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Editorial Board

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Editorial

Neural Stem and Progenitor Cells in Nervous System Function and Therapy

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For over two decades a significant proportion of neuroscience research has been dedicated to understanding the normal function of neural stem and progenitor cells, as well as developing novel ways to use them to achieve nervous system repair. These therapeutic stem and progenitor cell-based strategies can be broadly divided into three categories: activating endogenous neural stem/progenitor cells, cell transplantation, and the use of stem or progenitor cells to model disease. The progress being made in each of these key areas is addressed briefly within this review and by key contributions to this special issue.

Within the adult mammalian brain, including the human brain, neural stem cells (NSCs) are found in the subventricular zone and hippocampal dentate gyrus, where they divide and give rise to new neurons, in a process termed adult neurogenesis. These newly generated neurons are highly plastic and are important for many brain functions including learning and memory and mood. NSCs in the subventricular zone are also capable of generating astrocytes and oligodendrocytes [1, 2], and oligodendrocyte progenitor cells, which reside in all regions of the central nervous system, continue to generate new myelinating oligodendrocytes throughout life [3]. The activity of these proliferating populations markedly decreases with ageing [3, 4] and correlates with the age-related decline in cognitive performance. However, the fact that a large pool of quiescent precursor cells can be activated in the neurogenic niches of aged mice [5, 6], as well as the fact that oligodendrocyte progenitor cells are capable of spontaneously regenerating oligodendrocytes to replace myelin lost due to central nervous system injury or demyelination [7], offers hope that the endogenous pool of neural stem and progenitor cells can be activated to generate new cells even in the aged or injured nervous system. In this special issue, L. Harris et al. (2016) extensively review the biology and potential therapeutic applications of NSCs in the developing and adult cerebral cortex.

Studies in rodents have shown that behavioural interventions such as environmental enrichment and cognitive training and exercise can promote neurogenesis [8], and some types of learning have been shown to increase oligodendrogenesis [9]. Furthermore, a number of hormones, cytokines/chemokines, and growth factors have been shown to influence endogenous cell generation, including vascular endothelial growth factor, brain-derived neurotrophic factor, nerve growth factor, progesterone, and epidermal growth factor. In many cases these identified protein regulators have poor clinical efficacy due to poor stability, an inability to cross the blood-brain barrier, or significant off-target effects on other cell types; however it is possible that the next generation of drug-design and drug-delivery approaches will overcome some of these hurdles. In this special issue, L. Auderset et al. (2016) highlight the influence that the members of the low density lipoprotein receptor related protein family and their ligands have on neural stem and progenitor cell behaviour, and A. E. Cole et al. (2016) highlight the potential of bone morphogenic protein 4 and small molecule substitutes to direct endogenous neural stem and progenitor cells to generate glial cells following a central nervous system insult. The close proximity of the NSCs to the brain microvasculature also allows them to interact with peripheral immune system, a research area highlighted by O. Leiter et al. (2016).

The second major avenue of cell-based neural repair research is stem cell transplantation, which has been used for other clinical purposes since the 1960s. Stem cells from a variety of sources have been proposed and tested for the treatment of neurodegenerative disease. While mesenchymal stem cells have a limited ability to generate neural cell types, human embryonic stem cells can be expanded *in vitro* and retain their ability to differentiate into each of the major neural cell types. However, the benefits observed in response to stem cell transplantation in mouse models of neurodegenerative disease are often not the result of the transplanted cells differentiating into functional neurons or glia on a large scale. Instead they appear to promote neural regeneration by the secretion of paracrine factors. Neural and mesenchymal stem cells that are transplanted into mouse models of Alzheimer's dementia produce beneficial neurotrophins, which have an anti-inflammatory effect and reduce both amyloid and tau pathologies. Similarly, NSCs transplanted into rodents with experimental autoimmune encephalomyelitis, a model of inflammatory-mediated demyelinating disease, have been shown to attenuate inflammation and promote functional recovery [10]. The past decade has seen the evolution of protocols that produce more consistent and defined cell populations for transplants, making it more feasible that the cells can be engineered to maximize their paracrine influence and better abrogate disease pathology. In this special issue, X. Gao et al. (2016) have shown that NSCs engineered to overexpress glial cell line-derived neurotrophic factor enhance the immunomodulatory and neuroprotective potential of NSCs transplanted into the central nervous system of rats with chronic experimental autoimmune encephalomyelitis.

