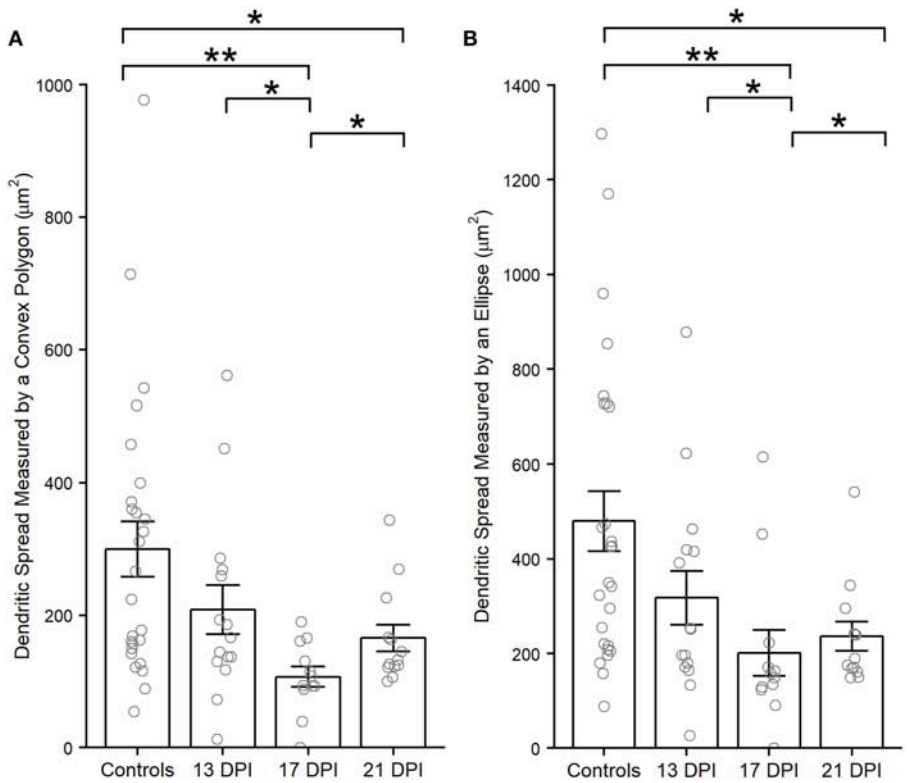


**FIGURE 6 |** Dendritic spreads measured using the convex polygon method **(A)** and the ellipse method **(B)**, of regenerated *nyx::mYFP*+ bipolar (BP) neurons, are reduced at 17 and 21 days post-injury (DPI) compared to controls. Column graphs show the mean of each group, open circles represent each individual, measured dendritic tree, and the error bars show the SEM. *$p < 0.05$, **$p < 0.01$ using Wilcoxon–Mann–Whitney tests. Control measurements are those shown in McGinn et al. (2018).

regenerated BP dendritic endpoint was counted as connecting to a blue sensitive cone, a ZPR1+ cone, or unassigned. We also re-analyzed cone contacts observed in control retinas, and at 60 DPI (McGinn et al., 2018) according to these criteria (**Figure 8F**; **Supplementary Table 3**). We then applied a Generalized Linear Model (GLM) to determine whether BP-cone connectivity patterns were different in control vs. regenerated retinas (i.e., do regenerated *nyx::mYFP* BPs sampled at any timepoint connect more or less frequently to any specific cone subtype than in control retina?). Based on this analysis, regenerated BPs sampled at 13, 17 DPI, and 60 DPI each showed patterns of photoreceptor connections that were statistically indistinguishable from controls ($p = 0.3844$, 0.2758, and 0.4534, respectively; GLM) (**Figure 8F**). Interestingly, the BPs sampled at 21 DPI were significantly different than controls ($p = 0.031$), apparently favoring ZPR1+ double cone contacts over blue cone contacts (**Figure 8F**), suggesting some plasticity of synaptic connections during regeneration.

Although the above analysis suggests that relatively normal connectivity may be restored at 13 and 17 DPI, it is again important to note that not all bipolar cells reconnected with visualized photoreceptors at these sampling times. Neurons with 1 or 0 projections reaching the OPL were excluded from the connectivity analysis due to the ambiguity of such connections.

## Axon Morphologies of Newly Regenerated BPs

We previously analyzed BP axons in regenerated retinas (at 60 DPI) through identification of stratification patterns and Sholl analysis of complexity of axon terminals (McGinn et al., 2018). In several cases in the present study, the IPL was difficult to clearly identify at 13, 17, and 21 DPI, and we were therefore unable to unambiguously subdivide the vague landmarks defining the IPL into the six typical substrata (Connaughton and Nelson, 2000), precluding analysis of axonal stratification. As discussed above, some BP axons observed in 13, 17, and 21 DPI regenerated retinas traversed long distances, branched into inappropriate retinal layers, or appeared to abruptly terminate (**Figures 3–5**, **Supplementary Table 2**; **Supplementary Figure 2**). Such axons were difficult and at times impossible to completely trace because axons would continue to traverse beyond the imaged region. Some of the more unusual-appearing BP axons from regenerating retina (which were traceable) were examined in further detail, and two are provided as 3-D projections in order to appreciate the context of each BP axon and surrounding regenerating retinal tissue (**Supplementary Figures 2B,D**), particularly in comparison with a BP axon residing within undamaged retina (**Supplementary Figure 2A**). One of these BPs had a cell body residing at the boundary of the GCL rather than within the INL

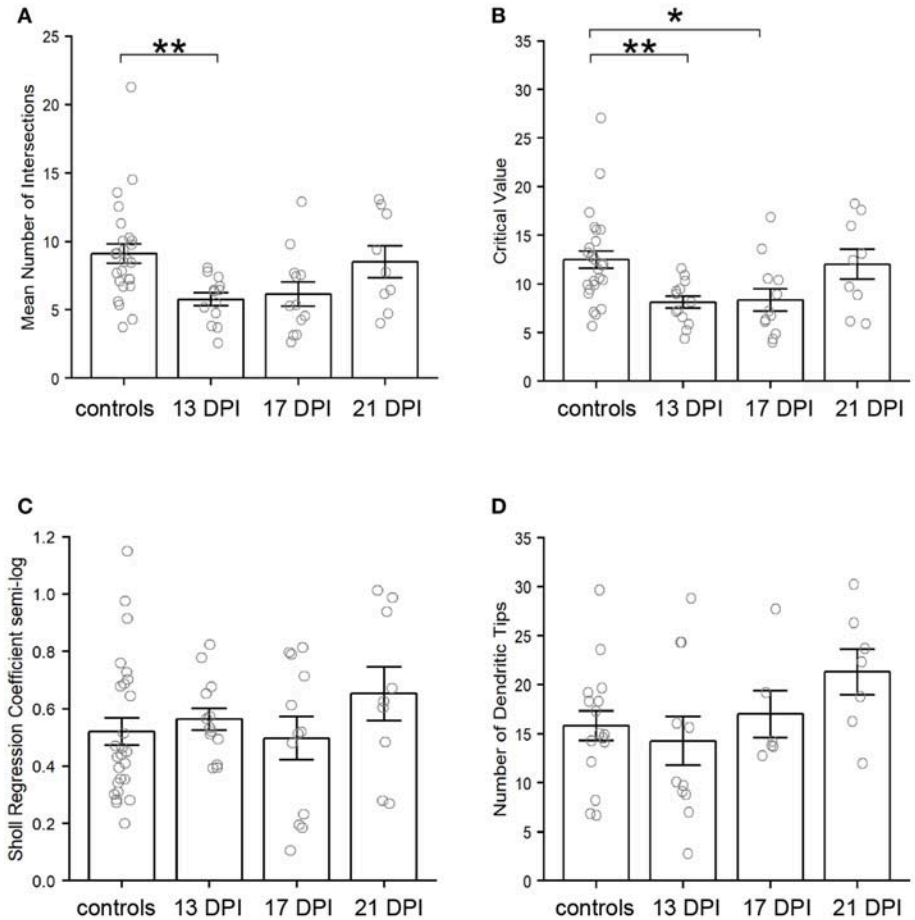


**FIGURE 7 |** Characteristics of *nyx::mYFP*+ bipolar (BP) neuron dendritic trees. **(A–C)** Outputs of Sholl analysis. **(A)** Average number of intersections is reduced at 13 days post-injury (DPI) but not at 17 and 21 DPI in comparison with controls. B. Sholl critical values are reduced at 13 and 17 DPI but not at 21 DPI in comparison with controls. C. Sholl regression coefficients are not significantly different across samples. **(D)** Numbers of dendritic tips (presumed photoreceptor connections) are not significantly different across samples. Bar graphs show the mean of each group, open circles represent each individual dendritic tree, and the error bars show the SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ using Wilcoxon–Mann–Whitney tests. Control measurements are those shown in McGinn et al. (2018).

(**Supplementary Figure 2B**), an abnormal position for this cell type that we previously documented for the rare BP sampled at 60 DPI (McGinn et al., 2018).

Because we were unable to completely trace many of the axons of newly regenerated BPs, we were not able to unambiguously measure axonal length as a potential metric of our qualitative observations. As an alternative, but distinctive analysis of axon characteristics, we performed Sholl analysis of axonal branching patterns for some of the BPs sampled from 13, 17, and 21 DPI retinas (**Supplementary Table 1**). The average number of intersections returned from Sholl analysis of axon branching indicated no significant differences among samples ($p = 0.8$) (**Figure 9A**). Similarly, the Sholl critical value reached control levels for regenerating BPs sampled at all regenerating time points ($p = 0.981$) (**Figure 9B**).The Sholl regression coefficient also showed no significant differences among samples ($p = 0.12$) (**Figure 9C**). These measureable axons therefore displayed axon branching characteristics similar to those of mature BPs.

# DISCUSSION

## Emergence of Regenerated BP Neurons

In this study, we report the neuronal morphologies and cone photoreceptor connectivities of regenerated BPs in adult zebrafish retinas from 13 to 21 days after a ouabain-induced lesion destroying only inner retinal neurons. After such a lesion (Nagashima et al., 2013; Sherpa et al., 2014; McGinn et al., 2018; Mitchell et al., 2018, 2019), regenerated BPs must extend axons and dendrites that locate and connect to their input partners (photoreceptors), which survived the lesion, as well as their output partners (amacrine and ganglion cells), which were destroyed and also regenerated following the lesion. We find that new PKCα+ and *nyx::YFP*+ BPs initially emerge by 13 DPI in reduced numbers compared to undamaged retinas, and that numbers of the PKCα+ BPs at 21 DPI show no evidence of a difference with numbers of these neurons in control retinas. This pattern of emergence of BPs is consistent with protracted and asynchronous regeneration of BPs, and/or with asynchronous

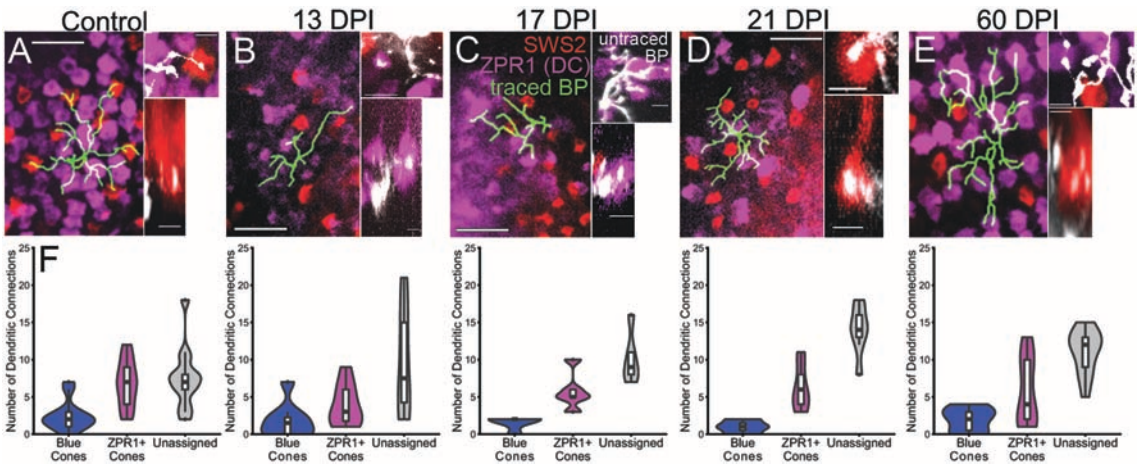


**FIGURE 8 |** Reestablishment of *nyx::mYFP*+ bipolar (BP) neuron dendritic connections with cone photoreceptor terminals. **(A–E)** Dendritic fields showing traced *nyx::mYFP*+ BPs (green) overlayed onto partial projections of blue-sensitive (red color) cone terminals and ZPR1+ (magenta color) red- and green-sensitive double cone terminals, from control **(A)**, 13 days post-injury (DPI) **(B)**, 17 DPI **(C)**, 21 DPI **(D)**, and 60 DPI **(E)** retinas. Control and 60 DPI images also appear in McGinn et al. (2018). Higher magnification views on right of each panel show untraced BP dendrites (white) forming connections with blue-sensitive (red color) and/or ZPR1+ (magenta color) red- and green-sensitive double cone terminals (Top images are z-projections; bottom images are partial projections from image stacks, showing resliced radial views; control and 60 DPI images also appear in McGinn et al. (2018), but with different pseudocoloring). **(F)** Distributions of dendritic connections to identified and unassigned photoreceptor subtypes for *nyx::mYFP*+ BPs in control, 13 DPI, 17 DPI, 21 DPI, and 60 DPI retinas. Shapes of violin plots were obtained by using a kernel density estimator to generate a smoothened histogram, mirrored along the x-axis, and then rotated. The width of each plot is determined by the proportion of bipolar cells making a given number of connections to that photoreceptor subtype at that point. In the boxplots within, the horizontal line inside each box represents the median, the top and bottom of the box represent the 25th and 75th percentiles, the whiskers represent the 1.5 interquartile range, and the filled circles represent outliers. Control vs. 13 DPI $p = 0.3844$, control vs. 17 DPI $p = 0.2758$, control vs. 21 DPI $p = 0.031$, control vs. 60 DPI $p = 0.4534$ (Generalized linear model). Scale bars in **(A–E)** = 10 μm for left panels, and 2.5 μm for right panels.

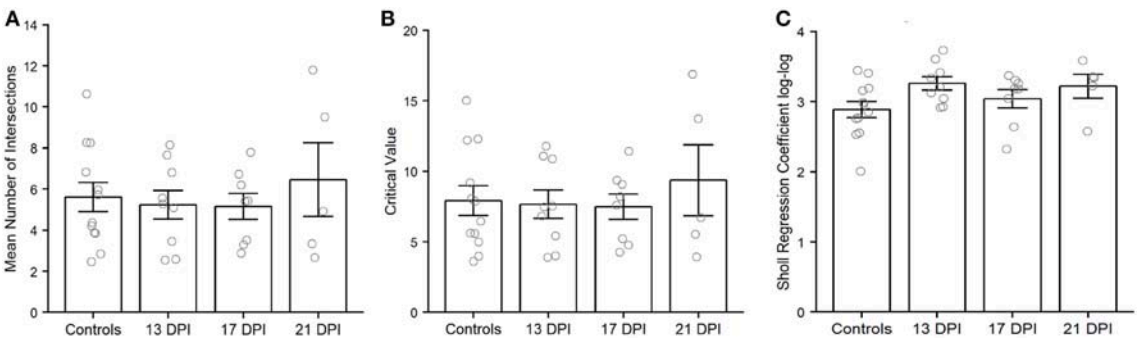


**FIGURE 9 |** Characteristics of *nyx::mYFP*+ bipolar (BP) neuron axonal arbors, using outputs of Sholl analysis. Average number of intersections **(A)**, Sholl critical values **(B)**, and Sholl regression coefficients **(C)** are not significantly different across samples. Column graphs show the mean of each group, open circles represent each individual dendritic tree, and the error bars show the SEM. Wilcoxon-Mann-Whitney tests did not reveal any significant differences for any of these measures. Controls are those shown in McGinn et al. (2018). DPI, days post-injury.

expression of BP markers. Our birthdating studies support the former interpretation, as they suggest steady production of both PKCα+ BPs and their postsynaptic partners, HuC/D+ amacrine and ganglion cells, over 6–13 DPI. Interestingly, this sequence of neuronal production during regeneration does not appear to recapitulate the sequence documented for embryonic retinal neurogenesis in the zebrafish, in which cells of the GCL are generated prior to those of the INL (Hu and Easter, 1999). Rather, during retinal regeneration, PKCα+ BPs and their post-synaptic partners are generated concurrently, and this regeneration occurs in a protracted manner, as has been shown for HuC/D+ amacrine and ganglion cells for even longer timeframes (Sherpa et al., 2014).

## General Attributes of Newly Regenerated BP Neurons

The morphologies of *nyx::mYFP*+ BPs sampled at 13, 17, and 21 DPI were in some cases highly unusual, with abnormal positions and sizes of apically and basally-projecting neurites, and/or laterally projecting axons, and/or no cone contacts. This is in contrast to BPs analyzed in our previous study of *nyx::mYFP*+ BPs, which were sampled at 60 DPI, in which the

majority of sampled BPs showed morphologies indistinguishable from controls (McGinn et al., 2018). In the present study, only a subset of analyzed BPs displayed a rather stereotypical appearance, closely resembling BPs sampled from undamaged retinas. One reason for such morphological differences in BPs compared at these different timepoints could be that relatively earlier regenerated BPs may be in the process of establishing their mature morphologies, with further growth and/or pruning or retraction taking place over 17–21 DPI. During mouse retinal development, apical and basal processes of differentiating BPs must retract as they elaborate their dendritic trees and axonal arbors, respectively (Morgan et al., 2006). Our staining and imaging procedures did not reveal, and perhaps may not be able to reveal, the presence of initial apical or basal processes that span the retina, as was found in mouse, but our findings do suggest regenerated zebrafish BP neurites may undergo pathfinding which extends beyond their potential targets, followed by refinement. Alternatively, mature BP morphologies may be manifest concomitantly with expression of the markers used to identify them in this study, and those BPs showing abnormal morphologies may be eliminated by cell death or clearance. Another possible explanation is that protracted regeneration of BPs and other inner retinal neurons may create a tissue environment that allows for more efficient neurite pathfinding over time (for example, due to increased or better organized spatial and directional cues), resulting in the later-regenerated BPs appearing more morphologically normal. We acknowledge that the PKCα+ BPs analyzed for birthdating represent a different population than the *nyx::mYFP*+ BPs we analyzed morphometrically (McGinn et al., 2018), and that both populations are heterogeneous (Schroeter et al., 2006; McGinn et al., 2018). Therefore, the protracted regeneration process of PKCα+ BPs may not be reflected by a similar time course for the *nyx::mYFP*+ BPs, and the apparent maturation dynamics and aberrant morphologies of the *nyx::mYFP*+ BPs may not be manifested by newly regenerated PKCα+ BPs. Finally, the heterogeneity of morphologies of newly regenerated *nyx::mYFP*+ BPs may represent distinct maturation strategies for different subpopulations of these BPs.

## Dendritic Attributes of Newly Regenerated BP Neurons

When measureable, BP dendritic tree sizes appeared to fluctuate over the 13–21 DPI timeframe. It is possible that this timeframe (and perhaps beyond) represents a time of modification of dendritic processes. Interestingly, dendritic tree attributes of the newly regenerated BPs also showed some statistically significant differences in comparison with controls, but dendrite attributes from BPs sampled at 21 DPI showed the most normal morphologies. For example, Sholl critical values of the 13 DPI and 17 DPI, but not of the 21 DPI, BP dendritic trees, were different from the controls. This suggests that dendritic branches are present at 13 DPI but may continue to be modified until 21 DPI, when they approach a more stable state. Similarly, the mean number of intersections was reduced for BP dendrites at 13 DPI, but not for those sampled at 17 DPI or 21 DPI. These results suggest that regenerating *nyx::mYFP* BPs sampled at 13 DPI have dendritic trees with fewer branches, shorter branches, or a combination of both, as compared with other sampling conditions. In general, the outputs of Sholl analysis indicate that the dendritic trees of sampled, regenerated BPs may undergo morphological changes consistent with maturation over the 13–21 DPI sampling time. It is interesting that this possible maturation process appears distinct from that of BP dendritic trees in developing retina. For example in mouse, BP dendritic field sizes are initially overextended and overlapping with each other, and then retract due to homotypic interactions with neighboring BPs (Lee et al., 2011). In larval zebrafish, developing *nyx::mYFP*+ BPs have also been observed to overextend their dendritic trees, but into the ONL and INL, rather than within the plane of the OPL (Schroeter et al., 2006).

Not all BPs showed visible connections to photoreceptors at 13 and 17 DPI. However, those that did, showed cone connectivities similar to those of control BPs. In addition, the numbers of presumed cone contacts (dendritic tips) were statistically similar across all timepoints. Together with the shifting dendritic field sizes and complexities discussed above, we hypothesize that regenerating BPs may make their connections to photoreceptors before their dendritic trees are mature, an event that could be likely in contexts where pre-synaptic photoreceptors are present during BP regeneration. In comparison, following cell-selective ablation of distinct subpopulations of BPs in larval retina, regenerated BPs display overall normal morphologies, but have larger dendritic trees and some errors in cone contacts (D'Orazi et al., 2016). It is possible that these regenerating larval BPs were analyzed prior to achieving a more mature state, as they were sampled only 13 days after ablation, and may represent a parallel sampling to the possibly immature BPs we describe here at 13 and 17 days after ouabain injury in adult fish. Similar phenomena have been described during embryonic retinal development in both mouse (Morgan et al., 2006) and zebrafish (Schroeter et al., 2006), in which the dendritic trees of differentiating BPs also appear to grow, elaborate beyond their intended targets, and then retract to their stable arbor morphologies.

An alternative interpretation for the observed differences in BP dendritic tree morphologies at 13 vs. 17 vs. 21 DPI, is that some of the regenerated BPs may undergo apoptosis or are cleared from the retina (or are less likely to be sampled), while new BPs continue to be generated and assume normal morphologies and connectivities. BP cell death alone as the explanation for the apparent loss of abnormal BPs between 17 and 21 DPI is somewhat unlikely for several reasons. Numbers of TUNEL+ cells in retinas over this timeframe are minimal compared to the initial (1–3 DPI) period of apoptosis due to ouabain damage (Fimbel et al., 2007). Furthermore, quantification of BPs suggests their accumulation rather than loss (**Figure 1**). Finally, Hitchcock and Cirenza (1994) found that amacrine cells were able to remodel and reintegrate into regenerated portions of the retina following surgical excision of a portion of goldfish retina. It is possible that regenerated BPs possess the same ability as regenerated amacrine cells to remodel or change their morphology accordingly.

### Axonal Attributes of Newly Regenerated BP Neurons

Among the more noteworthy abnormalities observed in the newly regenerated BPs were the unusual trajectories of some axons. In many cases axons traversed such long distances and/or entered an inappropriate layer, making complete documentation of their trajectories difficult or impossible. At the other extreme, some of the regenerated BPs displayed what appeared to be more than one basally-projecting neurite, or no basally-projecting neurite at all. These abnormalities were more frequently observed in the BPs sampled at 13 and 17 DPI in comparison with those at 21 DPI (**Supplementary Table 2**), and so may again represent immature states of the regenerated BPs prior to pruning/retraction or growth. Also noteworthy is that the newly regenerated BPs that could be sampled for Sholl analysis demonstrated axonal branching characteristics not significantly different from those of control BPs. One interpretation of these more normal BP axons is that they may represent those of the earliest-generated and therefore potentially more mature BPs. Such an interpretation is consistent with the findings for BP axon development in zebrafish larvae, in which active extension and retraction of axon filopodia takes place prior to the establishment of axon terminals in their appropriate sublaminae (Schroeter et al., 2006). Alternatively, the more normal axons may reside within an environment in which at least some of their postsynaptic partners have been more extensively regenerated, while the abnormal BP axons reside in regions with (or are regenerated at timepoints that display) a paucity of postsynaptic targets and plexiform structure, and lack the pathfinding cues that would guide them to the correct retinal location and synaptic partners. In support of this alternative, the absence of retinal ganglion cells and disruption of amacrine cell stratification during zebrafish larval development results in mis-targeting of BP axons (Kay et al., 2001).

## CONCLUSIONS

Following a lesion that destroys BPs and their postsynaptic partners, but spares their presynaptic partners, newly regenerated BPs are generated over a protracted timeframe and reappear from 13 to 21 DPI. Newly regenerated BPs display morphological characteristics consistent either with dynamic modifications of their dendritic and axonal arbors, or with heterogeneity in regenerated BPs dependent upon the time of regeneration, or some combination of these interpretations. In any case, generation and morphological differentiation of regenerated BPs is not a simple recapitulation of these processes during embryonic development, but distinctive to regeneration. Further, our data indicate that regeneration remains a dynamic process, as opposed to a single burst of genesis to replace lost neurons. Since photoreceptors are spared from this lesion, the presence and appropriate locations of photoreceptor synaptic terminals in regenerating retinas may provide long- or short-range cues to attract and pair with regenerated BP dendrites. The photoreceptors that survive this lesion have the capacity to support the a-wave of the electroretinogram (ERG) (McGinn et al., 2018), indicating that they remain physiologically active, and this activity may also provide targeting information for BP dendrites. In contrast, the absence or concomitant emergence of post-synaptic ganglion and amacrine cell dendrites leaves BP axons with potentially inappropriate (or absent) guidance information. These results are consistent with our previous study documenting faster recovery of visual function following inner retinal lesion compared to more extensive damage that includes photoreceptors (Sherpa et al., 2014).

Despite the presence of many morphologically abnormal regenerated BPs, the regenerated BPs that display dendritic trees and axons amenable to tracing and further analysis show characteristics that are quite similar to those of mature, undamaged BPs. Collectively our results suggest that the intrinsic mechanisms underlying the reestablishment of retinal circuitry during regeneration are robust, even in the context of a tissue-disrupting lesion. Further, the 13–21 DPI period following an inner retina-selective lesion appears to be an active time of both BP neurogenesis and the reestablishment of retinal circuitry. Among the next steps in the study of regeneration of retinal neurons and their connections will be to identify the cellular signaling and other processes that support functional rewiring (Angueyra and Kindt, 2018), as well as to determine the extent to which regenerated neurons are required in numbers and morphological "normalcy" to restore measureable physiological function. An evaluation of cell-specific transcriptomes at this time post-lesion may reveal signals that are specific for this rewiring (Sun et al., 2018a,b). In addition, microglia remain active over this period following retinal damage and regeneration (Mitchell et al., 2018), and have known roles in synaptic remodeling in other biological contexts (Schafer et al., 2012), making them excellent candidates as participants in the reestablishment of circuitry in the regenerated retina.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Care and Use Committee of the University of Idaho.

## AUTHOR CONTRIBUTIONS

TM and DS conceived of the study. TM, DM, and DS planned the experiments. TM, CG, and DM carried out the experiments and analyzed data. DL and NP contributed to data analysis. TM, DM and DS wrote the manuscript with approval of all authors.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00095/full#supplementary-material

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# Neural Stem Cell Regulation by Adhesion Molecules Within the Subependymal Niche

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In the mammalian adult brain, neural stem cells persist in neurogenic niches. The subependymal zone is the most prolific neurogenic niche in adult rodents, where residing stem cells generate large numbers of immature neurons that migrate into the olfactory bulb, where they differentiate into different types of interneurons. Subependymal neural stem cells derive from embryonic radial glia and retain some of their features like apico-basal polarity, with apical processes piercing the ependymal layer, and a basal process contacting blood vessels, constituting an epithelial niche. Conservation of the cytoarchitecture of the niche is of crucial importance for the maintenance of stem cells and for their neurogenic potential. In this minireview we will focus on extracellular matrix and adhesion molecules in the adult subependymal zone, showing their involvement not only as structural elements sustaining the niche architecture and topology, but also in the maintenance of stemness and regulation of the quiescence-proliferation balance.

**Keywords: subependymal zone, adhesion molecules, extracellular matrix, neural stem cell, quiescence, niche, adult neurogenesis**

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## THE NEUROGENIC ADULT SUBEPENDYMAL ZONE: A POLARIZED EPITHELIAL NICHE

In most adult tissues, stem cells dwell in specialized microenvironments called "niches" (Scadden, 2006). In the mammalian adult brain, the subependymal zone (SEZ), is a specialized niche that encompasses a thin layer of cells apposed to the ventricular wall, lined by ependymal cells (EC). Here, neural stem cells (NSC) coexist with their own progeny, supporting niche cells, neighboring blood vessels (BV), and a specialized extracellular matrix (ECM). In addition, they are in contact with the cerebrospinal fluid through a primary cilium at the end of a cytoplasmic process that traverses the ependymal layer through the center of a rosette of ependymocytes, named "pinwheel" (Mirzadeh et al., 2008; Lin and Iacovitti, 2015).

Residing NSC generate large numbers of immature neurons that migrate into the olfactory bulb (OB), to differentiate into several types of interneurons that contribute to refine the processing of olfactory information (Lim and Alvarez-Buylla, 2014; Merkle et al., 2014). The ceaseless production of neurons follows a hierarchical lineage where NSC divide to generate transit amplifying progenitors (TAP) and/or to self-renew. TAP cells can divide a few times more to expand future neural progeny to finally generate migrating neuroblasts (Ortega et al., 2013; Ponti et al., 2013). SEZ

NSC also contribute to gliogenesis, however, much less productively than to neurogenesis (Menn et al., 2006; Ortega et al., 2013; Sohn et al., 2015), at least in homeostatic conditions.

In early developmental stages, ectodermally derived neural progenitors organize in a neuroepithelial layer surrounding the ventricles, termed the ventricular zone (VZ). Within this polarized arrangement, neuroepithelial cells (and later radial glial cells, RGC), exhibit a characteristic bipolar radial morphology supported by two points of adhesion: at their apical end, neighboring cells adhere to the luminal surface of the VZ through adherens junctions (AJs) mediated mainly by N-cadherin, whereas they remain attached to the subpial ECM *via* integrin–laminin interactions through their basal end-feet (Meng and Takeichi, 2009; Hirano and Takeichi, 2012). Embryonically, NSC derive from a pool of RGC that become specified at mid-gestation (Tramontin et al., 2003; Merkle et al., 2004; Kriegstein and Alvarez-Buylla, 2009; Fuentealba et al., 2015; Furutachi et al., 2015), and are as well glial in nature, displaying several ultrastructural characteristics of astrocytes (Doetsch, 2003). Additionally, they retain the apico-basal polarity reminiscent of RGC, with apical processes piercing the ependymal layer, and a basal process extruding onto the traversing BV and contacting their basal lamina (BL). This cytoarchitecture, with NSC spanning these two important signaling compartments categorizes the SEZ as an epithelial niche (Mirzadeh et al., 2008; Shen et al., 2008; Kokovay et al., 2010) (**Figure 1**).

## THE ECM AS A NICHE COMPONENT

In most cellular contexts, cell-ECM adhesion mediated by receptors such as integrins provides not only physical support and positioning to the cells, but also initiates cellular responses mainly mediated by phosphorylation states and activities of cytosolic tyrosine kinases, which next regulate other kinases and scaffolding proteins to transduce signals (Morgan et al., 2007). By providing bidirectional connections (intracellularly, by the assembly of cytoskeleton and signaling complexes, and extracellularly, through interactions with ECM elements, and in some cases with counter-receptors on adjacent cell surfaces) integrins force spatial restrictions on signaling and ECM assembly, and so integrate cells with their microenvironment (Humphries et al., 2006). The adult SEZ is rich in ECM molecules, such as fibronectin, laminins-β1 and γ1, and chondroitin sulfate proteoglycans (Mercier et al., 2002; Marthiens et al., 2010) along with a layer of Tenascin-C that separates the SEZ from the adjacent striatum (Kazanis et al., 2007). Intriguingly, notwithstanding a relevance in embryonic and early postnatal NSC and neural progenitor proliferation and migration (Garcion et al., 2001), in the SEZ Tenascin-C deficiency does not affect NSC nor progeny (Kazanis et al., 2007).

Additionally, a specific feature of the SEZ ECM is the presence of "fractones," conspicuous ECM structures thought to be merely extended formations of the vascular BL, which now seem to be functionally and structurally independent, with their own relevance in the SEZ niche. They may appear either as thin branching lines (stems) or as round deposits (bulbs) frequently popping out at the center of pinwheels or scattered along the inner ependymal wall, and associated with $GFAP^{+}$-NSC (Nascimento et al., 2018; Sato et al., 2019). SEZ fractones contain laminins, N-sulfate heparan sulphate proteoglycans (HSPG), collagens-I/IV/VI, nidogen-1/2, agrin, netrin-4, and perlecan-1 (Mercier et al., 2002; Kerever et al., 2007; Douet et al., 2012; Mercier and Douet, 2014; Sato et al., 2019), and their source has been described as either ependymal (Nascimento et al., 2018) or originating from NSC themselves (Sato et al., 2019). Elimination of laminin-α5 from EC increased activation of NSC (Nascimento et al., 2018) and disrupting integrin-binding activities of laminins specifically in astrocytes (including NSC), decreased the number and size of fractones, although the effects of this disruption on NSC proliferation were not investigated *in vivo* (Sato et al., 2019). Interestingly, fractones can promote heparin-binding growth factor activity and influence cell proliferation in the SEZ by sequestering basic-FGF and BMP-4/7 from the extracellular milleu (Kerever et al., 2007; Douet et al., 2012; Mercier and Douet, 2014).

## ADHESION AT THE BASAL END: WHERE NSC MEET THE BLOOD VESSELS AND THEIR BASAL LAMINA

Around their basal process, RGC secrete a layer of ECM, to form the basement membrane, and transcriptome analyses of fetal human and embryonic mouse VZ, sub-VZ, and cortical plates, revealed elevated expression of genes related to cell adhesion and cell–ECM interactions (collagens, laminins, proteoglycans, and integrins) indicating their functional relevance (Fietz et al., 2012). Deletion of integrin function during brain development promotes process detachment, apoptosis, and altered neurogenesis (Graus-Porta et al., 2001; Campos et al., 2004; Belvindrah et al., 2007; Shen et al., 2008; Loulier et al., 2009; Fietz et al., 2010; Kazanis et al., 2010; Marthiens et al., 2010; Theocharidis et al., 2014; Chou et al., 2018), revealing altogether a structural role of integrin signaling within the niche, and in the maintenance of polarity, regulation of embryonic NSC pools asymmetric cell division, cortical expansion and neurogenesis.

Adult NSC are also polarized, and distinct stem-cell domains have been defined along their radial morphology. The basal domain corresponds to specialized long processes that contact directly BV, and the majority of dividing cells in the SEZ proliferate in the immediacy of BV which, ensheathed by their laminin-rich BL, provide the vascular compartment of the niche (Shen et al., 2008; Tavazoie et al., 2008). In fact, dividing NSC (and TAP) are directly associated with vasculature, establishing cell-ECM contacts with the endothelial BL (Shen et al., 2008). SEZ cells express differential levels of ECM receptors which appear to correlate with their mitotic status, rather than with a cell identity: for instance, quiescent NSC (qNSC) express low levels of α6β1-integrin, syndecan-1, and lutheran, whereas their levels increase in activated NSC (aNSC) and mitotic TAP (Shen et al., 2008; Kazanis et al., 2010; Morizur et al., 2018).

ECM *via* integrins regulates SEZ-cells proliferation and stemness, at least within this vascular context. Stromal-derived

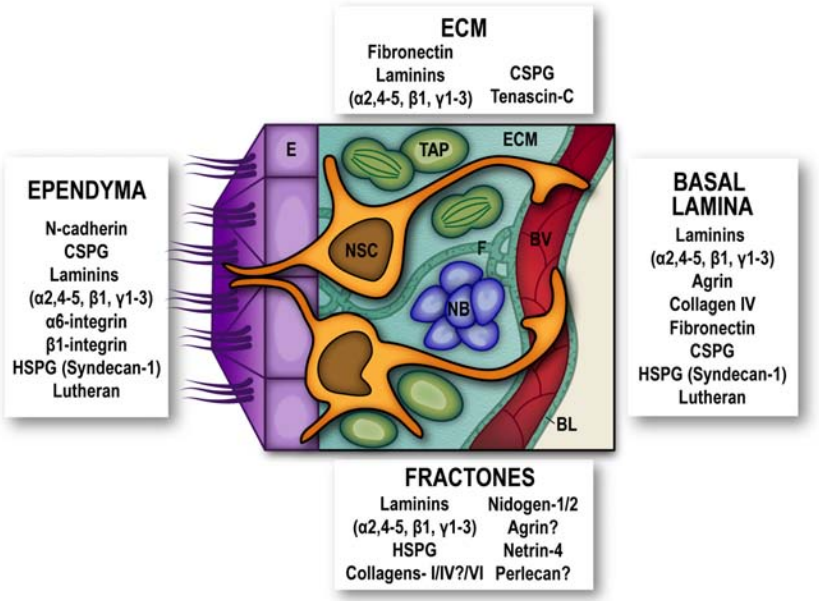


**FIGURE 1 |** Extracellular matrix and adhesion molecules are SEZ niche components. Schematic representation of the subependymal niche showing cellular populations (multiciliated ependymal cells –E, purple-, neural stem cells –NSC, orange-, transit amplifying progenitors –TAP, green-, neuroblasts –NB, blue- and blood vessels –BV, red-) and specific extracellular matrix proteins and adhesion molecules reported to be present subependymal zone extracellular matrix (ECM), in basal lamina (BL), fractones (F), and ependymal cells. Question marks are present where conflicting reports have been published. For the sake of clarity other niche elements such as microglia, non-neurogenic astrocytes and innervation have been omitted from the schematic. HSPG, heparan-sulfate proteoglycans; CSPG, chondroitin sulfate proteoglycans.

factor 1 (SDF1)- and CXC chemokine receptor 4 (CXCR4) upregulate EGFR and α6-integrin in aNSC and TAP, contributing to homing of SEZ progenitors to endothelial cells and to their proliferation by increasing their binding to laminin (Kokovay et al., 2010). *In vitro*, endothelial-derived laminin sustains proliferation and stemness of SEZ cells in brain endothelial-neurosphere cell co-cultures, process that is dependent on α6β1-integrin, *via* activation of the Notch and mTOR signaling pathways (Rosa et al., 2016). On the other hand, loss of integrin-linked kinase (ILK), enhances proliferation by over-activation of JNK (Porcheri et al., 2014). β1-integrin signaling suppresses astrocytic differentiation (Pan et al., 2014), and the carbohydrate-binding protein Galectin-1 interacts with β1-integrin to regulate the number of neural progenitors as well as new migrating neurons. This has a relevant implication on regeneration, as these new-born neurons enhanced recovery from behavioral deficits resulting from brain damage (Sakaguchi et al., 2006, 2010; Ishibashi et al., 2007). Contrasting downstream signals can be elicited by specific integrin ligands; transforming growth factor beta 1 (TGFβ1), an anti-inflammatory cytokine, exerts pro-neurogenic effects on SEZ NSC regulating a set of genes involved on the integrin pathway (Radice et al., 2015), and β8-integrin promotes proliferation of SEZ cells and in migrating neuroblasts, potentially through TGFβ1 as well (Mobley et al., 2009; Mobley and McCarty, 2011).

Although not considered strictly speaking as "cell adhesion," it is important to highlight that vascular endothelial cells can also provide NSCs with a pro-quiescence environment by means of direct cell-cell contacts of NSCs with BV, since it has been shown that endothelial ephrinB2 and Jagged suppress NSC proliferation while maintaining stemness (Ottone et al., 2014). Also, cell–cell interactions with other niche dweller populations work in a negative feedback loop to prevent NSC exhaustion. Niche-residing non-neurogenic astrocytes, for instance, secrete delta-like homolog (Dlk1) that binds its own membrane-bound isoform expressed in the surface of NSC to regulate their self-renewal (Ferron et al., 2011). Additionally, NSCs and TAPs express surface-bound Notch-ligand Dll1 to sustain quiescence in qNSC that specifically express Notch2 receptor (Kawaguchi et al., 2013; Llorens-Bobadilla et al., 2015).

## ADHESION AT THE APICAL END: REACHING THE EPENDYMAL WALL AND BEYOND

Distance from the EC/ventricle also seems to have a strong role limiting proliferation (Kazanis et al., 2010), indicating that elements involved in the maintenance of topology have functional roles in the SEZ neurogenic activity. According to their conserved apico-basal morphology, NSC directly contact the ependymal layer, with apical processes serving as grips that provide structural integrity. At the tip of the apical processes, a primary cilium pokes out from the ventricle wall serving as an antenna for the NSC (Mirzadeh et al., 2008).

Embryonic RGC bipolar radial morphology is supported at their apical tip through N-cadherin mediated AJs, to attach to the luminal surface of the VZ and neighboring cells (Hirano and Takeichi, 2012; Miyamoto et al., 2015). Compromised expression of N-cadherin in embryonic NSC leads to VZ/sub-VZ disruption, displacement of NSCs into the CSF, hydrocephalus, atypical neurogenesis and randomization of the intra-cortical structures and formation of periventricular heterotopias (Kadowaki et al., 2007; Gil-Sanz et al., 2014; Guerra et al., 2015; Jossin et al., 2017).

Attempts to inactivate N-cadherin specifically in postnatal/adult NSC have been made and, as in the embryo, N-cadherin-mediated adhesion is paramount to preserve the integrity of the adult SEZ and essential for the maintenance of NSC. hGFAP-Cre mice transgenic strain was used to evaluate the effect of N-cadherin in NSC from the SEZ. However, since both adult NSC and ependymocytes derive from RGC, which activate this promoter during development, unsurprisingly, both populations appeared drastically affected. The mutant displayed severe disassembly of the ependymal barrier concomitant with SEZ hyperplasia and increased proliferation of NSC, revealing that N-cadherin anchors act as quiescence signals (Porlan et al., 2014). In adult mice, acute inactivation of N-cadherin either in the whole SEZ or only in ECs showed an increase in NSC proliferation. In the case of ependymal inactivation, an additional denudation of the ventricle wall sheathing, in line with a previous report in which electroporation of a dominant-negative version of N-cadherin provoked ependymal loss and protrusion of the SEZ cells into the ventricle (Barnabe-Heider et al., 2008). These experiments clearly indicated that N-cadherin does indeed maintain the cytoarchitecture of the adult neurogenic niche and that this is functionally related to the activation status of residing stem cells (Porlan et al., 2014). E-cadherin, on the other hand, is the main component of the *zonula adherens* in non-neural epithelia, but maybe due to its secondary role in neural tissue, deletion of E-cadherin in the adult SEZ did not result in severe disruption of its cytoarchitecture, although still caused defects on NSC self-renewal (Karpowicz et al., 2009). Likewise, the lateral membrane adaptor protein Ank3 is critical for differentiation of EC and consequently, for neurogenesis (Paez-Gonzalez et al., 2011).

Integrins also contribute to the attachment of apical processes in embryonic RGC (Lathia et al., 2007; Loulier et al., 2009). In the adult SEZ, β1-integrin upregulates in NSC that activate to repopulate the niche after a depletion paradigm with antimitotic drugs. Also, injection of function-blocking antibodies in the adult ventricle in homeostasis results in disruption of the ventricular surface, increased TAP proliferation and invasion of neuroblast clusters within the ventricle, though NSC remained unaffected (Kazanis et al., 2010).

Preservation of quiescence and transition into an active proliferating state is an extremely regulated process. Therefore, is not surprising to find that extrinsic and intrinsic factors fine-tune the adhesive properties of the niche to retain stem cells and regulate their activation, adding another layer of complexity to the microenvironment. For example,

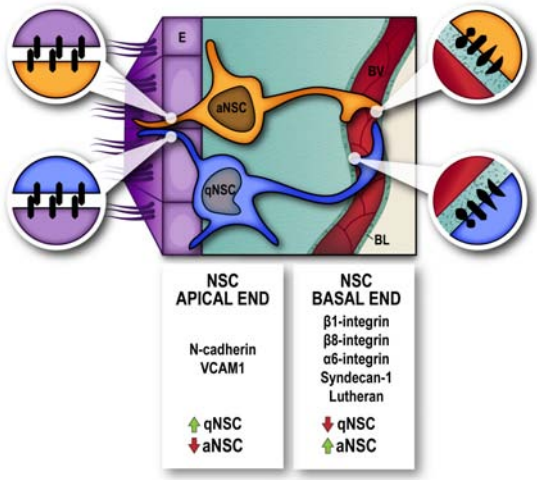


**FIGURE 2 |** Molecules involved in cell-cell adhesion and interaction with the niche extracellular matrix, actively participate in the regulation of neural stem cell dormancy/activation. Cell-cell junctions between Neural Stem Cells (NSC) and Ependyma (E) at the apical end (magnified circles, left) and Cell–Extracellular matrix interactions between NSC and endothelial basal lamina (BL) at the basal end (magnified circles, right), in the vicinity of blood vessels (BV), regulate the quiescence-activation state of NSC. High levels of N-cadherin and VCAM1 serve to maintain position and dormancy of quiescent neural stem cells (qNSC) within the niche by their apical end, whilst integrins α6/β1 and β8 and syndecan-1 and lutheran receptors are upregulated in neural stem cells that are actively proliferating (aNSC).