The final way in which neural stem and progenitor cells are being utilized to treat nervous system disorders is through the development of cell culture-based models of disease. For example, neural stem and progenitor cells can be differentiated into neurons, astrocytes, and oligodendrocytes in order to study specific human genetic mutations associated with neurodevelopmental or neurodegenerative disorders or expanded for the large-scale screening of novel pharmacotherapies. Human neural progenitor cells are ideally suited for these studies but are difficult to obtain. However, in this special issue, H. Fukusumi et al. (2016) demonstrate that human induced pluripotent stem cells can be derived from multiple sources, including dermal fibroblasts, cord blood cells, and peripheral blood mononuclear cells and readily differentiated into human neural progenitor cells. Moreover, N. Gunewardene et al. (2016) report that human induced pluripotent stem cells can be differentiated into neurons that are able to innervate cochlear hair cells, allowing them to be used to model auditory neuron replacement therapies *in vitro*.

This special issue highlights the rapid progress being made in neural stem and progenitor cell biology.

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References

[1] A. Kriegstein and A. Alvarez-Buylla, "The glial nature of embryonic and adult neural stem cells," *Annual Review of Neuroscience*, vol. 32, pp. 149–184, 2009.

[2] K. M. Young, T. Mitsumori, N. Pringle, M. Grist, N. Kessaris, and W. D. Richardson, "An Fgfr3-iCreER^{T2} transgenic mouse line for studies of neural stem cells and astrocytes," *Glia*, vol. 58, no. 8, pp. 943–953, 2010.

[3] S. Wang and K. M. Young, "White matter plasticity in adulthood," *Neuroscience*, vol. 276, pp. 148–160, 2014.

[4] H. G. Kuhn, H. Dickinson-Anson, and F. H. Gage, "Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation," *The Journal of Neuroscience*, vol. 16, no. 6, pp. 2027–2033, 1996.

[5] D. G. Blackmore, M. G. Golmohammadi, B. Large, M. J. Waters, and R. L. Rietze, "Exercise increases neural stem cell number in a growth hormone-dependent manner, augmenting the regenerative response in aged mice," *STEM CELLS*, vol. 27, no. 8, pp. 2044–2052, 2009.

[6] T. L. Walker, A. White, D. M. Black, R. H. Wallace, P. Sah, and P. F. Bartlett, "Latent stem and progenitor cells in the hippocampus are activated by neural excitation," *Journal of Neuroscience*, vol. 28, no. 20, pp. 5240–5247, 2008.

[7] K. A. Chamberlain, S. E. Nanescu, K. Psachoulia, and J. K. Huang, "Oligodendrocyte regeneration: its significance in myelin replacement and neuroprotection in multiple sclerosis," *Neuropharmacology*, 2015.

[8] G. Kempermann, *Adult Neurogenesis 2—Stem Cells and Neuronal Development in the Adult Brain*, Oxford University Press, New York, NY, USA, 2011.

[9] I. A. McKenzie, D. Ohayon, H. Li et al., "Motor skill learning requires active central myelination," *Science*, vol. 346, no. 6207, pp. 318–322, 2014.

[10] S. Pluchino, L. Zanotti, B. Rossi et al., "Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism," *Nature*, vol. 436, no. 7048, pp. 266–271, 2005.

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Review Article

Bone Morphogenetic Protein 4 Signalling in Neural Stem and Progenitor Cells during Development and after Injury

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Substantial progress has been made in identifying the extracellular signalling pathways that regulate neural stem and precursor cell biology in the central nervous system (CNS). The bone morphogenetic proteins (BMPs), in particular BMP4, are key players regulating neuronal and glial cell development from neural precursor cells in the embryonic, postnatal, and injured CNS. Here we review recent studies on BMP4 signalling in the generation of neurons, astrocytes, and oligodendroglial cells in the CNS. We also discuss putative mechanisms that BMP4 may utilise to influence glial cell development following CNS injury and highlight some questions for further research.