N-cadherin dependent activation of NSC can be modulated by the proteolytic activity of specific proteases, such as Mmp24-MT5 that cleaves N-cadherin to properly activate NSC under physiological and regenerative conditions (Porlan et al., 2014). Furthermore, in experimental demyelination, A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) processes N-cadherin in response to an activation signal initiated by EGFR (Klingener et al., 2014) again supporting the idea that dynamical regulation of adhesion to the niche by proteolysis can recruit NSC on demand for active proliferation. Interestingly, MMP12 another matrix metalloproteinase, regulates EC maturation and SEZ output modulating NSC quiescence (Shan et al., 2018).

Vascular Cell Adhesion Molecule-1 (VCAM1) is also highly expressed by NSC, specifically at their end-feet in the center of pinwheels to maintain NSC quiescence. Blocking VCAM1 function severely disrupts the niche structure, affecting ependymal cytoarchitecture and generating loss of pinwheels. This also produces an increase of OB neurogenesis, further showing that exogenous manipulation of the adhesive properties of the niche could indeed have an impact of neuronal progeny (Kokovay et al., 2012). Interestingly, VCAM1 increases as a response to IL-1β, signaling *via* NOX2-produced reactive oxygen species, to maintain NSC indicating that it can sense the environment, responding to chemokines involved in tissue repair (Kokovay et al., 2012).

## ADHESION AND REGULATION OF QUIESCENCE BY THE NICHE

Notwithstanding their potential for generating differentiated progeny, NSC display a functional quiescence in adulthood (Fuentealba et al., 2015; Furutachi et al., 2015), and in contrast to other tissues, adult NSC can be found at different states of activation (Daynac et al., 2013; Codega et al., 2014; Mich et al., 2014; Llorens-Bobadilla et al., 2015; Chaker et al., 2016; Kalamakis et al., 2019). Switch from qNSC to aNSC state can be induced by selective elimination of neural progeny (Daynac et al., 2013; Codega et al., 2014; Mich et al., 2014), and quiescence appears to be a mechanism to protect NSC pools throughout life and hence maintain homeostasis and tissue regeneration. Failure to restrict mitotic activation of NSC leads to premature depletion of the niche (Doetsch et al., 2002; Molofsky et al., 2003, 2005, 2006; Kippin et al., 2005; Marques-Torrejon et al., 2013; Porlan et al., 2013), and in the aged brain, NSC quiescence increases in what has been interpreted as a mechanism to avoid full exhaustion (Luo et al., 2006; Bouab et al., 2011; Silva-Vargas et al., 2016; Bast et al., 2018; Kalamakis et al., 2019). In light of all these evidences, the quiescent state of NSC is now considered as an actively regulated condition, in contrast to the classic vision of it being a mere passive quality. Interestingly, the niche appears to play an essential role in the regulation of NSC fate and number by controlling the reversible transition between the quiescent and active NSC compartments (Llorens-Bobadilla and Martin-Villalba, 2017; Basak et al., 2018; Kalamakis et al., 2019).

Many efforts have been made to reveal the molecular signature of qNSC, and strategies to prospectively isolate dormant NSC from the adult SEZ have used a combination of stem cell/progeny markers to analyze the transcriptome of non-proliferative vs. aNCS (Capela and Temple, 2002; Pastrana et al., 2009; Beckervordersandforth et al., 2010; Daynac et al., 2013; Codega et al., 2014; Mich et al., 2014; Llorens-Bobadilla et al., 2015; Chaker et al., 2016; Morizur et al., 2018). Transcriptome analyses have helped to disclose the integration of signals from the microenvironment that actively maintain the quiescent state. Not surprisingly, a very significant contribution of molecules involved in cell adhesion and interaction with the niche milleu have been found to actively maintain dormancy. Amongst the most represented GO categories in qNSC are cell–cell adhesion, ECM-response and anchorage-dependent niche signals, cell communication, and signaling receptors. Most genes enriched in qNSC encoded membrane-associated proteins, underscoring the key role played by the microenvironment in the regulation of the quiescent state in the adult SEZ. Of relevance, VCAM1 and N-cadherin previously reported as regulators of NSC quiescence (Kokovay et al., 2012; Porlan et al., 2014) were specifically found increased in prospectively isolated qNSC, as well as other cadherins, protocadherins, neural cell adhesion molecule 1/2 and ECM-components, whereas syndecan-1 was overexpressed specifically in aNSC (Morizur et al., 2018) as previously described (Kazanis et al., 2010). Interestingly, syndecans play their functions as cell surface receptors by acting as both adhesion and docking receptors, and thus are capable of regulating both intra- and extracellular activities and can recruit soluble growth factors, matrix metalloproteinases, chemokines and cytokines to the cell surface (Kwon et al., 2012) (**Figure 2**).

## CONCLUDING REMARKS

Within the SEZ niche ecosystem, NSC necessarily communicate with their neighboring cellular populations and surrounding matrix, and such interaction is essential for the maintenance of stem cell identity and control of the timing and mode of cell division. Indeed, cell-to-cell contacts and cell-ECM adhesion not only provide tissue integrity, cell orientation and topology, but actively foster NSC self-renewal and maintenance by either placing stem cells in proximity of different signaling sources, or directly participating in the signaling process, since most adhesion molecules act as receptors and signal transductors. Many of the signaling cues that maintain NSC positioning, and some of the molecular mechanisms that trigger the switch from dormancy toward proliferation in physiological and pathological conditions to promote tissue regeneration, are starting to emerge. Amongst these, ECM and cell adhesion molecules play a crucial role, highlighting that the niche allows a relative plasticity whose manipulation provides an important window for regeneration.

## AUTHOR CONTRIBUTIONS

EP conceived the manuscript. EP and JMM-R wrote the manuscript.

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# Reactive Disruption of the Hippocampal Neurogenic Niche After Induction of Seizures by Injection of Kainic Acid in the Amygdala

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Adult neurogenesis persists in the adult hippocampus due to the presence of multipotent neural stem cells (NSCs). Hippocampal neurogenesis is involved in a range of cognitive functions and is tightly regulated by neuronal activity. NSCs respond promptly to physiological and pathological stimuli altering their neurogenic and gliogenic potential. In a mouse model of mesial temporal lobe epilepsy (MTLE), seizures triggered by the intrahippocampal injection of the glutamate receptor agonist kainic acid (KA) induce NSCs to convert into reactive NSCs (React-NSCs) which stop producing new neurons and ultimately generate reactive astrocytes thus contributing to the development of hippocampal sclerosis and abolishing neurogenesis. We herein show how seizures triggered by the injection of KA in the amygdala, an alternative model of MTLE which allows parallel experimental manipulation in the dentate gyrus, also trigger the induction of React-NSCs and provoke the disruption of the neurogenic niche resulting in impaired neurogenesis. These results highlight the sensitivity of NSCs to the surrounding neuronal circuit activity and demonstrate that the induction of React-NSCs and the disruption of the neurogenic niche are not due to the direct effect of KA in the hippocampus. These results also suggest that neurogenesis might be lost in the hippocampus of patients with MTLE. Indeed we provide results from human MTLE samples absence of cell proliferation, of neural stem cell-like cells and of neurogenesis.

**Keywords: neural stem cells, hippocampal neurogenesis, seizures, gliosis, amygdala**

## INTRODUCTION

In the hippocampus of most mammals, including humans (Eriksson et al., 1998; Moreno-Jiménez et al., 2019), neurogenesis continues postnatally (Altman and Das, 1965) and throughout adulthood due to the existence of a population of neural stem cells (NSCs) with neurogenic (Seri et al., 2001) and gliogenic potential (Encinas et al., 2011). Neuronal activity is a major regulator of adult hippocampal NSCs which respond differentially to different levels of neuronal activity. Tonic

gamma-aminobutyric acid (GABA) promotes quiescence of NSCs while its reduction promotes their activation (entry into the cell cycle) (Song et al., 2012). As neuronal activity increases above physiological levels in experimental models of electroconvulsive therapy (electroconvulsive shock, ECS) (Segi-Nishida et al., 2008; Jun et al., 2015) or epilepsy (Huttmann et al., 2003; Indulekha et al., 2010) NSCs get activated in increasing numbers which in turn accelerates their depletion in the long term (Sierra et al., 2015) after an initial boost of neurogenesis (Parent et al., 1997). Furthermore, in an experimental model of mesial temporal lobe epilepsy (MTLE), that characterizes by seizures being originated in the hippocampus and related structures, NSCs undergo a profound alteration of their neurogenic program. Shortly after seizures NSCs transform into reactive NSCs (React-NSCs) and later into reactive astrocytes that contribute to hippocampal sclerosis, a pathological hallmark of MTLE consisting of neuronal death and reactive gliosis. React-NSCs get activated massively switching to a symmetric manner of cell division and transform into a reactive-like multibranched and thickened phenotype with overexpression of nestin and GFAP (Sierra et al., 2015). As a result neurogenesis is lost. The rodent MTLE model is based on a single injection of the glutamate receptors agonist kainic acid (KA) in the hippocampus (hcMTLE) (Bouilleret et al., 1999). Thus it could be argued that the strong effects observed in the neurogenic niche could be due to the direct effect of KA. Thus we wondered how NSCs and the neurogenic cascade would be affected in an alternative model of MTLE (aMTLE) based on the intra-amygdalar injection of KA (Mouri et al., 2008). In addition, we showed that in a similar fashion to what we found in the mouse models of MTLE, cell proliferation and neurogenesis are lost in the hippocampus of MTLE patients.

## MATERIALS AND METHODS

### Animals

Nestin-GFP transgenic mouse line (on a C57BL/6 background) was used for all procedures addressing tissue analysis (Sierra et al., 2015) except for three wild type mice used for the injection tracking (see below). At least three mice were used in each control (PBS) group and at least five were used for the aMTLE group per time point. All procedures on these mice were approved by the University of the Basque Country (EHU/UPV) Ethics Committee (Leioa, Spain) and the Comunidad Foral de Bizkaia (CEEA: M20/2015/236). C57BL/6 mice were used for the EEG studies. The procedures were approved by the Comité Ético Científico para el Cuidado de Animales y Ambiente, CEC-CAA of the Universidad Pontificia de Chile and the bioethics committee of the Chilean (Comision Nacional de Investigación Científica y Tecnólogica, CONICYT). Five mice were used in each control (PBS) and aMTLE group.

### Intra-Amygdalar Injection

PBS or KA was injected into the right basolateral-amygdala using the following coordinates: AP −1.4 mm, ML −3.1 mm, and DV −4.7 mm, using a dose of 100 nL of sterile PBS, or KA at 13 mM (1.3 nmol). Three mice were injected with fluorescein (CSFE) and sacrificed 24 h after to test the accuracy of the stereotaxic injection (**Figure 1**).

### Electroencephalographic Recordings

Neuronal activity was recorded by using a 32-channel silicon probe (Right hemisphere: A1 × 32-Poly3-6 mm-50-177; left hemisphere; 32 channel-4 shank silicon probe, Buzsáki 32. Neuronexus, mean resistance 1 MΩ) stained with DiI (for a subsequent anatomical identification). Electrodes were located as close as possible to the dorsal CA1 stratum pyramidale, for which the electrode was descended 0.8–1 mm, until ripple oscillations were visually detected online. Electrical activity was recorded with an electrical amplifier (Intan RHD 2132 amplifier board connected to an RHD2000 evaluation system; Intan Technologies). Local field potential (LFP; sampling rate 20 kHz) were digitally filtered between 0.3–2 kHz.

### 5-Bromo-2′-deoxyuridine (BrdU) Administration

BrdU was administered intraperitoneally (four injections 2 h-apart)on the 2nd day after the intra-amygdalar injection (four injections 2 h-apart).

### Immunohistochemistry and Cell Quantification

Experiments were performed essentially as described before following methods optimized for the use in transgenic mice (Encinas et al., 2006, 2011; Encinas and Enikolopov, 2008; Sierra et al., 2015).

### Image Capture

All fluorescence immunostaining images were collected employing a Leica SP8 laser scanning microscope and their corresponding manufacturer's software following protocols optimized for stereotaxic quantification and quantitative image analysis (Encinas et al., 2011; Sierra et al., 2015).

### Human Tissue

Human samples from individuals with MTLE. Freshly resected hippocampi from adult drug-resistant MTLE patients were obtained from the Basque Biobank at the Cruces University Hospital (Bilbao, Spain) with the patient's written consent and with approval of the University of the Basque Country Ethics committee (CEISH/154/2012).

### Statistical Analysis

SigmaPlot (San Jose, CA, United States) was used for statistical analysis. For the analysis of pairs of groups a Student's *t*-test, a Mann–Whitney Rank Sum test or a One-column sample test were performed.

For an extended description of see sections "Materials and Methods" and "**Supplementary Material and Methods**" in **Supplementary Material**.

## RESULTS

We first confirmed the accuracy of the coordinates used to target the basolateral nucleus of the amygdala by injecting fluorescein (CFSE) and co-staining for DAPI and the neuronal marker Tbr1 to assure identification of the exact area (**Figure 1A**). Then we analyzed the effect on neuronal activity of the intra-amygdalar injection of KA to confirm its validity as a model of MTLE to study alterations of hippocampal neurogenesis. In hcMTLE a single injection is enough to trigger an initial set of seizures and then spontaneous seizures become chronic (Bouilleret et al., 1999; Sierra et al., 2015). Here we confirmed the existence of initial seizures behaviorally (using the Racine scale) on the day of the surgery and then registered neuronal activity by electroencephalographic recordings (EEG) through bilateral electrode insertion in the hippocampus (**Figure 1B** and **Supplementary Figure 1**) at a later time point. Seizures (**Figure 1B**), as well as ripples (**Figure 1C**) and epileptiform discharges (**Figure 1D**), were registered in both the ipsi and contralateral hippocampus of aMTLE mice 2 months after the KA injection. The frequency of the epileptiform activity was quantified by automatic detection of epileptiform events set up using baseline activity under anesthesia in control (PBS-injected) and aMTLE mice (**Supplementary Figure 1**). The

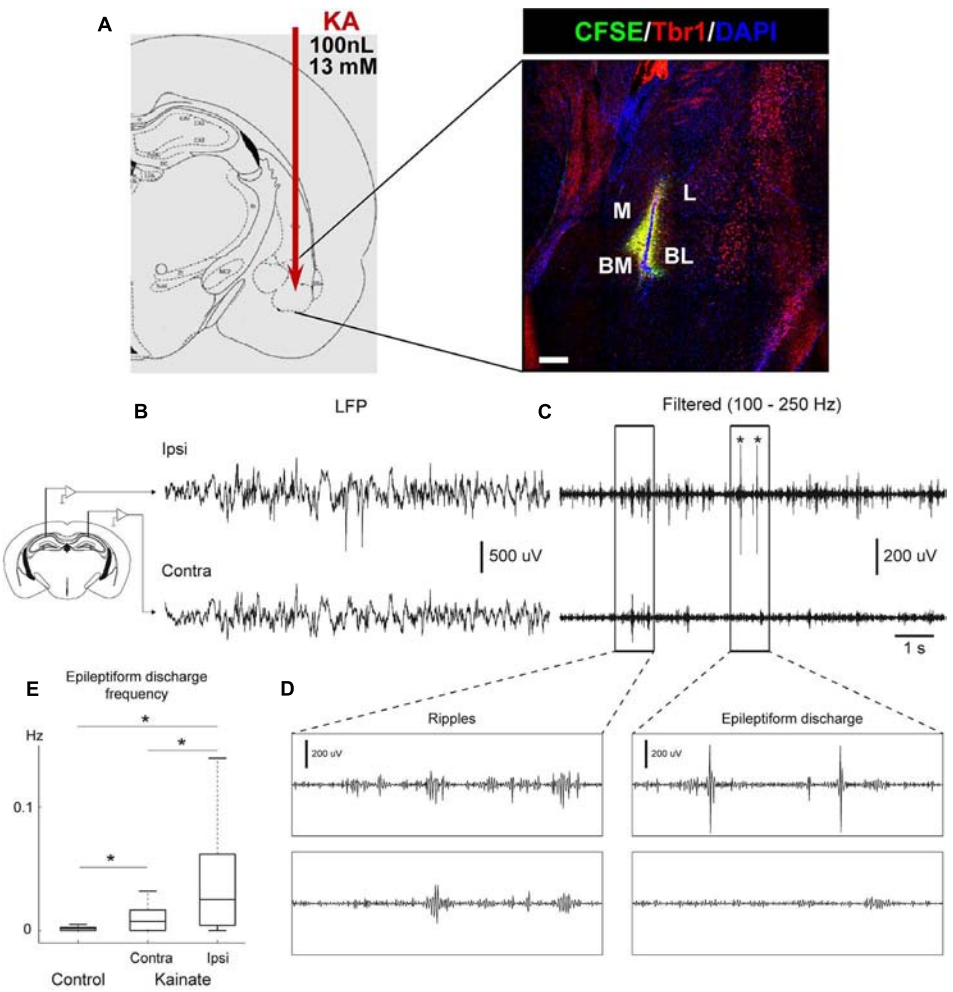


**FIGURE 1 |** Intra-amygdalar injection of KA induces seizures and epileptiform activity in the ipsi and contralateral hippocampus. **(A)** KA was stereotaxically injected via canula into the basomedial basolateral nucleus of the amygdala (BM/BL, 100 nL at a concentration of 13 mM). The accuracy of the injection was tested by injecting fluorescein (CFSE) and sacrificing mice 24 h later. Co-staining with Tbr1 and DAPI was performed to assure identification of the BL. M, medial nucleus. BM, basomedial nucleus. L, lateral nucleus. CEP, endopiriform cortex. Scale bar is 50 μm in **(A)**. **(B)** Intrahippocampal electrophysiological recordings performed 2 months after KA injection. Local field potential (LFP) activity recorded in the hippocampal pyramidal layer from one electrode in the ipsilateral hemisphere (KA injected side, Ipsi, upper panel) and one electrode in the contralateral hippocampus (uninjected side, Contra, lower panel) in a urethane anesthetized mouse. **(C)** Filtered LFP (100–250 Hz). Asterisks depict epileptiform discharges. **(D)** Zoomed in activity depicts wave ripple complex during basal activity (left) and epileptiform discharge (right) for filtered signal shown in **(C)**. **(E)** Epileptiform discharge events were automatically detected in both control and KA mice (see section "**Supplementary Material**" for details). Epileptiform events were more abundant in KA mice ($n$ = 52 sessions, 5 animals) compared to control mice ($n$ = 33 sessions, 5 animals). Moreover, in KA mice, the hemisphere ipsilateral to KA injection (Ipsi) had a higher rate of epileptiform events compared to contralateral (Contra) hemisphere (Kruskal–Wallis test, $P = 1.049 \times 10^{-7}$).

frequency of the epileptiform events was significantly higher in the contralateral hippocampus of aMTLE mice ($n$ = 5) than in controls (ipsilateral, $n$ = 5). In the ipsilateral hippocampus of aMTLE mice ($n$ = 5) epileptiform events were significantly more frequent than in the control and the contralateral aMTLE hippocampus (**Figure 1E**).

We proceeded to analyze the dentate gyrus of mice injected either with PBS or KA in the amygdala and sacrificed 1 week later (**Figure 2**). We observed an overall overexpression of GFAP and Nestin-GFP typical of reactive gliosis (**Figure 2A**). The number of React-NSCs, as previously described for hcMTLE (Sierra et al., 2015), was drastically increased in the aMTLE mice compared to the PBS-injected mice (**Figure 2B**). React-NSCs presented several thickened prolongations emerging from the soma, had lost the fine broccoli-like apical arborization, overexpressed Nestin-GFP and GFAP and frequently moved into the granule cell layer (GCL) away from the subgranular zone (SGZ) (**Figure 2E**). Quantification of these morphological parameters is shown in **Figure 3**. To evaluate cell proliferation we administered BrdU on the second day (four injections, 2 h apart) after the intra-amygdalar injection of KA or PBS. The overall number of BrdU-labeled cells was significantly increased in the SGZ + GCL (**Figures 2C,D**) as well as in the hilus (**Figures 4A,B**) where astrocytes (GFAP-positive, Nestin-GFP-negative cells) and reactive astrocytes (Nestin-GFP/S100ß-positive cells) accounted for most of the BrdU-labeled cells (**Figures 4C–F**). As expected, BrdU incorporation by React-NSCs (Nestin-GFP/GFAP-positive and negative for S100ß) was significantly higher than their normal NSCs counterparts of the PBS-injected mice (**Figures 2E–G**) both in percentage (**Figure 2F**) and in total numbers (**Figure 2G**). Astrocyte proliferation was almost absent in the PBS animals but was increased in the KA mice (**Figure 2H**). We quantified, also in the SGZ + GCL, the presence of reactive astrocytes (Nestin-GFP/S100ß-positive cells, **Supplementary Figure 2**) to evaluate the development of gliosis in the dentate gyrus. Reactive astrocytes were mostly absent in the PBS mice whereas their proportion among total astrocytes (**Figure 2I**) as well as their total number (**Figure 2J**) increased in the aMTLE mice. Together these results show the early transformation of NSCs into React-NSCs in response to the local neuronal hyperexcitation triggered by the intra-amygdalar injection of KA (**Figure 5E**). Furthermore, we confirmed the induction of React-NSCs (**Figure 5A**) as well as incremented NSCs/Reactive-NSCs activation (entry into the cell cycle) (**Figure 5B**), and overall cell proliferation (**Figures 5C,D**), in the contralateral dentate gyrus (all measurements performed in the SGZ + GCL). Branching (**Figures 5F,G**), as well as thickening of the processes (**Figures 5H,I**), morphological hallmarks of React-NSCs, were significantly increased in aMTLE. Finally, we analyzed cell death in the SGZ + GCL, another hallmark of hippocampal sclerosis in MTLE, and found that it was significantly increased in the ipsilateral and the contralateral hippocampus of the aMTLE mice compared to the PBS-injected ones (**Supplementary Figure 3**). As in hcMTLE we found that in aMTLE reactive gliosis and induction of React-NSCs, as well as cell death, extended along the whole septo-temporal length of the dentate gyrus. Neuroblasts (DCX-positive cells) with abnormal morphology and location were found in both the ipsi and contralateral dentate gyrus (data not shown).