1. Introduction

Neural stem cells (NSCs) are self-renewing, multipotent progenitor cells that can generate neurons as well as the two major glial cell types, oligodendrocytes and astrocytes [1, 2]. Altman and Das first speculated about the possibility of postnatal neurogenesis generated by an unidentified pool of undifferentiated cells located around the ventricular and germinal zones in young rats [3]. This has since been attributed to NSCs migrating from stem cell niches located in the subventricular zone (SVZ, also known as the subependymal zone) [4]. Adult NSCs share common features with astrocytes [5], and can be identified by nestin, glial fibrillary acidic protein (GFAP), and Sox2 expression [6, 7]. Adult NSCs are derived from embryonic radial glia-like cells (RGCs) during development [8] and are specified at approximately E11.5 in murine embryogenesis [9]. Adult NSCs can give rise to neural precursor cells (NPCs), which include neuroblasts [10] and glial precursor cells such as oligodendrocyte progenitor cells (OPCs) [11, 12]. Neurogenesis also occurs in the hippocampal subgranular zone (SGZ) of the dentate gyrus from precursor cells with stem-like properties. Whether or not these SGZ progenitor cells are "true" stem cells has been debated [13, 14]. There is evidence that they do not self-renew indefinitely but can give rise to all neuronal subtypes through sequential differentiation [13, 15]. These two regions are currently the only known source of NSCs in the mammalian brain [2, 14].

Several key signalling pathways govern the regulation of NSC maintenance and specification in the adult CNS. These include WNT/β-catenin [16], Sonic hedgehog (Shh) [17, 18], fibroblast growth factor (FGF) [19], and bone morphogenetic protein (BMP) signalling [20], with degrees of crosstalk between many of these pathways [16, 21–23]. This review will examine the role of BMP signalling in NSC specification in the developing, adult, and injured CNS. In particular, it will focus on the role of BMP4, which has a particularly well-characterised effect on glial development [24]. SVZ NSCs have been better characterised in regard to BMP4 signalling compared to SGZ NSCs [20] and will be discussed in this review at the expense of the latter.

2. BMP4 Signalling Is a Complex, Tightly Regulated System

BMPs are the largest class in the transforming growth factor β (TGF-β) superfamily, with at least 20 structurally distinct members. Aside from their eponymous functions in bone and cartilage formation, they also have defined roles in cellular and developmental processes including proliferation and

differentiation, cell-fate determination, and apoptosis [25]. A protein preparation contributing to osteogenesis was first isolated from decalcified bone extracts and studied for its stimulating effect on chondrocytes, osteoblasts, and osteoclasts by Urist in 1965 [26]. It was initially unclear as to whether a single protein within this mixture was responsible, but subsequent studies by Urist and others lead to the characterisation of several proteins described as "bone morphogenetic proteins" due to their critical role in bone formation [27, 28]. Their contribution to vertebrate development has since been shown to be so extensive that several researchers have suggested that the name "body morphogenetic proteins" would better describe their significance [29, 30]. Within this broad and heterogeneous family, BMP4 in particular has many critical roles in the development of the nervous system during embryogenesis [20]. Furthermore, BMP4 reemerges as an important factor regulating neural cell fate determination during adulthood and following CNS injury.

BMP4 was purified and cloned by Wozney et al. in 1988 and was originally known as BMP-2B due to its DNA sequence similarity to BMP2 [31]. Structurally, human BMP4 is a highly conserved, 116-residue protein that is posttranslationally cleaved from a 408-residue preproprotein. The functional BMP4 peptide chain (from residues 292 onwards) is highly conserved between human, mouse, rat, and zebrafish [32]. The C-terminus contains seven conserved cysteine residues that are glycosylated, leading to the formation of a characteristic cysteine knot structure; this domain allows BMP4 to assemble into a biologically active homodimer and form heterodimers with other BMPs [33].

After synthesis at the endoplasmic reticulum and posttranslational modifications in the Golgi apparatus, the BMP4 peptide chain is proteolytically cleaved and dimerization occurs at the Mad homology (MH2) domain. BMP4 also has a unique secondary cleavage site that governs whether it is subsequently secreted as a short, soluble isoform or longer isoform that is tethered to the extracellular matrix (ECM) [34, 35]. Thus, BMP4 can have local or regional effects depending on cleavage of this secondary prodomain, the exact mechanisms of which remain unclear and are likely to be context-dependent [29]. It can also be carried via matrix vesicular transport, although the exact isoform of BMP4 transported remains unknown [36].

Given the variety of cell types and tissues that it influences, the BMP signalling network is a fittingly diverse affair. BMP4 signalling is transduced through the canonical TGF-β family pathway [37–40]. This involves glycosylated BMP4 forming homodimers in the extracellular space or extracellular matrix and subsequent binding to a membrane-bound receptor complex. This complex is classically comprised of two BMP Type I serine-threonine kinase receptors, of which there are two classes, BMPRIA (or ALK3) and BMPRIB (or ALK6), and two of a single class of Type II receptor, BMPRII. All three receptors contain two conserved functional domains flanking a typical transmembrane domain: an N-terminal extracellular ligand-binding domain for BMP homodimer interaction and a C-terminal intracellular kinase domain. Structurally similar receptors may also act as receptors for BMP4. Activin Receptor Type 1 (ACVR1) can act as a Type I receptor for BMP4 under certain contexts [41]. Similarly, Activin Receptor Type II (ActRII) and Activin Receptor Type IIB (ActRIIB) can act as Type II receptors, with similar binding affinities for BMP4 in certain tissues [42, 43].

Signalling may occur through two mechanisms: preformed complexes (PFCs) of Type I/Type II receptors binding to BMP4 homodimers or initial binding of BMP4 homodimers to the high affinity Type I receptor, which then recruits the Type II receptor to the complex (BMP-Induced Signal Complex or BISC) [44]. Comparatively, BISC signalling is reliant upon cholesterol-enriched regions of the plasma membrane to facilitate BISC formation, whereas PFC signalling does not. However, PFC signalling does appear to require clathrin-mediated endocytosis of the receptor complex to transmit downstream signalling [45]. In general, BMP4 has much higher affinity for its Type I receptors than the Type II receptor [46–49]; direct binding to the Type II receptor is less common. In the canonical BMP signalling pathway, upon binding of the BMP4 homodimer to the receptor complex, conformational changes allow the constitutively active Type II receptor to phosphorylate a conserved glycine/serine box on the Type I receptor kinase domain. This activated Type I receptor then propagates the signal downstream by phosphorylation of the SMAD (signalling mothers against decapentaplegic [50]) family of intracellular signalling molecules (see Figure 1).

BMP4 signalling through complexes comprised of BMPRA/IB and BMPRII preferentially phosphorylates receptor-associated SMAD1, SMAD5, and SMAD8 (known as the R-SMADs) [29], as opposed to SMAD2 and SMAD3. These activated R-SMADs can each form heteromeric complexes with Co-SMAD4, which translocates to the nucleus and acts as a transcription factor (TF), binding cooperatively with other TFs and interacting with specific regulatory DNA sequences to control gene expression [51, 52]. In certain contexts, activated BMPRIA/B may also signal through the p38/mitogen-activated protein kinase (MAPK) pathway in a SMAD-independent manner [53]. Other SMAD-independent or noncanonical BMP signalling pathways have been documented in various applications [54]: these will be selectively discussed as they pertain to neural stem and precursor cell differentiation.

Precise spatiotemporal regulation of BMP signalling is vital due to the many roles that BMPs exert during development and adulthood in multiple tissue-specific processes. As such, the BMP4 signalling pathway can be regulated by numerous extracellular and intracellular factors. Several endogenous extracellular inhibitors of BMP4 have been classified, including noggin [55], chordin [56], FSTL1 [57], DAN (NBL1) [58], and gremlin [59] (for review, see Mulloy and Rider, 2015 [60]). Secretion of noggin, follistatin, and chordin by specialised groups of cells known as organisers is particularly crucial during development to balance the dorsalising effects of BMPs during gastrulation [20, 49]. At a receptor-ligand level, several receptor cobinding partners can enhance or inhibit BMP4 homodimer ligand binding to regulate downstream signalling. For example, BAMBI (BMP and activing membrane-bound inhibitor) is a BMP receptor analogue with similar extracellular protein binding sites as the