In order to investigate the effects of seizures on the neurogenic niche in the longer term we studied animals that were sacrificed 6 weeks after the intra-amygdalar injection of KA (**Figure 6**). We first observed a marked gliosis in the dentate gyrus characterized by a massive generation of reactive astrocytes and/or React-NSCs. In sharp contrast with the PBS-injected mice, multibranched cells strongly expressing Nestin-GFP and GFAP were prominently distributed in the hilus, the SGZ, the GCL and to a lower extent into the molecular layer of the aMTLE mice (**Figure 6A**). The number of React-NSCs, described as Nestin-GFP/GFAP cells (with the morphological criteria explained before, **Figure 2**) was significantly increased in the KA mice (**Figure 3B**). We next quantified the number of BrdU cells (BrdU was administered in day 2 after the KA injection, the same as in the 1 w experiments) to assess differentiation. There were significantly more BrdU-positive cells in the SGZ + GCL of KA mice most likely reflecting the initial increase of cell division found at the 1-week time point (**Figure 6C**). BrdU-positive cells were also observed in the hilus (**Figure 6D**) where most of them were reactive astrocytes (**Figure 7**). We next assessed the BrdU-labeled population by cell types in the SGZ + GCL. The proportion of NSCs or React-NSCs in the KA mice, defined as Nestin-GFP (and GFAP)-positive cells lacking S100ß expression that were labeled with BrdU was significantly increased in the aMTLE animals (**Figures 6E,L**). As the total number of BrdU cells was increased, this translated in a significant increase in the total number of BrdU-labeled NSCs/React-NSCs (**Figures 6F,L**). BrdU-labeled reactive astrocytes (defined as S100ß and Nestin-GFP-positive cells) were absent in the control mice whereas they were abundant in the KA mice as observed by relative proportion over the total BrdU-positive population (**Figure 6G**) and in total number as quantified in the SGZ + GCL (**Figure 6H**). Reactive astrocytes were absent in the hilus of PBS-injected mice but were abundant in aMTLE mice (**Figure 7**). Due to the prominent reactive gliosis (**Figure 7A**) and the notorious increase in cell proliferation (**Figure 7B**) observed in the hilus the generation of astrocytes (**Figures 7C,D**) and of reactive astrocytes (**Figures 7E,F**) was assessed also in this region. A significant increase in astrogliogenesis and reactive astrogliogenesis was confirmed. Finally, we analyzed neurogenesis by colocalization of BrdU with the neuronal marker NeuN (**Figure 6K**). We found that neurogenesis was greatly impaired. The proportion of BrdU/NeuN-positive cells among the total BrdU population was significantly diminished (**Figure 6I**). In spite of the larger population of BrdU-positive cells, the total number of BrdU/NeuN-positive cells was also significantly decreased in the KA mice (**Figure 3J**). We checked also the expression of DCX, the specific marker of neuroblasts/immature neurons and confirmed the almost total absence of neurogenesis associated with reactive gliosis in the dentate gyrus (**Supplementary Figures 4A,B**). Noteworthy, the few DCX-positive cells that were found in the KA mice presented the morphological abnormalities that characterize aberrant neurogenesis (**Supplementary Figures 4A,B**). These results are very similar to those we reported for hcMTLE

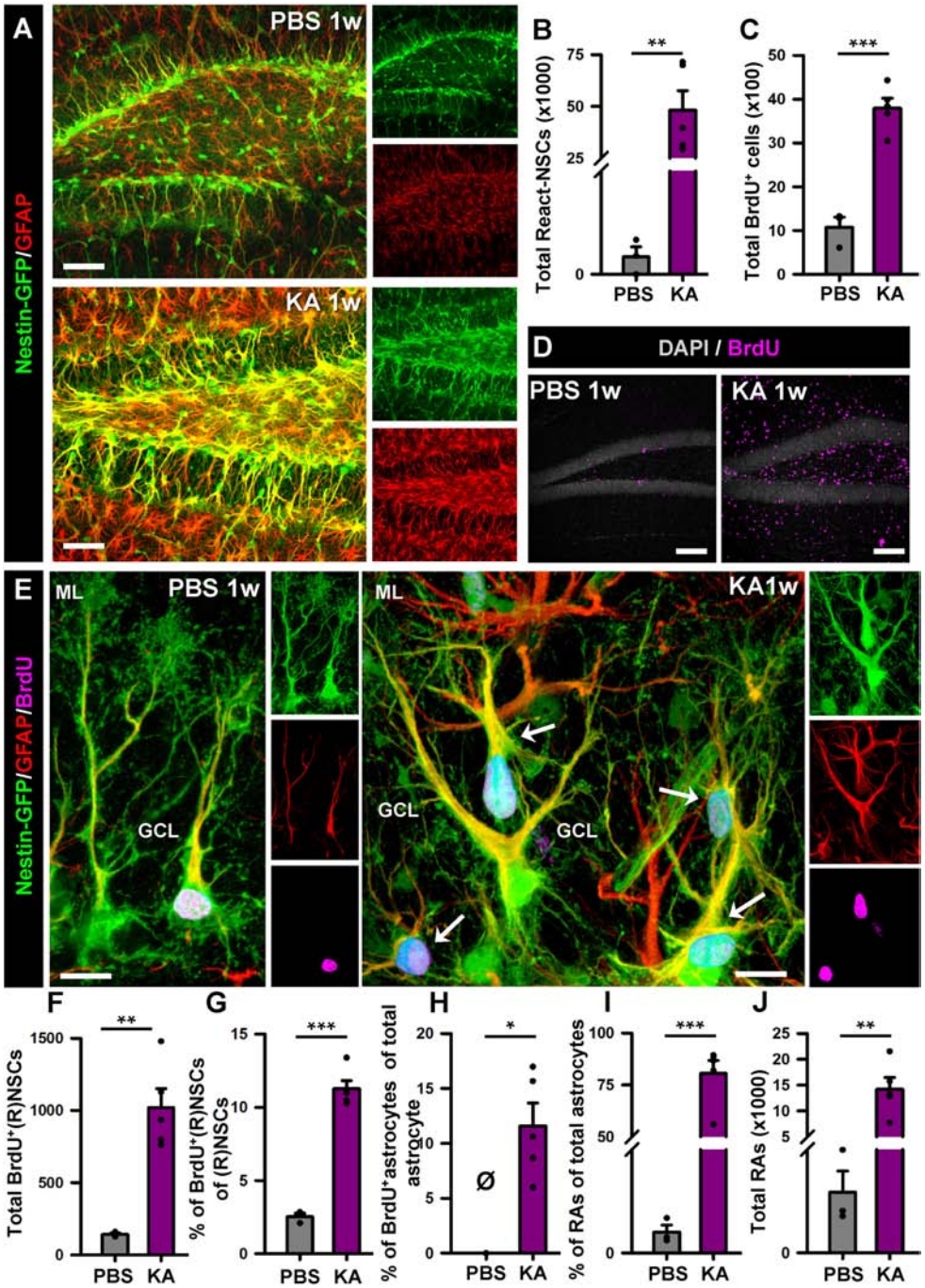


**FIGURE 2 |** Intra-amygdalar injection of KA induces gliosis and React-NSCs in the dentate gyrus in the short term (1 week). BrdU (four injections, 2 h-apart) was injected the 2nd day after the KA injection. **(A)** Representative confocal microscopy images showing the reactive gliosis developed in the dentate gyrus. **(B)** Quantification of the number of React-NSCs (Nestin-GFP/GFAP-positive, S100ß-negative cells with reactive morphology). **(C)** Quantification of the number of BrdU-positive cells. **(D)** Representative confocal microscopy images for BrdU staining. **(E)** Representative confocal microscopy images showing a NSC in a control mouse and React-NSCs in a KA mouse labeled with BrdU (arrows). **(F–I)** Quantification of: **(F)** total number of BrdU-labeled NSCs or React-NSCs (Nestin-GFP/GFAP-positive, S100ß-negative); **(G)** percentage of BrdU-label NSCs or React-NSCs among the total number of NSCs/React-NSCs; **(H)** percentage of BrdU-labeled astrocytes (BrdU/GFAP-positive, Nestin-GFP-negative cells) among the total number of astrocytes (GFAP-positive Nestin-GFP-negative); **(I)** percentage of reactive astrocytes (Nestin-GFP/S100ß-positive cells) among the total number of astrocytes; and **(J)** total number of reactive astrocytes. All quantifications are referred to the SGZ + GCL. For quantifications regarding the hilus refer to (**Supplementary Figure 3**). Scale bar is 50 μm in **(A)** and **(D)**; and 10 μm in **(E)**. $n = 3$ for PBS and 5 for KA mice. $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$ by Student's *t*-test **(B,C,G)**; One-column sample *t*-test **(H)** and Mann–Whitney Rank Sum test **(I,J)**. Bars show mean ± SEM. Dots show individual data. The same settings for image acquisition were used for PBS and KA samples.

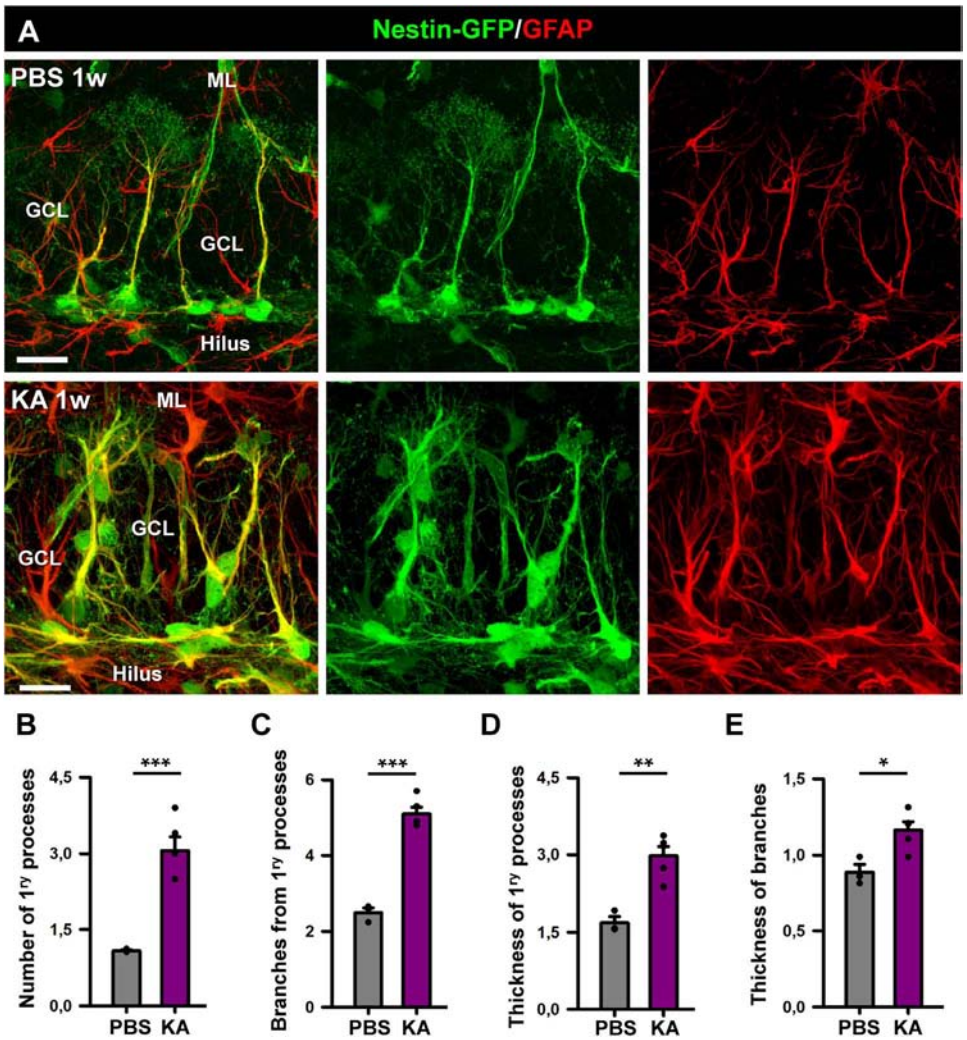


**FIGURE 3 |** Morphological parameters of React-NSCs. **(A)** Confocal microscopy images of NSCs in a control mouse (upper row) and React-NSCs in a KA mouse (lower row). **(B–E)** Quantification of number of primary processes (those directly emerging from the soma) **(B)**; number of secondary processes (those emerging from the primary processes) **(C)**; thickness of the primary process **(D)**; and secondary processes **(E)**. $n = 5$ per group. $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$ by Student's *t*-test. Bars show mean ± SEM. Dots show individual data.

and show how the neuronal activity of the hippocampus and related structures is an essential regulator of NSC activity and how seizures cause a drastic disruption of the hippocampal neurogenic niche.

We thus have observed in both aMTLE and the hcMTLE (Sierra et al., 2015) that reactive gliosis associates with abolished neurogenesis. We next investigated whether the same effect takes place in the human hippocampus. We analyzed hippocampi resected for therapeutic purposes from three patients (38, 46, and 56 years-old, all with hippocampal sclerosis ILAE type 1) of drug-resistant MTLE. We collected the samples as soon as they were removed from the patients to assure optimal conditions of tissue fixation. We studied the dentate gyrus and observed apparent granule cell dispersion (GCD) with the classical enlargement and loss of density of the GCL (**Figure 8A**). We also confirmed the presence of reactive gliosis using GFAP and S100β (**Figure 8B**) to identify astrocytes which presented the classical morphology of reactive astrocytes (**Figure 8C**). Importantly no cell resembling a putative NSC (radial morphology with soma located in the SGZ or lower part of the GCL) was found (**Figure 8C**). We cannot, however, make the claim that this result means that NSCs has transformed into React-NSC and ultimately into reactive astrocytes as reported to occur in the rodent models. We addressed the existence of cell division by using the mitosis marker Ki67. Ki67-positive cells were extremely rare in the dentate gyrus with none or just one cell found per human sample (**Figure 8D**). We also assessed the presence of neuroblasts (young migrating neurons) using immunostaining for DCX. No DCX-positive cell was found in the dentate gyrus of any of the human samples. In some samples a few scattered DCX-positive cells were found outside of the dentate gyrus (**Figure 8E**). The absence of cell proliferation and neurogenesis in the hippocampal

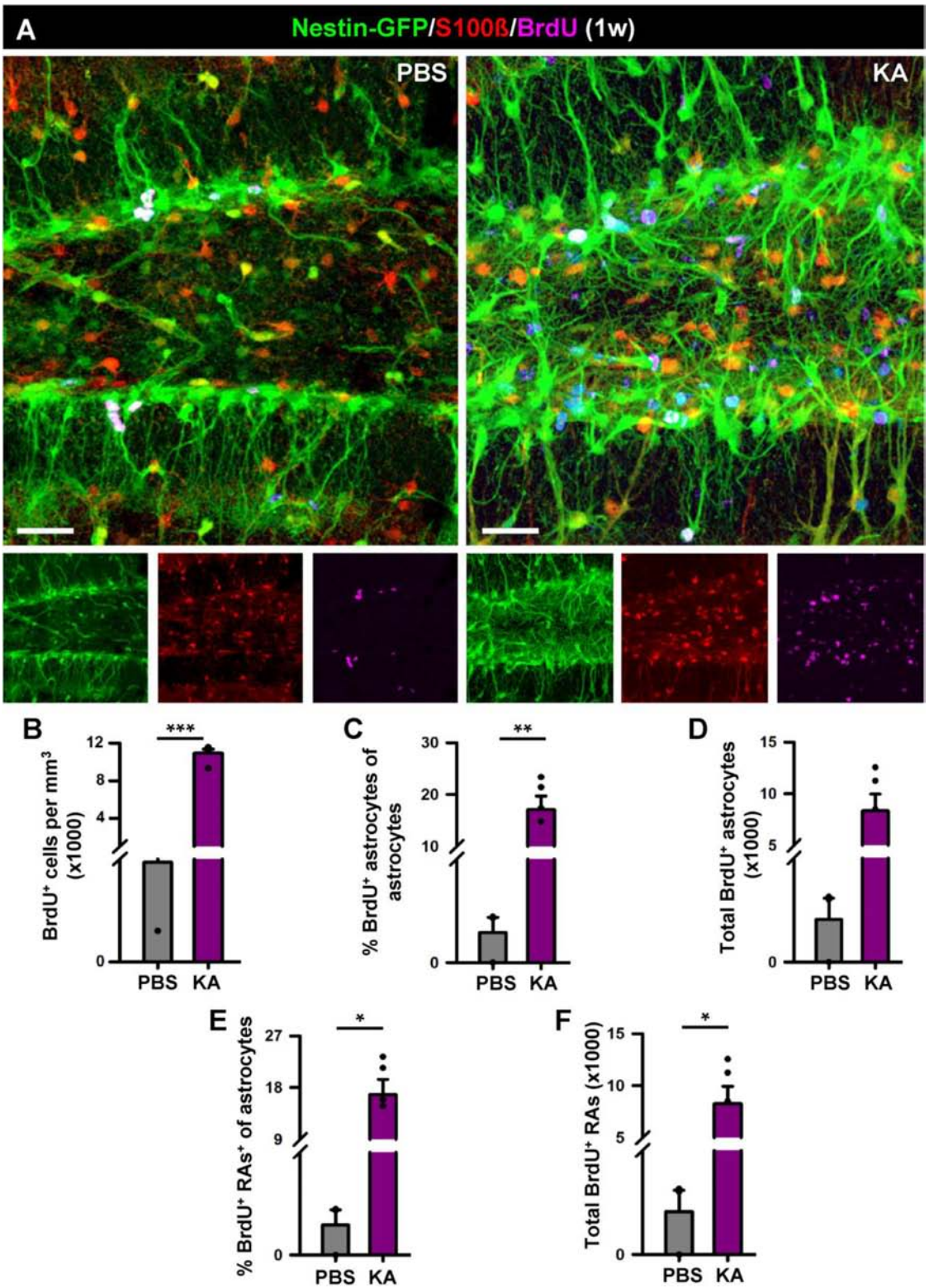


**FIGURE 4 |** Development of reactive gliosis in the hilus in the short term (1 week). **(A)** Representative confocal images of PBS (left panel) and KA (right) mice after staining for Nestin-GFP, BrdU and S100β to assess reactive gliosis. **(B–F)** Quantification of the density of $BrdU^+$ cells **(B)**; percentage of BrdU-labeled astrocytes among astrocytes **(C)**; total number of BrdU-labeled astrocytes **(D)**; percentage of BrdU-labeled of reactive astrocytes among astrocytes **(E)**; total BrdU-labeled reactive astrocytes **(F)**. Scale bar is 20 μm. $n = 5$ per group. $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$ by Student's *t*-test **(C,E)** and Mann-Whitney Rank Sum test **(B,D,F)**. Bars show mean ± SEM. Dots show individual data.

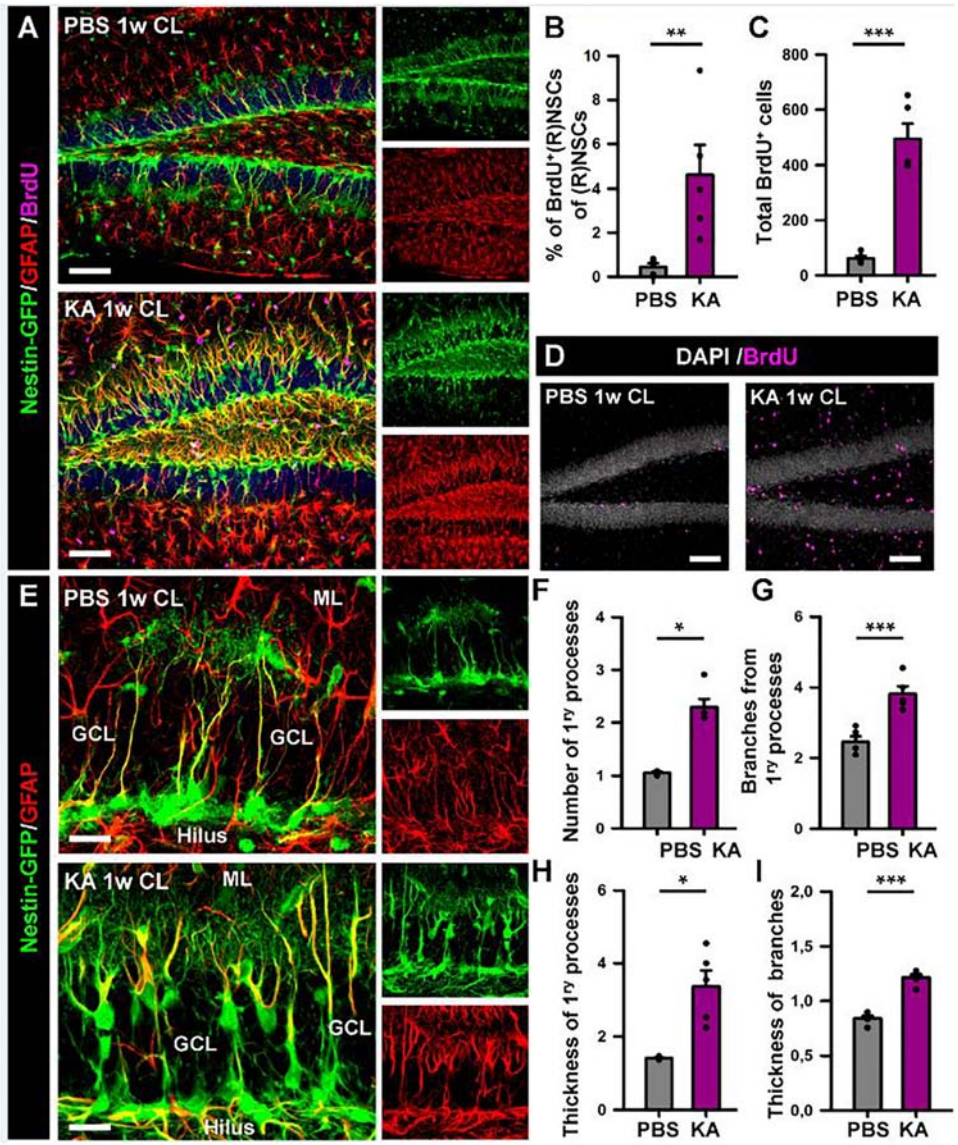


**FIGURE 5 |** Intra-amygdalar injection of KA induces gliosis and React-NSCs in the contralateral dentate gyrus. BrdU (four injections, 2 h-apart) was injected the second day after the KA injection. Animals were sacrificed 1 week after the PBS or KA injection. **(A)** Representative confocal microscopy images showing the reactive gliosis developed in the dentate gyrus. **(B)** Quantification of the percentage of BrdU-label NSCs or React-NSCs among the total number of NSCs/React-NSCs. **(C)** Quantification of the total number of BrdU-positive cells in the SGZ + GCL. **(D)** Representative confocal microscopy images for BrdU staining. **(E)** Confocal microscopy images of NSCs in a control mouse (upper row) and React-NSCs in a KA mouse (lower row). **(F)** Quantification of number of primary processes (those directly emerging from the soma); **(G)** Number of secondary processes (those emerging from the primary processes); **(H)** thickness of the primary process; and **(I)** secondary processes. $n = 5$ per group. Scale bar is 50 μm in **(A)** and 10 μm in **(E)**. $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$ by Student's *t*-test **(B,G,I)** and Mann–Whitney Rank Sum test **(C,F,H)**. Bars show mean ± SEM. Dots show individual data.

neurogenic niche of patients of MTLE are agreement with what we have observed herein and in our previous study of hcMTLE (Sierra et al., 2015).

## DISCUSSION

We had shown before that seizures induced in an experimental model of MTLE by intrahippocampal injection of MTLE have a profound effect on NSCs. Very soon after seizures NSCs become React-NSCs, i.e., they become multi branched with thicker processes overexpressing nestin and GFAP; lose their fine arborization in the molecular layer; migrate from the SGZ and closer to the molecular layer and get activated (enter mitosis) with much higher rate (Sierra et al., 2015). Finally, React-NSCs after several weeks become reactive astrocytes indistinguishable, at least by biomarker expression, morphology and location, to those derived from parenchymal astrocytes.

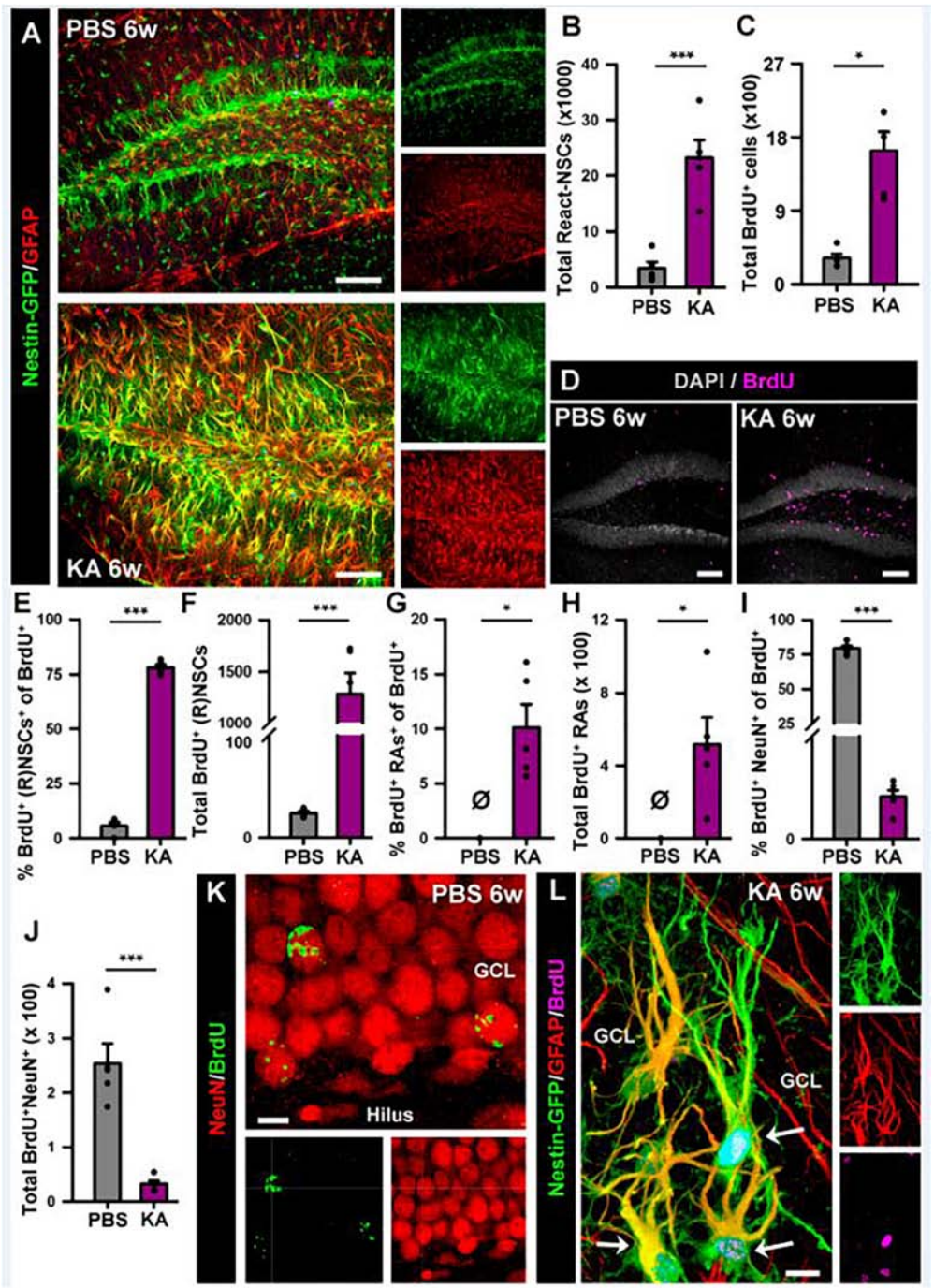


**FIGURE 6 |** Intra-amygdalar injection of KA induces gliosis and React-NSCs in the dentate gyrus in the long term (6 weeks). BrdU (four injections, 2 h-apart) was injected the 2nd day after the KA injection. **(A)** Representative confocal microscopy images showing the reactive gliosis developed in the dentate gyrus. **(B)** Quantification of the number of React-NSCs (Nestin-GFP/GFAP-positive, S100ß-negative cells with reactive morphology). **(C)** Quantification of the number of BrdU-positive cells. **(D)** Representative confocal microscopy images for BrdU staining. **(E–I)** Quantifications of: **(E)** percentage of BrdU-labeled NSCs (in PBS) or React-NSCs (in KA) among total BrdU-positive cells; **(F)** Total number of BrdU-labeled NSCs/React-NSCs; **(G)** Percentage of BrdU-positive reactive astrocytes (BrdU/Nestin-GFP/S100ß-positive cells) among total BrdU-positive cells; **(H)** Total number of BrdU-labeled reactive astrocytes; **(I)** Percentage of BrdU-labeled NeuN-positive neurons among total BrdU-positive cells; **(J)** Total number of BrdU-labeled neurons. All Quantifications are referred to the SGZ + GCL. For quantifications for the hilus refer to **Supplementary Figure 4**. **(K)** Representative confocal microscopy image of BrdU-labeled neurons in the GCL of a control mouse. **(L)** Representative image of two BrdU-labeled React-NSCs (arrows) in the GCL of a KA mouse. Scale bar is 50 μm in **(A)** and **(D)**; and 10 μm in **(K)** and **(L)**. $n = 5$ in both groups. $^{***}p < 0.001$, $^{**}p < 0.005$, $^{*}p < 0.05$ by Student's *t*-test **(B,E)**; One-column sample *t*-test **(G,H)** and Mann–Whitney Rank Sum test **(C,I,J)**. Bars show mean ± SEM. Dots show individual data. The same settings for image acquisition were used for PBS and KA samples.

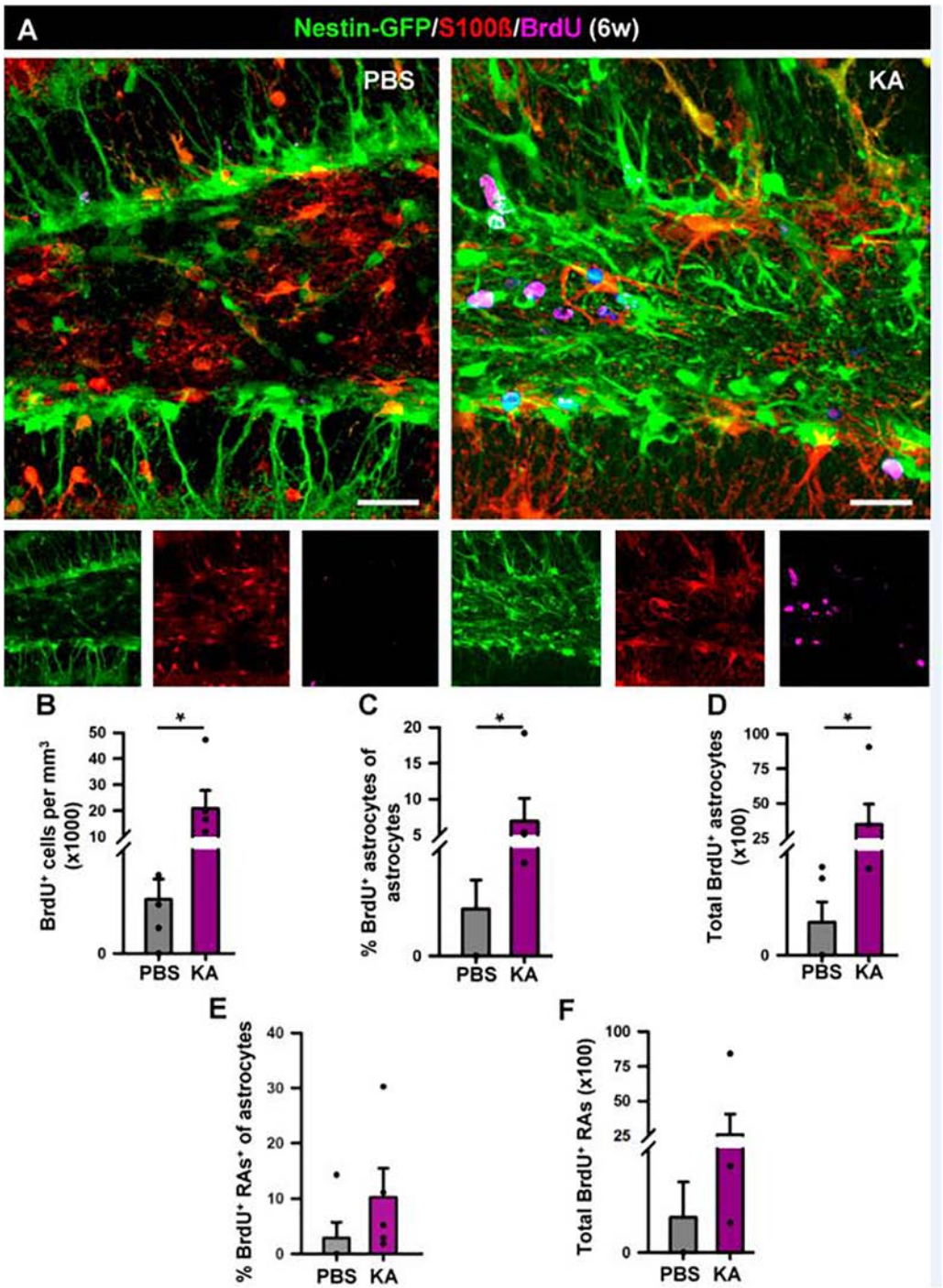


**FIGURE 7 |** Development of seizure-reactive gliosis in the hilus in the longer term (6 weeks). **(A)** Representative confocal images of PBS (left panel) and KA (right) mice after staining for Nestin-GFP, BrdU, and S100ß to assess reactive gliosis. **(B–F)** Quantification of **(B)** density of $BrdU^+$ cells; **(C)** Percentage of BrdU-labeled astrocytes among astrocytes; **(D)** Total number of BrdU-labeled astrocytes; **(E)** percentage of BrdU-labeled reactive astrocytes among astrocytes; **(F)** total BrdU-labeled reactive astrocytes. BrdU (four injections, 2 h-apart) was administered on the 2nd day after KA injection. Scale bar is 20 μm. $n = 5$ per group. $^*p < 0.05$ by Mann–Whitney Rank Sum test. Bars show mean ± SEM. Dots show individual data.

It could be argued that the injection of KA in hcMTLE could have a direct effect on NSCs. We now provide evidence that neuronal hyperexcitation of the hippocampal circuits triggered by the injection of KA in a separated, although connected, structure such as the amygdala provokes a very similar reactive reaction into the neurogenic niche of the dentate gyrus. 1 week

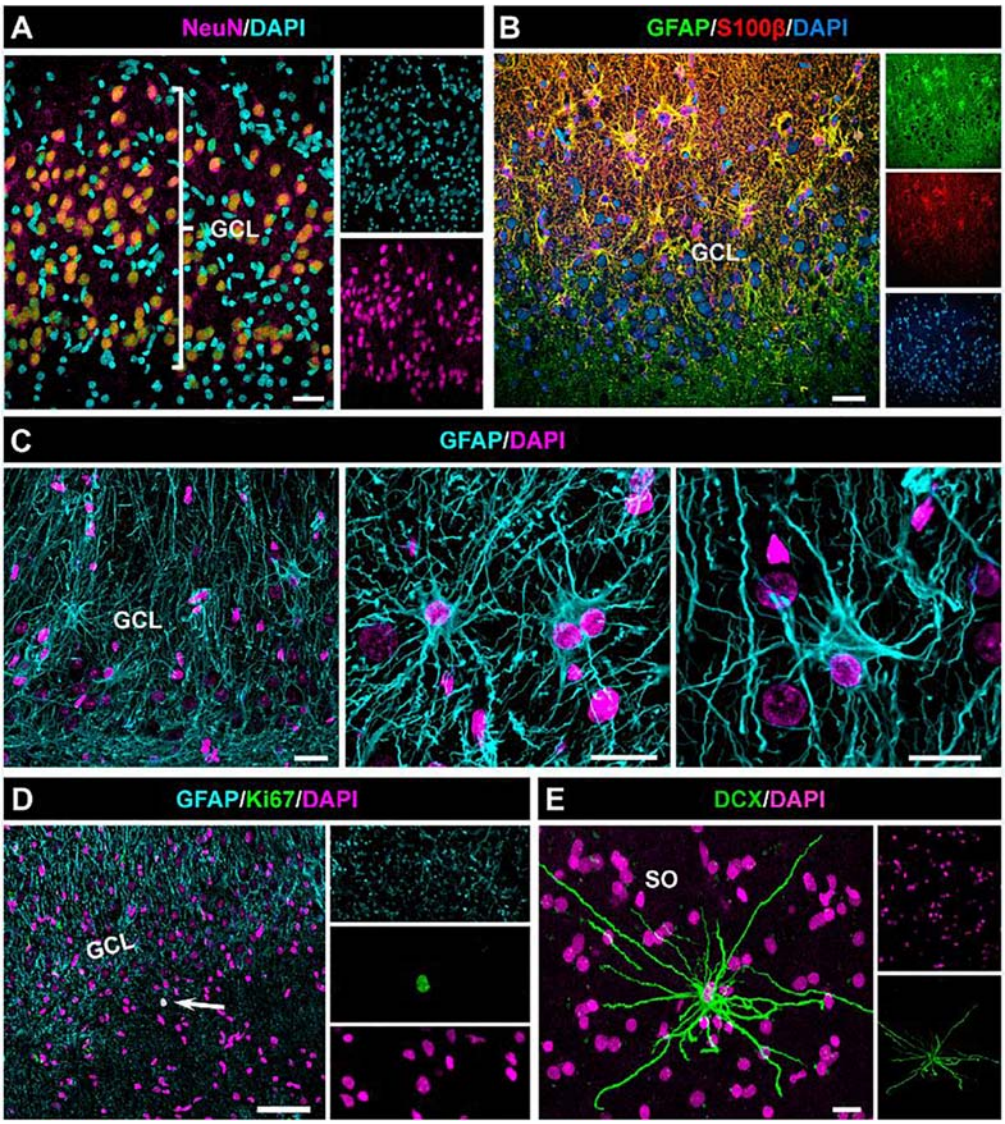


**FIGURE 8 |** Absence of cell proliferation and neurogenesis in the hippocampus of drug-resistant MTLE patients. Unilateral resection of the hippocampus was performed for therapeutic purposes. Confocal microscopy images showing the characteristic dispersion of the GCL **(A)** and the reactive gliosis **(B)** which characterize MTLE. **(C)** Images of reactive astrocytes in the neurogenic niche. **(D)** Cell proliferation was extremely rare in the dentate gyrus. **(E)** A DCX-positive cell found in the *stratum oriens* (SO) of the hippocampus (CA3). No DCX-positive cell was found in the dentate of any of the samples. Scale bar is 15 μm in **(A)** and **(B)**; 20 μm in **(C)** and 10 μm in **(D)**.

after the intra-amygdalar injection of KA, the induction of React-NSCs with higher rate of activation and reactive gliosis were clear (**Figure 2**). In the longer term, 6 weeks after the injection of KA, the reactive gliosis was further developed and neurogenesis was almost abolished. The few neurons that were found presented the characteristic aberrant features (abnormal dendrites and location) described in other models of MTLE (Parent et al., 2006). Further, the aMTLE model allows further manipulations in the hippocampus. For instance we here present data from electrode arrays used to monitor neuronal activity over time to validate the model. In a similar fashion to hcMTLE (Bouilleret et al., 1999; Sierra et al., 2015), spontaneous seizures and epileptiform activity were recorded several weeks after the initial episodes of seizures triggered by the injection of KA suggesting that aMTLE mice become chronically epileptic.

The aMTLE model can be used in combination for instance with optogenetic manipulation and viral vector injections in the hippocampus without the surgery for the KA injection interfering with these manipulations. This work also provides confirmation for pro-gliogenic and anti-neurogenic effects of seizures in MTLE. Thus the cognitive functions associated with neurogenesis will be abolished or lost. Hippocampal neurogenesis is has been shown to participate in spatial and associative learning (Saxe et al., 2006; Dupret et al., 2008; Farioli-Vecchioli et al., 2008;

Imayoshi et al., 2008; Clelland et al., 2009; Deng et al., 2009), as well as in the responses to stress and depression (Snyder et al., 2011). It could then be argued that part of the cognitive problems found in MTLE patients, such as memory impairment (Gargaro et al., 2013) and anxiety and depression (Heuser et al., 2009) as neuropsychiatric comorbidities could be attributed to impaired neurogenesis. We here show that the neurogenic niche of MTLE patients lacks cell proliferation and neurogenesis (**Figure 4**). A previous report, using similarly prepared samples, reported that cell proliferation (assessed by Ki67 staining) and neurogenesis (assessed by DCX immunostaining) are present in the adult human dentate gyrus and decline with age and could be similar or slightly lower in TLE (Fahrner et al., 2007). Although there has been intense debate regarding the presence of neurogenesis in the adult human hippocampus with reports in favor (Eriksson et al., 1998; Spalding et al., 2013; Boldrini et al., 2018) and against (Cipriani et al., 2018; Sorrells et al., 2018) a new report shows the existence of abundant neurogenesis in the adult, and even in the aged, human hippocampus by overcoming the technical obstacles associated with working with human tissue by tightly controlling for the *port-mortem* fixation conditions of the tissue (Moreno-Jiménez et al., 2019). An even newer work supports the persistence of cell proliferation and neurogenesis (again measured by DCX immunostaining) even in aged human brains (Tobin et al., 2019). One of the main articles arguing against the existence of adult human hippocampal neurogenesis uses epileptic tissue (Sorrells et al., 2018). Although in some experimental models of epilepsy boosted neurogenesis is found (Parent et al., 1997, 1998), longer-term studies reported depleted neurogenesis in rodents (Hattiangady et al., 2004) in accordance with data from humans (Mikkonen et al., 1998; Mathern et al., 2002; Pirttilä et al., 2005).

We conclude that NSCs respond swiftly to surrounding neuronal hyperexcitation and that when this activity is in the form of seizures, NSCs abandoned neurogenesis and switch to a reactive gliogenesis. Reactive gliosis, which have been proposed to be key to the development of secondary recurrent seizures (Devinsky et al., 2013) and neuroinflammation are being considered as targets for therapeutic efforts to fight epilepsy. Although the React-NSCs functional contribution to gliosis and neuroinflammation remains to be explored, because of the loss of neurogenesis, and arguably of its physiological functions and capacity to regenerate the dead neuronal population, we propose that React-NSCs should be considered targets as well.

## DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

## ETHICS STATEMENT

All experiments for tissue analysis were done using the Nestin-GFP transgenic mouse line. All the animals were housed with *ad libitum* food and water access, in a 12:12 light:dark cycle. Nestin-GFP transgenic mouse line, kindly provided by Dr. Grigori Enikolopov at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, United States), were crossbred with C57BL/6 mice for at least 10 generations (Mignone et al., 2004). All procedures were approved by the University of the Basque Country (EHU/UPV) Ethics Committee (Leioa, Spain) and the Comunidad Foral de Bizkaia (CEEA: M20/2015/236). All procedures followed the European Directive 2010/63/EU and NIH guidelines. For electrophysiological recordings, all procedures involving animals were performed in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals, reviewed and approved by university (Comite Etico Cientifico para el Cuidado de Animales y Ambiente, CEC-CAA) and national (Comision Nacional de Investigacion Cientifica y Tecnologica, CONICYT) bioethics committees. Experiments were carried out with 2 months old mice (C57Bl/6 J) in accordance with the Ethics Committee (protocol CEBA 13-014). Human samples from individuals with MTLE. Freshly resected hippocampi from adult drug-resistant MTLE patients were obtained from the Basque Biobank at the Cruces University Hospital (Bilbao, Spain) with the patient's written consent and with approval of the University of the Basque Country Ethics committee (CEISH/154/2012). The patient's anonymity was preserved for this study. Sample MTLE030 corresponded to a 38-year-old male, MTLE049 to a 56-year-old male, and MTLE52 to a 46-year-old female. All of them with hippocampal sclerosis ILAE type 1.

## AUTHOR CONTRIBUTIONS

TM-G participated in the experimental design, performed the experiments and data analysis, and prepared the figures regarding mouse tissue. SM-S participated in the experimental design, performed the experiments and data analysis, and prepared the figures regarding mouse and human tissue. NE participated in the experimental design, performed the experiments and data analysis, and prepared the figures regarding EEG recordings. RV-M participated in the experimental design, and performed the experiments and data analysis regarding mouse tissue. AM provided medical expertise on epilepsy and her participation was necessary for obtaining the human tissue. LZ, LG, and AS initiative and participation were necessary for obtaining the human tissue. PF participated in the overall project design and in experimental design, and performed the data analysis regarding the EEG recordings. JE designed the project, participated in the experimental design, performed the experiments and data analysis, prepared the figures, provided funding, and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2019.00158/full#supplementary-material

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# Type 1 Interleukin-4 Signaling Obliterates Mouse Astroglia *in vivo* but Not *in vitro*

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Recent findings suggest that reduced neurogenesis could be one of the underlying reasons for the exacerbated neuropathology in humans, thus restoring the neural stem cell proliferation and neurogenesis could help to circumvent some pathological aspects of Alzheimer's disease. We recently identified Interleukin-4/STAT6 signaling as a neuron–glia crosstalk mechanism that enables glial proliferation and neurogenesis in adult zebrafish brain and 3D cultures of human astroglia, which manifest neurogenic properties. In this study, by using single cell sequencing in the APP/PS1dE9 mouse model of AD, we found that IL4 receptor (*Il4r*) is not expressed in mouse astroglia and IL4 signaling is not active in these cells. We tested whether activating IL4/STAT6 signaling would enhance cell proliferation and neurogenesis in healthy and disease conditions. Lentivirus-mediated expression of IL4R or constitutively active STAT6VT impaired the survival capacity of mouse astroglia *in vivo* but not *in vitro*. These results suggest that the adult mouse brain generates a non-permissive environment that dictates a negative effect of IL4 signaling on astroglial survival and neurogenic properties in contrast to zebrafish brains and *in vitro* mammalian cell cultures. Our findings that IL4R signaling in dentate gyrus (DG) of adult mouse brain impinges on the survival of DG cells implicate an evolutionary mechanism that might underlie the loss of neuroregenerative ability of the brain, which might be utilized for basic and clinical aspects for neurodegenerative diseases.

**Keywords: interleukin-4, STAT6, astroglia, mouse, Alzheimer's disease, neurogenesis, regeneration, zebrafish**

## INTRODUCTION

Alzheimer's disease (AD) is a progressive and yet irreversible neurodegenerative disease. It is characterized by a progressive loss of neurons due to the Amyloid-mediated neurotoxicity that leads to a memory loss, cognitive decline, and eventually inability to perform simple tasks (Beyreuther and Masters, 1997; Selkoe, 2001, 2002, 2003; Brown et al., 2005; Blennow et al., 2006; Harman, 2006). Alzheimer's pathology manifests due to malfunctioning of several cell types including neurons, immune cells, neurovascular compartment and astroglia (De Strooper and Karran, 2016;

Scheltens et al., 2016). The pathology in the neural compartments leads to loss of synaptic connections and eventually to neuronal death while immune cells cause a chronic inflammatory environment and exacerbate neuronal loss (Heneka et al., 2015; Heppner et al., 2015; Jay et al., 2015; Liddelow et al., 2017). Modulation of inflammatory environment and efforts to retain the synaptic integrity during the course of AD are promising approaches to revert the neuropathological changes of the disease, yet other cellular paradigms such as neurogenesis could be involved in manifestation of AD phenotypes (Amor et al., 2010; Rodriguez and Verkhratsky, 2011; Heneka et al., 2013; Nisbet et al., 2015; De Strooper and Karran, 2016; Dzamba et al., 2016; Scheltens et al., 2016; Kizil, 2018). Recently, several studies suggested that in AD patients neurogenesis is significantly reduced compared to healthy individuals (Rodriguez et al., 2008; Rodriguez and Verkhratsky, 2011; Tincer et al., 2016; Kizil and Bhattarai, 2018; Choi and Tanzi, 2019; Moreno-Jimenez et al., 2019). This might indicate that impaired neurogenesis, a physiological phenomenon that has not been investigated extensively in AD, could be a factor in the manifestation of AD pathology (Cosacak et al., 2015; Tincer et al., 2016; Kizil, 2018; Kizil and Bhattarai, 2018; Choi and Tanzi, 2019; Cosacak et al., 2020). Indeed, one of the early symptoms of AD in mouse models is reduced neural stem cell proliferation and neurogenesis (Haughey et al., 2002a,b; Ziabreva et al., 2006), and increased neurogenesis – when experimentally coupled to neuronal survival in AD mouse brains – can revert the cognitive decline (Choi et al., 2018). These findings suggest that enhancing neurogenesis might be a way to counteract AD progression by "regenerating" neurons. However, our knowledge of the molecular mechanisms by which neural stem cells could enhance their proliferation and neurogenic ability in disease conditions is limited.

We identified that in a zebrafish model of AD, Amyloid-mediated pathology induces neural stem cell proliferation and subsequent neurogenesis and integration of newborn neurons into the brain despite the prevalent neurodegenerative toxicity (Bhattarai et al., 2016, 2017a,b, 2020; Cosacak et al., 2019). Our findings suggested that IL4 could be a mechanism to enable neural stem cell plasticity and neurogenesis in AD conditions. To test this hypothesis, we generated a 3D hydrogel culture model where astroglia from fetal human cortex or iPSC-derived neural stem cells were encapsulated and exposed to aggregated amyloid (Papadimitriou et al., 2018; Celikkaya et al., 2019). We found that IL4/STAT6 signaling could revert the reduced proliferative and neurogenic ability of human astroglia upon Amyloid toxicity in 3D hydrogels *in vitro*. Based on these results, we hypothesized that IL4/STAT6 signaling could also enhance neurogenesis in mouse brains *in vivo* during health and in AD.

We conceptualized that if a molecular program is active in zebrafish astroglia and this program is involved in regenerative neurogenesis, it would be interesting to see whether this program is also active in mammalian astroglia *in vivo*, and whether it has a similar role (e.g., regenerative neurogenesis). In our previous studies, we found that a toxicity-specific neuron–glia interaction through Interleukin-4 (*il4*) that is expressed by immune cells and dying neurons, and its receptor *il4r*, which is specifically expressed in the neural stem cells with radial glial identity enable toxicity-induced neurogenesis through STAT6 signaling (Bhattarai et al., 2016, 2020; Cosacak et al., 2019). This naturally led us to investigate whether mouse astroglia expressed *Il4r*, and if not, whether the astroglial proliferation and neurogenesis would enhance after activating the signaling *in vivo*. This approach can be generalized as a workflow for future studies (**Figure 1**).

IL4 is an anti-inflammatory chemokine that plays a key and complex role in polarization of the microglia and resolution of the inflammation (Rolling et al., 1996; Chen et al., 2003; Kelly-Welch et al., 2003; Lyons et al., 2007). After the onset of inflammation- for instance in disease states – IL4 downregulates pro-inflammatory cytokines TNF and IL1β (Hart et al., 1989). Such a relief of inflammatory environment was suggested to ameliorate the disease-associated outcomes such as pro-inflammatory milieu, neuronal death, or excitotoxicity (Suzumura et al., 1994; Garg et al., 2009) and act as a neuroprotective mechanism. In neurons, long-term potentiation is enhanced after IL4 during aging and AD conditions in rodents *in vivo* (Maher et al., 2005; Kiyota et al., 2010). In mouse AD and amyloidosis models, the role of IL4 is controversial. Synaptic degeneration alleviates when key inflammasome component NLRP3 is knocked-out in mice and these mice increase the expression of *Il4*. However, the increase in synaptic integrity is possibly not a direct consequence of the enhanced *Il4* expression but rather the microglial dynamics (Heneka et al., 2013). Overall, IL4 has a beneficial role on the homeostatic functions of the brain and it ameliorates AD symptoms by suppressing the inflammation and producing a permissive environment (Maher et al., 2005; Nolan et al., 2005; Lyons et al., 2007, 2009; Clarke et al., 2008; Gadani et al., 2012; Barrett et al., 2015).

The effect of IL4 on the proliferative potential and neurogenic ability of astroglia is unclear. According to one study a viral mediated overexpression of murine IL4 in the APP/PS1 mouse model of AD leads to a reduction of amyloid induced gliosis and amyloid peptide deposition together with improvement of neurogenesis (Kiyota et al., 2010). Yet, the worsening of AD-like symptoms upon overexpression of murine IL4 was also proposed by another study that used another mouse model for AD – TgCRND8 (Chakrabarty et al., 2012). So far, IL4 signaling was not investigated specifically in astroglia and the studies addressing the changes in neurogenesis after IL4 peptide injection into the mouse brain resulted in varying outcomes due to its direct effects on the immune environment and microglia. In our zebrafish Amyloid toxicity model, microglia is activated rapidly concomitant to the upregulation of *il4* expression, the prevalence of which overlaps with the neurogenic burst and morphological changes in the microglia (Bhattarai et al., 2016). We believe that determining the cell types expressing Interleukin-4 receptor (*Il4r*) would provide a further understanding on the confounding roles of IL4 in the complex milieu of the mouse brain. Additionally, enhancing IL4 signaling in astrocytes would help addressing any cell autonomous effects of this signaling pathway. Finally, investigating the neuroregenerative response in a complex mammalian brain system would help generating models that could better resemble the human brains and could contribute to designing clinical avenues for neuroregenerative therapies.

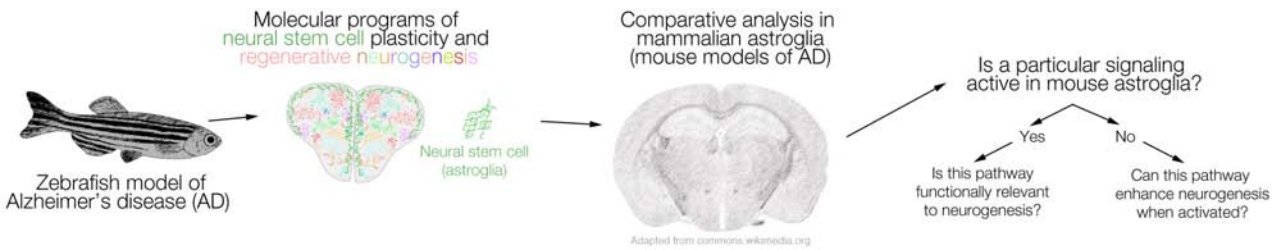


**FIGURE 1 |** A schematic representation of the comparative logic between zebrafish and mammalian models of Alzheimer's disease (AD). The regenerative neurogenic ability of zebrafish brain can be harnessed to elucidate the mechanisms, which can be further tested in mouse models of AD. If a particular signaling that enables regeneration in zebrafish brain is active also in mouse brains (e.g., in neural stem cells or progenitors), functional relevance to neurogenesis can be tested. If such a program ceases to exist in mammalian brains, activation of the pathway or its components can be tested as to whether they induce the regenerative neurogenic ability in mammalian brains. We propose this workflow as a stringent comparative analysis pipeline between zebrafish and mammalian brains.

Therefore, in our study, we aimed to determine (1) whether IL4R is expressed in mouse astroglia by performing single cell sequencing and immunohistochemical stainings, and (2) whether active IL4R signaling could affect the proliferative and neurogenic ability of astroglial cells in mouse brains. Since astrocytes are the primary sources of new neurons by acting as neural stem cells in special niches (Doetsch et al., 1999; Doetsch and Scharff, 2001; Alvarez-Buylla et al., 2002; Doetsch, 2003a,b), investigating the effects of certain signaling pathways in these cell types hold the promise for a yet-elusive "induced regeneration" response of the mammalian brains.

# MATERIALS AND METHODS

## Ethics Statement

All animal experimental procedures were approved by Landesdirektion Sachsen, under license number TVV 87/2016, and followed the safety regulations of DZNE Dresden and TU Dresden. All precautions were taken to minimize animal suffering and to reduce animal numbers. Wild type and age-matched APP/PS1dE9 (Janus et al., 2015) animals were used for this study.

## Lentiviral Construct Production

The generation of HIV-1 pseudo-typed virus was achieved by a co-transfection of three plasmids in HEK293T cells: (I) pCD/NL-BH – packaging plasmid that contains the Gag, Pol, Rev, and Tat genes; (II) pczVSV-Gwt – envelope plasmid that encodes the VSV-G protein; and (III) p6NST90 – transfer vector plasmid (Dirk Lindemann, University Clinic Dresden, Germany) that contained the genes of interest: the IL4R or STAT6VT. The sequences for the full length of hIL4R and hSTAT6VT together with T2A were cloned into the backbone of the HIV transfer vector using the following primers: hIl4r_FW 5′–3′; hIl4r_RV 5′-accggtaaagggccgggattctcctcca-3′; hIl4r_NLS_FW 5′-ACCGGTgcggccatgggtctcacctcccaactgctt-3′; hIl4r_NLS_RV 5′-CACGTCACCGCATGTTAGAAGACTTCCTCTGCCCTCgctcg aacactttgaatatttct-3′; Stat6VT_FW 5′-accggtgccaccatgtctctgtgg ggtctggtct-3′; Stat6VT _RV 5′-accggtaaagggccgggattctcctccacgtc accgcatgttagaagacttcctctgccctcccaactggggttggccct-3′. The plasmid for hSTAT6VT was a gift from Mark H Kaplan (Indiana University) (Kaplan et al., 2007; Sehra et al., 2010).

The generation of p6NST90-based replication-deficient lentivirus particles and transduction of target cells were based on a permit by the Sächsisches Staatsministerium für Umwelt und Landwirtschaft (Az. 54-8452/78/10). For transfection, 5 million HEK293T cells were seeded in a 10 cm dish in 8 ml of DMEM (10% heat-inactivated FBS, 1% Pen/Strep). For one virus preparation, we used 18–21 dishes. After 24 h post-seeding, for every dish 1 ml of pre-warmed blank DMEM without FBS and Pen/Strep was mixed with 5 μg of each of the three plasmids (pCD/NL-BH, pczVSV-Gwt and p6NST90 with cloned transgenes). Next, 45 μl of polyethylenimine (PEI, 1 mg/ml) were diluted in 1 ml of blank DMEM per dish. The PEI solution was added rapidly to the plasmid solution, and incubated for 30 min at room temperature. Fresh DMEM with 15% heat-inactivated serum and 1% Pen/Strep were added to each dish (4 ml/dish) and the transfection mixture was added on top. At 30 h post-transfection, media was changed by adding 5 ml DMEM (1% Pen/Strep, no FBS) to each dish.

At 48 h post-transfection the supernatants were collected, filtered and concentrated by ultracentrifugation. The generated viral pellets were then re-suspended in PBS as documented before (Stirnnagel et al., 2010; Ho et al., 2012). The presence of viral particles was tested using Lenti-X$^{TM}$ GoStix$^{TM}$ Plus (Takara Cat.-No. 631280) and the presence of GFP expression in transfected or transduced target cells was verified by fluorescent microscopy.

## Culture of Adult Mouse Neural Stem/Progenitor Cells (NSPCs)

Adult neural stem/progenitor cells were isolated from the dentate gyri (DG) of 3 month-old WT mice following an optimized version of an established protocol (Hagihara et al., 2009; Walker et al., 2009; Walker and Kempermann, 2014). Mice were sacrificed by cervical dislocation. DG from both hemispheres were microscopically dissected on ice in PBS containing Pen/Strep. Tissues were then further minced using a scalpel and transferred to 1.5 mL tubes for dissociation using Neural Tissue Dissociation Kit from Miltenyi Biotec. The dissociated single cell suspensions were plated in a

PDL/Laminin coated 25 $cm^2$ culture flask and incubated at 37°C with 5% $CO_2$. Cells were expanded and passaged as monolayers in a complete Neurobasal Media. Media was exchanged every 48 h. Only passages 8–12 were used during the experiments.

## Stereotaxic Injections of Lentiviral Vectors and Astroglia

The viral injections into wild type mouse brains were carried out in an S2-approved laboratory. All the regulated precautions were met to prevent the direct contact of personnel with viruses and to avoid infecting the animal during the operation. The procedure was carried out according to previously established protocol (Artegiani et al., 2011). During the entire surgery the mice were anesthetized using a mix of oxygen and isoflurane (49:1) (Baxter – HDG9623) flow and placed on a pre-warmed heat-pad to prevent hypothermia. 1 µl of the respective virus was injected at the coordinates: (a) ± 1.6 mm mediolateral, −1.9 mm anterior–posterior and −1.9 mm dorsoventral from the Bregma for the hippocampus at 200 nl/min speed; and (b) ± 1.0 mm mediolateral, −1.0 mm anterior–posterior and −0.8 mm dorsoventral from pia for the cortex at 50 nl/min speed. Contralateral hemispheres of wild type animals were used for injecting Lv-UbiC:GFP or Lv-UbiC:IL4R-GFP viruses. Brains were isolated at 2 weeks post-injection after the last BrdU injection. BrdU was administered intraperitoneally in the concentration of 50 mg/kg of body weight three times 6 h apart. The transplantation procedure is an independent experiment and technically was performed as described for the virus injection.

For the transduction of astroglia before transplantation, cells were seeded in a 24-well plate coated with PDL (100 µg/ml) and Laminin (0.01% w/v). After 48 h cells reach 70–80% confluency. The respective virus was then added to each well ($10^9$ infection units). Cells were incubated with the virus for 24 h at 37°C with 5% $CO_2$, after which the media was exchanged and cells were allowed to grow till 90% confluency. At this point cells were ready for either transplantation. Transduced cells were trypsinized immediately before transplantation to avoid keeping cells on ice for longer than 1 h. 1.5 µl of $1 \times 10^5$ cells/µl suspension was manually delivered to each hemisphere at 100 nl/min speed. Virus titers were $10^9$ infection units per milliliter for injection into the mouse brain and transduction of cells *in vitro*. Mice were sacrificed 1 week after injection.

## Immunohistochemistry

Mice were anesthetized with an intraperitoneal injection of a mixture of Ketamine (100 mg/kg) and Xylazine (10 mg/kg) and then transcardially perfused with NaCl 0.9% followed by cold freshly prepared 4% PFA. Brains were further post-fixed in 4% PFA overnight at 4°C. 40 µm-thick free-floating sections were made on a microtome and collected in six consecutive series in a cryo-preservation solution [0.1M Phosphate buffer, 25% (v/v) ethylene glycol, 25% (v/v) glycerol]. One serial group of free-floating sections were washed in PBS, blocked in PBS + (10% donkey or goat serum, 0.2% TritonX, 1x PBS) for 1 h at RT and incubated overnight at 4°C with the desired primary antibody of defined dilution in PBS + (3% donkey or goat serum, 0.2% Triton-X, 1x PBS). Sections were washed three times within 1 h and incubated for another hour at RT with the respective secondary antibody (1:500) coupled to a desired fluorophore. After short while, wash samples were then incubated in DAPI diluted in PBS (1:5000) for 10 min. Another series of washes were done and samples were mounted on the charged glass slides. After mounting, slides were left to dry and covered with a coverslip using Aqua Mount.

The fixed cell cultures were permeabilized with 0.1% Triton-X in PBS for 5 min at RT followed by 10 min blocking with 5% goat or donkey serum and 0.1% Triton-X in PBS at RT. Cells were then washed with PBS and primary antibodies of the required dilutions in PBS were added. Cells were incubated for 1 h at RT, washed three times for 5 min with PBS. Secondary antibodies diluted in PBS (1:500) were added and cells were incubated for another hour at RT followed by DAPI treatment for 10 min (1:5000 in PBS). At the end cells were washed three times for 5 min in PBS. At this point cells were either kept in PBS in 4°C or proceeded to imaging.

Samples were imaged on a ZEISS fluorescent microscope with ApoTome using 10x/0.45 20x/0.4 40x/0.95 objectives. Images were acquired using ZEN software and analyzed using ZEN and FIJI software (version 2.0.0.).

## Single-Cell Sequencing

The DGs from WT and APP/PS1 mice were dissected in ice cold PBS with Pen/Strep and the cell dissociation was done using Neural Tissue Dissociation Kit (P) (Miltenyi Biotech) as described (Bhattarai et al., 2016). Cells were sorted by (BD FACS Calibur™) flow cytometry using Propidium Iodide cell viability dye to exclude dead cells. Subsequently, alive cells were directly loaded onto a 10x A-chip after mixing them with reverse transcriptase master mix. GEM generation, cDNA synthesis and amplification (for eight cycles) as well as library preparation was performed with Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v2 (10x Genomics) according to the manufacturer's protocol (Zheng et al., 2016). Read alignment and read counts were done by Cell Ranger 2.1.0. For data analysis Seurat R package (Butler et al., 2018; Farrell et al., 2018) was used as described in Cosacak et al. (2019). In total, two replicates from the same cell mix were processed and sequenced. All count matrices were imported by Read10X function of Seurat and uniquely named to trace back cells if required. In a first step cells with either more than 10000 UMI and less than 1000 UMI, or less than 500 and more than 2500 unique genes were filtered out, likewise cells with more than 6% mitochondrial genes. Further, genes found in less than 10 cells were excluded. The remaining cells and genes were used for downstream analysis for all samples. The data was normalized using the "LogNormalize" method, data scaled with "scale.factor = 1e4," nUMI, nGene and batch effects

were regressed out. For each datasets variable genes were found with FindVariableGenes with the following options mean.function = ExpMean, dispersion.function = LogVMR, x.low.cutoff = 0.125, x.high.cutoff = 10, y.cutoff = 0.5. The top 1000 most variable genes from every sample (determined by Seurat) were merged. Then, the intersection of these genes with all genes in each samples were used for CCA analysis. The two Seurat objects and the variable genes found above were used to generate a new Seurat object with RunCCA function, using num.ccs = 30. The canonical correlation strength was calculated using num.dims = 1:30 and the samples were aligned using dims.align = 1:20. The cell clusters were found using aligned CCA and 1:10 dims, with resolution 0.5. Each cell cluster named based on the markers. 1,324 cells from wild type and 1,429 cells from APP/PS1dE9 mouse hippocampi were analyzed. The raw data BAM files and matrices can be found in GEO[1] (accession number: GSE140793). All R scripts are available on kizillab.org/resources.

[1]https://www.ncbi.nlm.nih.gov/geo

## Animal Maintenance

Mice were housed in a 12 h alternating light/dark cycle with food and water *ad libitum*. All animal experimental procedures were approved by local authorities, and all reasonable precautions were taken to minimize animal suffering and to reduce animal numbers. BrdU (Bromodeoxyuridine) was administered intraperitoneally in concentration of 50 mg/kg of body weight three times with 6 h intervals. Depending on the question, animals were sacrificed from 12 h to 2 weeks after the third injection. Animals were sacrificed by Ketamin/Xylazine mixture and perfused using filtered 0.9% saline solution followed by a 4% PFA.

## Quantification and Statistical Analyses

The cells that were positive for BrdU were only counted when they appeared in the two-cell layer thick area adjacent to the SGZ of the DG. Cells were counted for 1/6th of the entire mouse brain and extrapolated for the whole brain. Five animals per condition were used. Student's *t*-test were used with the significance level ($\alpha = 0.05$). Graphs represent mean and standard deviation.

## List of Antibodies Used

| Antigen | Host | Subtype | Dilution | Vendor | Cat. No. |
|---|---|---|---|---|---|
| Acetylated Tubulin | Mouse | IgG2b | 1:500 | Sigma | T6793-0.2ML |
| Beta-III Tubulin | Mouse | IgG2A | 1:250 | R&D | MAB1195 |
| DCX | Rabbit | IgG | 1:250 | Abcam | ab207175 |
| GFAP | Rabbit | IgG | 1:1000 | Abcam | ab7260 |
| GFP | Chicken | IgY | 1:2000 | Abcam | ab13970 |
| IL4 Receptor | Mouse | IgG2a | 1:250 | Santa Cruz | sc-28361 |
| Ki67 | Mouse | IgG2b | 1:1000 | Abcam | ab86373 |
| Ki67 | Rabbit | IgG | 1:500 | Abcam | ab16667 |
| NeuroD1 | Mouse | IgG2a | 1:500 | Abcam | ab60704 |
| Nestin | Mouse | IgG1 | 1:500 | Santa Cruz | sc-23927 |
| Olig2 | Rabbit | IgG | 1:1000 | Mybiosource | MBS502172 |
| pSTAT6 | Rabbit | IgG | 1:100 | Thermo Fisher S. | PA5-36690 |
| pSTAT6 | Rabbit | IgG | 1:100 | Sigma | SAB4504546 |
| Sox2 | Mouse | IgG1 | 1:100 | Santa Cruz | sc-365823 |
| Sox2 | Goat | IgG | 1:100 | Santa Cruz | sc-17319 |

| Secondary antibodies | Target class | Fluorophore | Dilution | Vendor |
|---|---|---|---|---|
| Donkey Anti-Goat | IgG (H + L) | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Donkey Anti-Mouse | IgG (H + L) | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Donkey Anti-Rabbit | IgG (H + L) | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Chicken | IgG (H + L) | Alexa 488,555,647 | 1:500 | Novus Biologicals |
| Goat Anti-Mouse | IgG (H + L) | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Mouse | IgG1 | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Mouse | IgG2a | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Mouse | IgG2b | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Mouse | IgG (H + L) | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Mouse | IgG1 | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Rabbit | IgG (H + L) | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |
| Goat Anti-Rat | IgG (H + L) | Alexa 488,555,647 | 1:500 | Thermo Fisher S. |

## RESULTS

### Aβ42 Reduces the BrdU-Positive Cells in the Neurogenic Zone of the Dentate Gyrus and Increases Reactive Gliosis

Transgenic AD mouse models display accumulation of amyloid and reactive gliosis (Chen et al., 2000; Donovan et al., 2006; Rodriguez et al., 2008; Lithner et al., 2011; van Tijn et al., 2011; Janus et al., 2015). To confirm these findings, we determined the accumulation of amyloid by performing immunolabelling for 4G8 – a widely used antibody that detects the amino acid residues 18–23 in Aβ peptides in abnormally processed isoforms as well as precursor forms – in 3, 6, and 12-month-old mice (WT and APP/PS1dE9) (**Figure 2**). In 3-month-old mice there was no immunoreactivity against 4G8 detected in the hippocampus. At 6 months of age, the first signs of 4G8-positive aggregations were observed and at 12 months the accumulation was widespread and abundant (**Figures 2A–C**). WT animals did not show any signs of plaques at 12 months of age (**Figure 2D**). To determine the level of reactive gliosis, the brains were immunolabeled against the GFAP that marks the astroglia. Compared to WT animals, the GFAP-positive activated astroglia with a distinctive morphology (increased in size as well as ramification and thickness of processes) were evident in the double transgenic animals as early as 3 months (**Figures 2A,B,D**). By 12 months a pronounced astrogliosis was observable that coincided with the plaque stage of Aβ42 (**Figure 2C**). These results confirm previous findings in this animal model (Donovan et al., 2006; He et al., 2013; Heneka et al., 2013; McClean and Holscher, 2014; Unger et al., 2016) and indicate that 12-month-old mice can be used to investigate the role of IL4/STAT6 signaling in the astroglia of diseased brain that manifests amyloid pathology.

To determine how the proliferation of neural stem/progenitor cells change in APP/PS1dE9 animals as compared to controls, we performed BrdU pulses as described in Section "Materials and Methods," performed BrdU immunolabeling stainings and stereologically quantified the proliferating cells at the stem cell niche of the hippocampus as described before (Kempermann et al., 2003). In WT animals, the levels of BrdU-positive proliferating neural stem/progenitor cells (NSPCs) declined with the age (**Figure 3**). The decline in the APP/PS1 animals however was more pronounced (**Figure 3**). The overall difference between the levels of proliferation in wild type and APP/PS1dE9 animals becomes statistically significant at 12 months where the strongest accumulation of Aβ and gliogenesis was observed (**Figures 2**, **3**). This finding was also consistent with previous reports where NSPC proliferation reduces in AD mouse brains (Poirier et al., 2010; Mu and Gage, 2011; Tincer et al., 2016; Unger et al., 2016; Baglietto-Vargas et al., 2017; Choi et al., 2018; Teixeira et al., 2018; Choi and Tanzi, 2019).

### *Il4ra* Is Not Expressed in Mouse Astroglia

To identify the expression of Il4 receptor (*il4ra*) in the hippocampus, we performed single cell sequencing from wild type and APP/PS1dE9 mouse brains at 12 months of age (**Figure 4**). After clustering and identification of cell types (astroglia/NSPCs "AG/NSC," oligodendrocytes "OD," microglia "MG," T-cells "TC," pericytes/endothelial cells "PC/EC," neurons "N," **Figure 4**), we investigated the expression of *Il4ra* and found that only immune cells (MG, TC) that express the receptor while AG/NSCs are negative for *Il4ra* (**Figure 4**). To confirm our single cell sequencing results, we performed immunolabeling against IL4R in astroglia (GFAP-positive cells), in SOX2-positive cells, and in immune cells (Iba1-positive) (**Figure 5**). We indeed found that IL4R is expressed only in immune cells in mouse hippocampus. Based on these results, we hypothesized that if IL4R expression was induced in AG/NSCs, proliferation and neurogenesis could be enhanced similar to the zebrafish brain (Bhattarai et al., 2016) and *in vitro* in 3D human astroglia cultures (Papadimitriou et al., 2018).

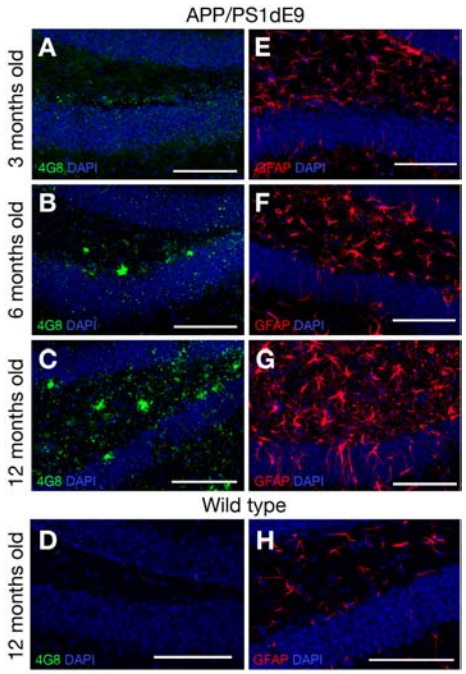


**FIGURE 2 | (A–C)** 4G8 immunolabeling on brain sections of APP/PS1dE9 AD mouse model (3, 6, 12 months old respectively). **(D)** 4G8 staining on wild type mouse (12 months old). **(E–G)** GFAP immunolabeling on brain sections of APP/PS1dE9 mouse (3, 6, 12 months old respectively). **(H)** GFAP staining on wild type mouse (12 months old). $n = 2$ animals. Scale bars: 100 μm.

### The Overexpression of IL4R in the Adult Mouse DG Using Viral Expression Vectors

To overexpress the IL4R in mouse astroglia, we generated lentivirus particles containing the human IL4R under the uniquitous promoter UbiC (LV-UbiC:IL4R-GFP) and used the empty GFP-expressing lentivirus backbone as control (LV-UbiC:GFP, **Figure 6A** and **Supplementary Figure S1**). To test the efficiency of transduction, we cultured adult

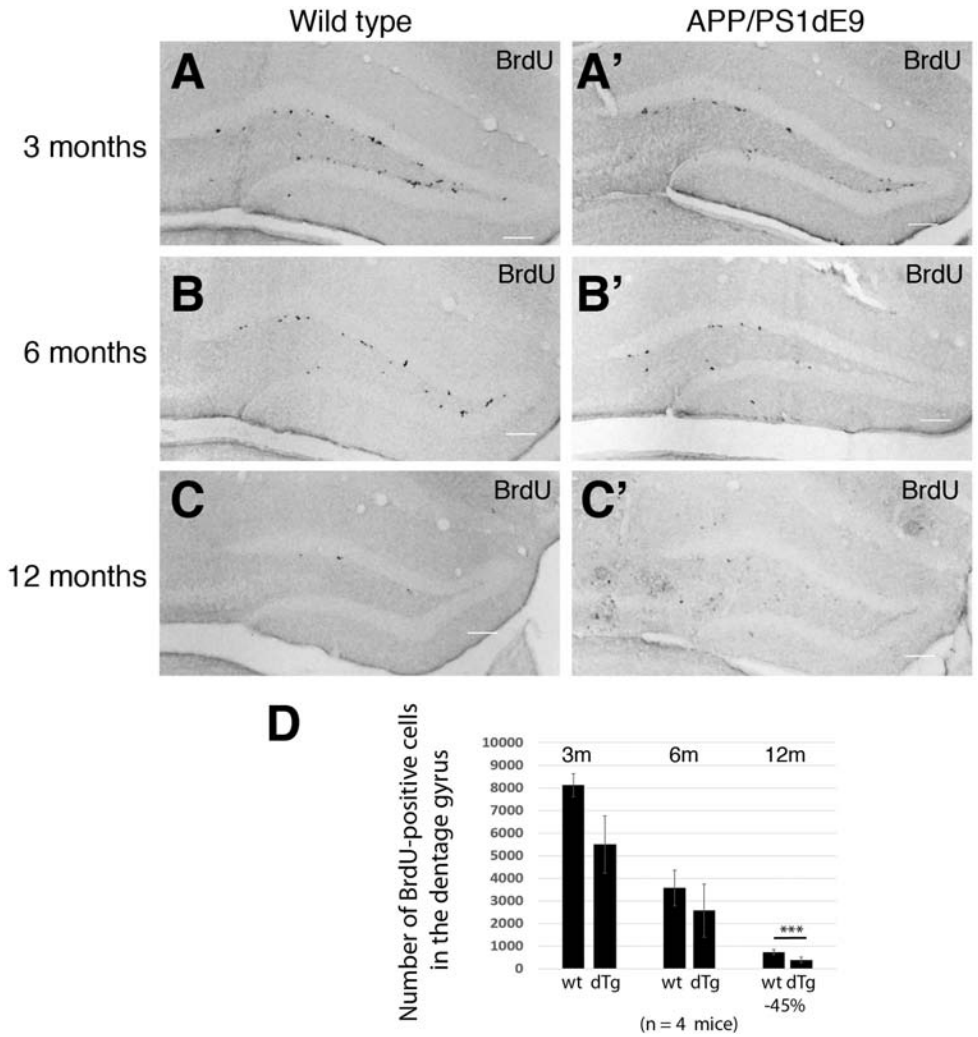


**FIGURE 3 | (A–C)** BrdU immunostaining on cross sections of wild type mouse hippocampus at 3, 6, and 12 months of age. **(A′–C′)** BrdU immunostaining on cross sections of APP/PS1dE9 mouse hippocampus at 3, 6, and 12 months of age. **(D)** Quantification of total BrdU positive cells per mouse hippocampus. Proliferation reduces significantly at 12 months of age in APP/PS1dE9 mouse. *n* = 4 animals. Scale bars: 100 μm.

mouse dentate gyrus progenitors (**Figure 5A**) that can form neurons *in vitro* after growth factor withdrawal (**Supplementary Figure S2**). These cells expressed GFAP and SOX2 (**Figure 6B**) but not IL4R (**Figure 6C**). Transduction with LV-UbiC:GFP-IL4R resulted in strong expression of IL4R and this did not change the viability of the cells (**Figure 6D**). LV-UbiC:GFP transduction displayed a similar efficiency of GFP expression (**Figure 6E**). We concluded that lentiviral particles expressing GFP or IL4R could transduce mouse astroglia and lead to the expression of the IL4R and GFP *in vitro*.

When we injected LV-UbiC:GFP into adult mouse dentate gyrus (**Figure 7A**), we observed transduction in the subgranular zone (SGZ) and we could target glial cells (**Figures 7B,C**). However, the injection of LV-UbiC:IL4R-GFP into the brains of wild type animals consistently resulted in a considerable lower number of transduced cells (**Figures 7D,E**) and we hardly saw any GFP-positive glia (**Figure 7F**). When we performed LV injection into the cortex, we observed that IL4R virus resulted always in lower number of transduced cells independent of the relative titers (data not shown). These results suggested that the astroglia expressing IL4R survive *in vitro* but not *in vivo*, proposing a non-permissive environment that impinges on the survival of the IL4R-expressing glia *in vivo*.

## Transplantation of Transduced Adult NSPCs

Since overexpression of IL4R via lentivirus injection impaired the survival of cells, we designed an alternative strategy to first transduce the mouse astroglia *in vitro* and then transplant these cells into the hippocampus or the cortex of the mouse brains. Regarding our experimental conditions, it could be

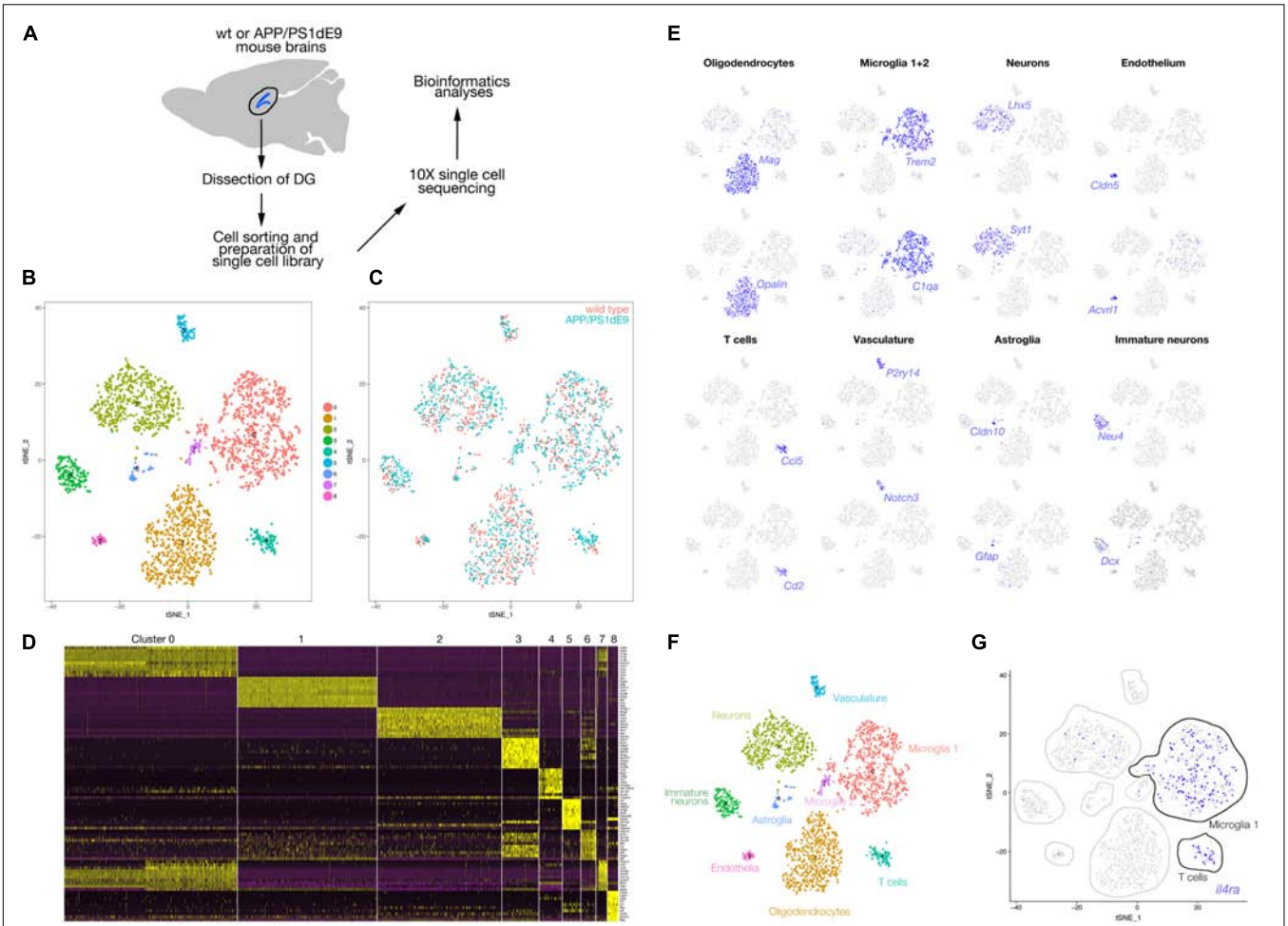


**FIGURE 4 | (A)** Schematic overview of the single cell sequencing procedure. Dissected dentate gyri (DG) of hippocampi were dissociated into single cells, sorted by flow cytometry. Cells were subjected to 10X library preparation, followed by 3′ end transcriptome sequencing and bioinformatics analyses. **(B)** tSNE plot after cell clustering. **(C)** Cells from wild type and APP/PS1dE9 mouse DG plotted in different colors on the tSNE supporting a similar cell type composition and distribution. **(D)** Heat map for cell clusters showing top10 marker genes. **(E)** Feature plots for several genes known to be markers of various cell types. Cell clusters are named according to the marker gene expression: *Mag* and *Opalin* for oligodendrocytes, *C1qa* and *Trem2* for microglia, *Lhx5* and *Syt1* for neurons, *Cldn5* and *Acvrl1* for endothelial cells, *Ccl5* and *Cd2* for T cells, *P2ry14* and *Notch3* for vasculature, *Cldn10* and *Gfap* for astroglia, *Neu4* and *Dcx* for immature neurons. **(F)** Named cell clusters on tSNE plot. **(G)** Expression of *Il4r* on tSNE plot. IL4 receptor is expressed mainly in microglia and T cells. Very few neurons also express the receptor. *Il4r* is not expressed in astroglia.

possible that IL4R-expression after direct transduction by the virus injection might affect the astroglia because of the injection paradigm, the presence of the virus in the tissue. To eliminate these probabilities we used the transplantation paradigm. Additionally, we used an alternative strategy to express a constitutively active form of STAT6 (STAT6VT; Kaplan et al., 2007; Sehra et al., 2010) that would keep the IL4/STAT6 signaling continuously active. We specifically tried this because provided that the outcomes of IL4R expression and STAT6VT expression would be similar, we could be more confident about a specific effect of IL4R signaling on astroglia (by these two independent modes of activating the IL4R signaling).

Mouse astroglia were transduced with the virus, collected at 2 days after transduction to allow the expression of the gene of interest (GFP and STAT6VT), transplanted into the hippocampal and the cortical region of 12-month old wild type mice and the brains were analyzed 1 week after the transplantation. Hippocampal transplantations did not yield any integration into the region (data not shown) but cortical transplantations did. Therefore, we continued with the analyses of the cortical transplantations. To determine which cell types are formed by transplanted transduced astroglia, we performed immunolabeling against OLIG2 (oligodendrocytes), GFAP (astrocytes), and NeuN (neurons). The transplantations resulted in a varying number of integrated GFP-positive cells as determined by immunostaining for GFP. The majority of the transplanted control astroglia (LV-UbiC:GFP-transduced) yielded in GFAP-positive astrocytes (**Figure 8A**) while a minor fraction of transplanted cells

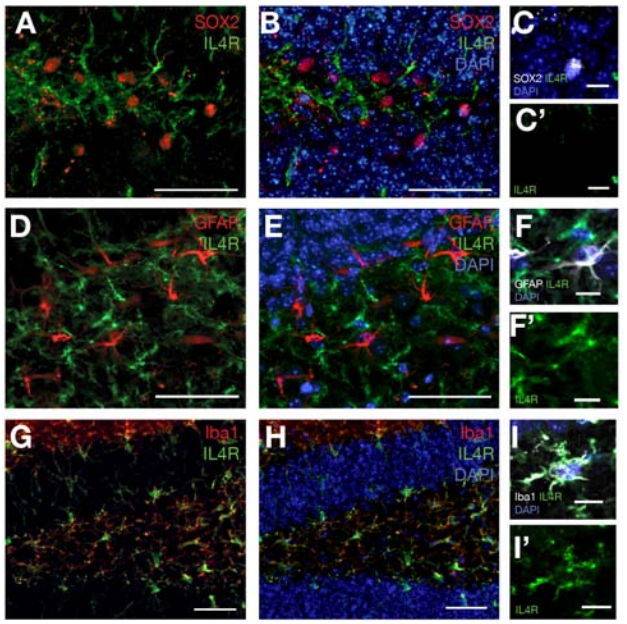


**FIGURE 5 | (A)** SOX2 and IL4R immunostaining on brain sections of 12 months old wild type mouse. **(B)** DAPI added to **(A)**. **(C)** SOX2-positive cells do not express IL4R. **(C')** IL4R fluorescence channel alone. **(D)** GFAP and IL4R immunostaining on brain sections of 12 months old wild type mouse. **(E)** DAPI added to **(D)**. **(F)** GFAP-positive cells do not express IL4R. **(F')** IL4R fluorescence channel alone. **(G)** Iba1 and IL4R immunostaining on brain sections of 12 months old wild type mouse. **(H)** DAPI added to **(G)**. **(I)** Iba-positive cells express IL4R. **(I')** IL4R fluorescence channel alone. $n = 3$ animals. Scale bars: 100 μm **(A,B,D,E,G,H)** and 25 μm elsewhere.

formed oligodendrocytes (**Figure 8B**). We also observed a rather small fraction of transplanted LV-UbiC:GFP-transduced glia formed neurons with extended processes (**Figure 8C**). When LV-UbiC:STAT6VT-GFP-transduced astroglia were transplanted, we found that the transplanted cells did not form extended processes and the number of GFP-positive cells were significantly lower than the control transplantations and they displayed round, fragmented morphology with no processes (**Figure 8D**). A rather small minority of the UbiC:STAT6VT-GFP-transduced cells expressed oligodendrocyte marker OLIG2 (**Figure 8E**) and GFAP (**Figure 8F**), while no neurons were observed. This pattern was consistent in APP/PS1dE9 mouse brains where the difference in transplantation efficiency was apparent between transplanted control astroglia (**Figure 8G**, LV-UbiC:GFP-transduced) and STAT6VT-expressing astroglia (**Figure 8H**, LV-UbiC:STAT6VT-GFP-transduced). Altogether, these results suggest that expression of IL4R or STAT6VT in astroglia impairs the survival of these cells *in vivo* but not *in vitro*.

To test our hypothesis, we performed TUNEL staining to detect apoptotic cells after direct transduction of LV-UbiC:GFP or LV-UbiC:IL4R-GFP or transplantation of transduced astroglia in wild type animals (**Figure 9**). Unlike the transduction with LV-UbiC:GFP (**Figures 9A–B″**), transduction with LV-UbiC:IL4R-GFP resulted in TUNEL-positive astroglia (**Figures 9C–D″**). Similarly, while the transplantation of LV-UbiC:GFP-transduced astroglia did not lead to TUNEL-positive glia (**Figures 9E–F″**), LV-UbiC:IL4R-GFP-transduced astroglia displayed TUNEL reactivity (**Figures 9G–H″**). These results support our findings that expression of IL4R in astroglia leads to cell death.

## DISCUSSION

The restoration of neural tissue is of clinical importance especially in neurodegenerative diseases. However, in mammals, the neurogenic ability declines with aging and the pool of the stem cells decreases (Ekdahl et al., 2003; Monje et al., 2003; Borsini et al., 2015). Additionally, during disease progression, neural stem cell proliferation and neurogenesis reduce even further. In AD patients, neurogenesis reduces dramatically and the decrease in adult neurogenesis could be a contributing factor to the pathology (Tincer et al., 2016; Hollands et al., 2017; Kizil and Bhattarai, 2018; Teixeira et al., 2018; Choi and Tanzi, 2019; Moreno-Jimenez et al., 2019; Cosacak et al., 2020). Therefore, studying regenerating organisms such as the zebrafish to learn how to counteract the pathology-suppressed neurogenic ability, and neural stem cell plasticity could be a promising approach to develop circuit resilience and brain repair in AD. Based on our previous findings (Bhattarai et al., 2016; Cosacak et al., 2017, 2019; Papadimitriou et al., 2018), we proposed that IL4 could be a factor to coax mammalian astroglia *in vivo* to become proliferative and neurogenic in disease conditions.

In our previous work, we found that zebrafish uses IL4 signaling as crosstalk to activate glial cells toward proliferation and neurogenesis (Bhattarai et al., 2016). In 3D cultures of human neural stem cells and astroglia, IL4 receptor (IL4R) is expressed and IL4 can revert the AD-associated reduction of astroglia proliferation and neurogenesis (Papadimitriou et al., 2018). These results suggested that IL4 signaling could be used to enhance neurogenesis and proliferation of astrocytes in a cell-autonomous manner by activating this signaling in astroglia. In the current study, we found that type 1 IL4/STAT6 signaling obliterates astroglia *in vivo* in mouse brains. This correlates with previous findings that IL4 could promote apoptosis through a caspase-dependent mechanism in microglia (Soria et al., 2011). IL4 was reported to promote differentiation, proliferation, and survival of different tumor cells through its interaction with IL4R (Koller et al., 2010; Venmar et al., 2014; Kim et al., 2016). For instance *IL4R* is a biomarker for various aggressive forms of glioblastoma multiforme (Puri et al., 1994; Joshi et al., 2001; Scheurer et al., 2008; Gadani et al., 2012). Additionally, glioblastoma cells would evade apoptosis in correlation with *Il4r* expression and enhance growth unlike healthy astroglia (Barna et al., 1995; Debinski, 1998). This suggests that type 1 IL4 signaling in glia induces apoptosis unless a neoplastic transformation evades apoptosis and allows IL4 signaling to promote proliferation of astroglia, which happens *in vivo* in zebrafish and *in vitro* in mammalian cells. It should also be noted that the

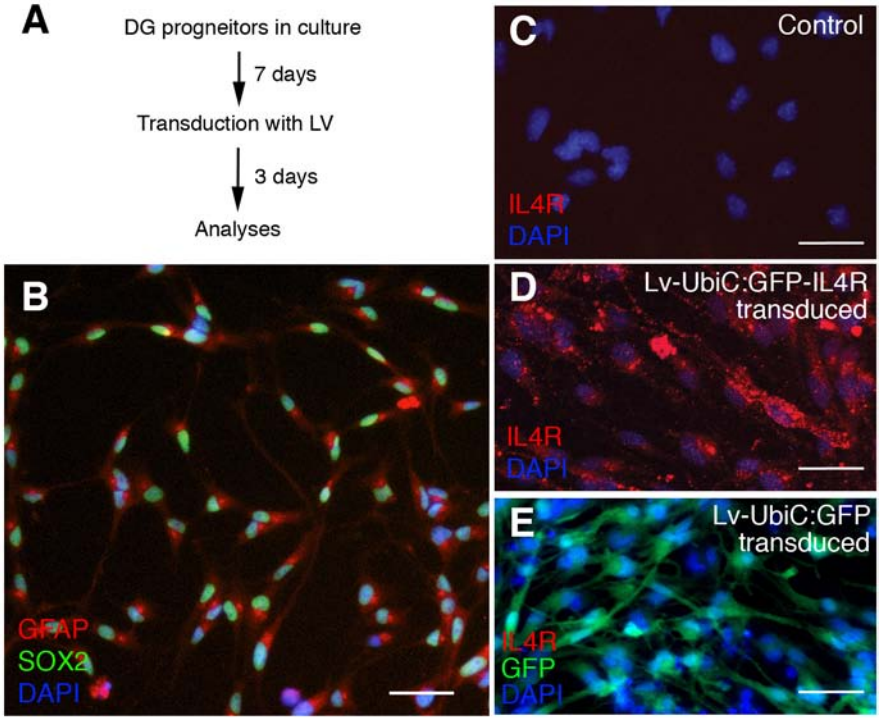


**FIGURE 6 | (A)** Schematic view of cell culture and transduction of adult mouse dentate gyrus astroglia. **(B)** Immunostaining for GFAP and SOX2 in control cultures. **(C)** Immunostaining for IL4R in control cultures. **(D)** Immunostaining for IL4R after transduction with Lv-UbiC:IL4R-GFP. **(E)** Immunostaining for IL4R and GFP after transduction with Lv-UbiC:GFP. Scale bars: 25 μm.

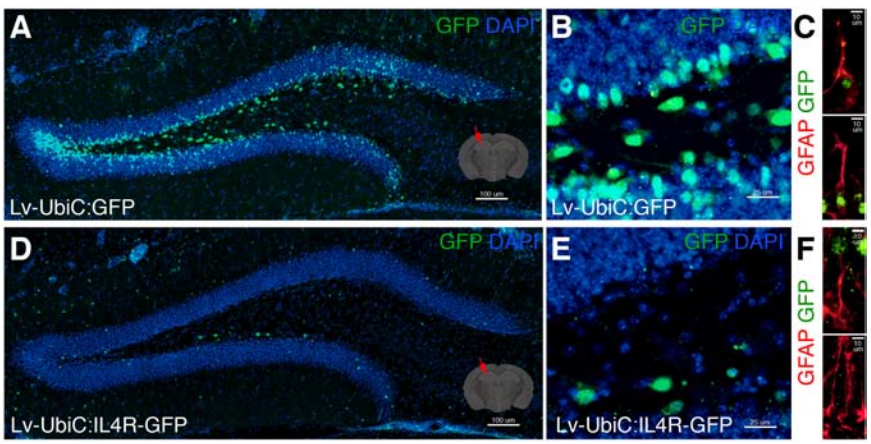


**FIGURE 7 | (A)** GFP immunostaining on coronal section of wild type mouse brain after transduction of Lv-UbiC:GFP. **(B)** Close up image from **(A)**. **(C)** GFAP and GFP immunostaining showing Lv-UbiC:GFP-transduced astroglia. **(D)** GFP immunostaining on coronal section of wild type mouse brain after transduction of Lv-UbiC:IL4R-GFP. **(E)** Close up image from **(D)**. **(F)** GFAP and GFP immunostaining showing Lv-UbiC:IL4R-GFP-transduced astroglia. *n* = 3 wild type animals. Scale bars: 100 μm **(A,D)**, 25 μm **(B,E)**, and 10 μm **(C,F)**.

effects of type 1 IL4R signaling on astroglia might be non-cell autonomous as many cells express the receptor after transduction. Additionally, the non-permissive environment hypothesis can include the effects of other cells in the brain on IL4R-expressing astroglia. Further research is needed to clarify this aspect.

We suggest that evolutionarily, mammalian brains developed a non-permissive environment for astroglia that have active IL4 signaling for its potential effects on hyper-proliferation. Under apoptosis-evading conditions of tumors, IL4 receptor (IL4R) is promoting proliferation and blockage or hypomorphic nucleotide polymorphisms in IL4R

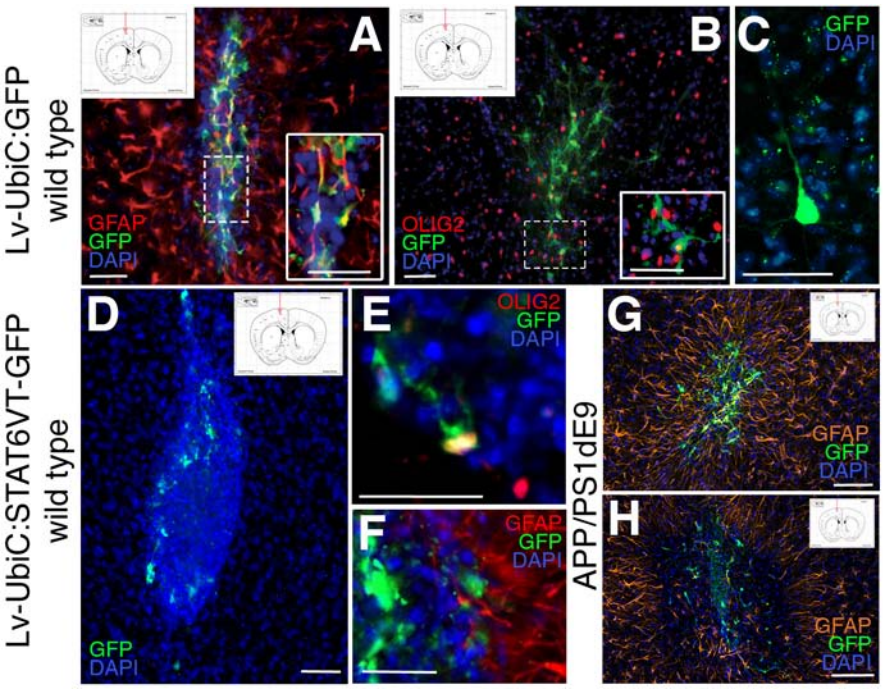


**FIGURE 8 | (A)** Immunostaining for GFAP (red) and GFP after transplantation of astroglia transduced with Lv-UbiC:GFP into wild type mouse cortex. **(B)** Immunostaining for OLIG2 (red) and GFP after transplantation of astroglia transduced with Lv-UbiC:GFP into wild type mouse cortex. **(C)** Immunostaining for GFP after transplantation of astroglia transduced with Lv-UbiC:GFP into adult mouse cortex shows neuronal morphologies. **(D)** Immunostaining for GFP after transplantation of astroglia transduced with Lv-UbiC:STAT6VT-GFP into adult mouse cortex. **(E)** Immunostaining for OLIG2 (red) and GFP after transplantation of astroglia transduced with Lv-UbiC:STAT6VT-GFP into wild type mouse cortex. **(F)** Immunostaining for GFAP (red) and GFP after transplantation of astroglia transduced with Lv-UbiC:STAT6VT-GFP into wild type mouse cortex. **(G)** Immunostaining for GFAP (orange) and GFP after transplantation of astroglia transduced with Lv-UbiC:GFP into APP/PS1dE9 adult mouse cortex. **(H)** Immunostaining for GFAP (orange) and GFP after transplantation of astroglia transduced with Lv-UbiC:STAT6VT-GFP into APP/PS1dE9 adult mouse cortex. *n* = 3 animals. Schematic information on injection locations presented in the insets. Scale bars: 50 μm.

reduce the aggressiveness of glial tumors (Scheurer et al., 2008). Therefore, our work suggests that an evolutionary divergent role for signaling pathways (such as the IL4 signaling) in astroglia might underlie the disparity between the proliferative and neurogenic properties of mouse and zebrafish astroglia in health and disease. This difference might have functional ramifications in the regenerative outputs of zebrafish and mouse brains.

We would like to note that investigating complex diseases of humans in non-human model organisms is challenging. Recapitulation of the pathological culprits of a disease faithful to the human pathology is unlikely to fully succeed in a model due to inherent physiological differences between the cells of humans and other organisms, even rodents (Qiu et al., 2016; Hodge et al., 2019). However, reductionist models of human diseases in appropriate organisms are quite powerful for addressing particular aspects of pathologies or for designing experimental treatment options that may defy that particular disease. For instance, zebrafish proposed many signaling pathways that could be harnessed for enhanced responses in tissue counterparts in humans (Zon, 1999; Tomasiewicz et al., 2002; Poss et al., 2003; Rubinstein, 2003; Lieschke and Currie, 2007; Newman et al., 2010; Diep et al., 2011; Kizil et al., 2012a,b; Kyritsis et al., 2012; Gemberling et al., 2013; MacRae and Peterson, 2015; Mokalled et al., 2016; Papadimitriou et al., 2018; Celikkaya et al., 2019; Cosacak et al., 2019; Reinhardt et al., 2019). Other disease models in zebrafish yielded in useful information on the pathological mechanisms and led to the development of promising drugs (Cully, 2019).

A peculiarity of zebrafish that is appealing to us is its regenerative ability. Provided that the molecular basis of regenerative neurogenesis is understood in the zebrafish brain, we may have the chance to pinpoint what is missing in mammalian brains and how this "gap" can be filled. Our current study counts among the first such comparative approaches, which we believe will become a norm and will flourish as the reliability of zebrafish disease models prove to be of high relevance to humans. We also emphasize that as a general comparative analysis pipeline, using the experimental data acquired from the disease models; humanized models such as 3D cell cultures can be employed for the validity of the findings in human cells. Finally, the differences in the inherent complexity and the cellular physiology of mammalian brains may render such comparative analysis challenging and the findings in zebrafish may not be directly applicable to mammals in some cases. Such

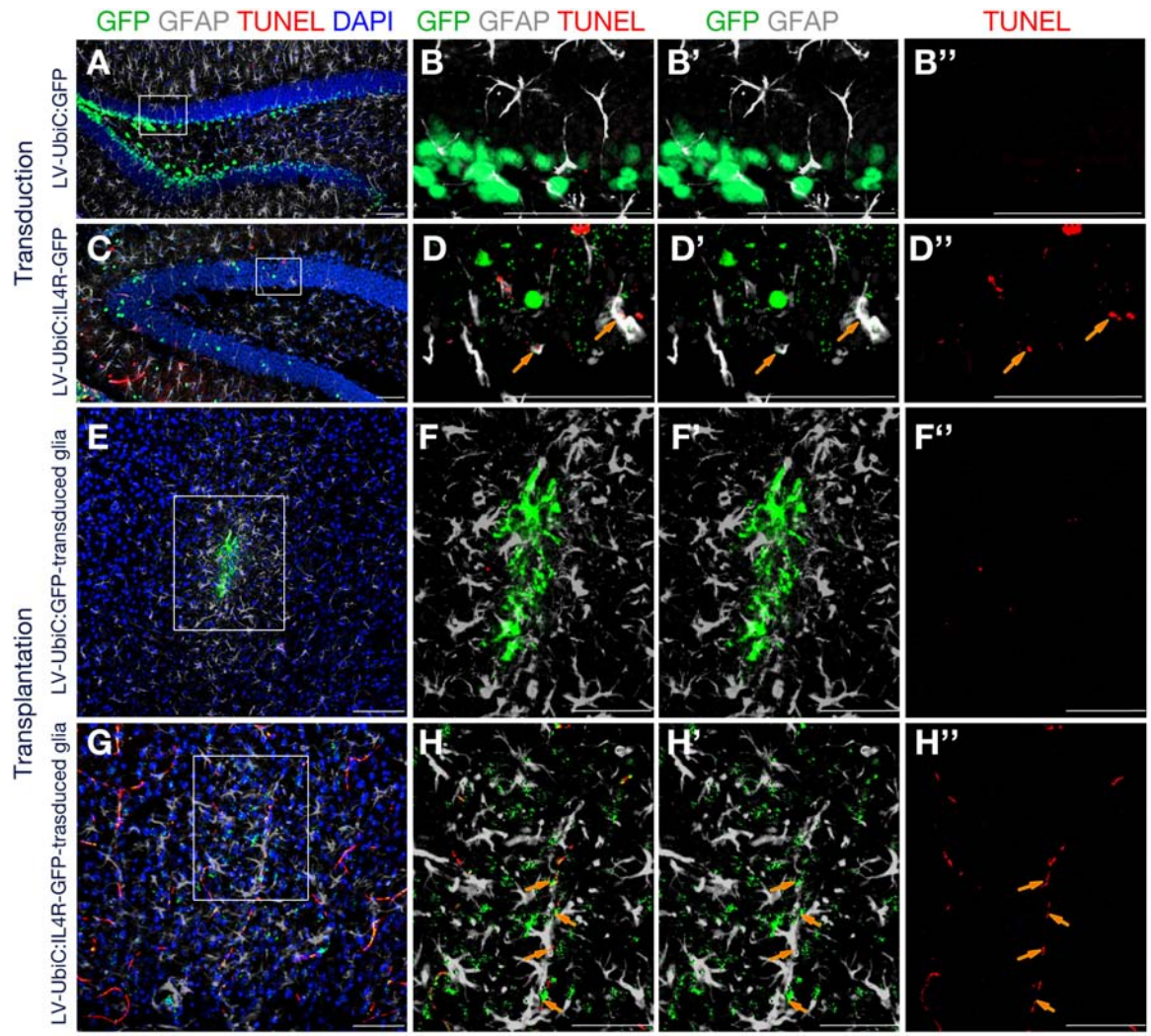


**FIGURE 9 |** Immunostaining for GFAP (gray) and GFP (green) coupled to TUNEL staining (red). **(A)** Wild type mouse hippocampus transduced with Lv-UbiC:GFP. **(B)** Higher magnification of the framed region in **(A)** without DAPI. **(B′)** Overlaid GFP and GFAP channels. **(B′′)** TUNEL staining as single fluorescence channel. **(C)** Wild type mouse hippocampus transduced with Lv-UbiC:IL4R-GFP. **(D)** Higher magnification of the framed region in **(D)** without DAPI. **(D′)** Overlaid GFP and GFAP channels. **(D′′)** TUNEL staining as single fluorescence channel. **(E)** Wild type mouse cortex transplanted with Lv-UbiC:GFP-transduced astroglia. **(F)** Higher magnification of the framed region in **(E)** without DAPI. **(F′)** Overlaid GFP and GFAP channels. **(F′′)** TUNEL staining as single fluorescence channel. **(G)** Wild type mouse cortex transplanted with Lv-UbiC:IL4R-GFP-transduced astroglia. **(H)** Higher magnification of the framed region in **(G)** without DAPI. **(H′)** Overlaid GFP and GFAP channels. **(H′′)** TUNEL staining as single fluorescence channel. Orange arrows show TUNEL-positive, transduced glia. All animals are WT. $n \geq 3$ wild type animals. Scale bars: 100 μm.

incongruences will also enhance our understanding from an evolutionary standpoint as to why and how mammalian brains lost their regenerative power, and which cell types and in what specific context must be nudged to become regenerative. This audacious workflow will surely increase the comparative power of the findings in zebrafish and validate the reliability of the use of this model in AD research.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GEO GSE140793.

## ETHICS STATEMENT

The animal study was reviewed and approved by Landesdirektion Sachsen, Germany. Permit number: TVV 87/2016.

## AUTHOR CONTRIBUTIONS

VM and CK conceived and designed the experiments and wrote the manuscript. VM performed the experiments and acquired the data. AD, SR, and AP contributed to the generation of sequencing data. PB, TS, KB, NG, and RL contributed to the experimental procedures or provided samples. SP prepared virus particles.

MC analyzed the single cell sequencing data. VM, TS, PB, SP, NG, RL, and CK edited the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00114/full#supplementary-material

**FIGURE S1 |** Lentiviral backbone that is used for generating Lv constructs for IL4R and STAT6VT. Genes of interests were cloned into upstream region in frame to EGFP.

**FIGURE S2 | (A)** Schematic workflow for testing neurogenicity of dentate gyrus progenitors in culture. **(B)** Immunostaining for TUBB3 and GFAP at 1 week after growth factor withdrawal. **(C)** Immunostaining for TUBB3 and GFAP at 2 weeks after growth factor withdrawal. **(D)** Quantification charts indicating the relative abundance of GFAP-positive astroglia and TUBB3 positive neurons.

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