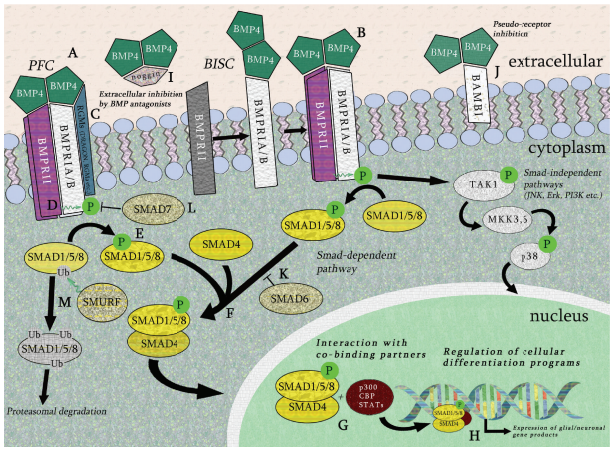

Figure 1: General BMP4 cellular signalling pathway. BMP4 dimers may bind to preformed complexes (PFCs, A), in which BMPRI Type I and Type II receptors are already bound at the cell surface, or by firstly binding to the Type I receptor and inducing the Type II receptor to the complex (BMP-induced signalling complex or BISC, B). Repulsive guidance molecules (RGMa, DRAGON, etc.) may enhance binding of the BMP4 dimer to the Type I receptors in both PFC and BISC binding (C). In the canonical SMAD-dependent pathway, the constitutively active Type II receptor kinase domain phosphorylates a glycine-serine-rich area known as the "GS box" on the Type I receptor (D). The activated Type I receptor sequentially phosphorylates receptor-associated SMADs (SMAD1, SMAD5, and SMAD8 in the case of BMP4) (E). These receptor-associated SMADs then form complexes with Co-SMAD4 (F), enter the nucleus, and further associate with cobinding partners including p300, CBP, STATs, and others (G). These heteromeric complexes then act as transcription factors to regulate the expression of neuronal and glial gene products (H). Extracellular inhibitors of the BMP4, such as noggin, bind BMP4 prior to receptor binding (I). Pseudoreceptors such as BAMBI bind BMP4 dimers but do not propagate downstream signalling activity to a lack of an intracellular kinase domain (J). Other inhibitory intracellular factors include SMAD6, SMAD7, and SMURF. SMAD6 competes with SMAD4 for binding to receptor-associated SMADs (K), SMAD7 blocks the kinase domain of BMP Type I receptors (L), and SMURF mediates ubiquitination and subsequent proteasomal degradation of receptor SMAD1, SMAD5, and SMAD8 (M). Other SMAD-independent pathways may be activated by BMP4, such as MAPK/p38, JNK, Erk, and PI3K (MAPK/p38 pathway shown in this figure).

Type I receptors, but lacking a concomitant kinase domain. This pseudoreceptor competitively binds BMP4 homodimers but prevents further downstream phosphorylation [61]. Another factor, DRAGON, is a glycosylphosphatidylinositol-anchored protein from the repulsive guidance molecule (RGM) family. This protein associates with both types of receptors at the external cell membrane and binds directly to BMP4, enhancing its binding to the receptor complex [62]. A DRAGON homologue, repulsive guidance molecule A (RGMa) enhances binding of BMP2 and BMP4 to BMP Type I receptors, leading to activation of BMP-SMAD signalling [63]. Both DRAGON and RGMa are expressed in the murine neural tube during embryogenesis [62, 63], corresponding to the increased role of BMP-SMAD signalling during this process [64].

At an intracellular level, inhibitory SMAD7 is a cytosolic factor that stably binds to the activated Type I receptors [65] and prevents R-SMADs from being phosphorylated. Downstream of BMP ligand-receptor interactions, SMAD molecules themselves are also subject to regulation. Inhibitory SMAD6 competitively binds with SMAD4 to disrupt the formation of the R-SMAD/SMAD4 TF complex [66]. SMAD specific E3 ubiquitin protein ligases (also known as Smad Ubiquitin Regulatory Factor or SMURF) 1 and 2 are factors that modulate levels of ubiquitinated cytosolic R-SMADs [67]. They may also cooperatively bind with inhibitory SMADs such as SMAD7 to target receptor degradation [68].

Heterodimerisation of BMPs is another extracellular method of signalling regulation [29, 69, 70]. The shared cysteine knot domain allows BMP members to form heterodimers with other BMPs [71]. For instance, BMP4 may bind with BMP7 to form a BMP4-BMP7 heterodimer; this is thought to promote more effective signal transduction in certain applications [72]. The degradation pathway of the BMP4 homodimer and other heterodimers after binding remains unclear.

The specificity of downstream transcriptional control of gene expression by BMP4 is largely dependent on the particular ligand-receptor combinations at the cellular membrane [29]. Several factors can contribute to this interaction: the composition of BMP dimer ligand, whether signalling occurs through the PFC model or BISC model, and the relative contributions of the two different Type I receptors in mediating downstream activity. In particular, variable signalling through BMPRIA/BMPRII complexes versus BMPRIB/BMPRII complexes has been shown to occur at different stages in development, affecting unique downstream targets and regulating diverse cellular processes [38, 73]. Studies using fibroblastic, myoblastic, and osteoblastic cell lines have also shown that BMP2 signalling occurring through PFC complexes preferentially activates SMAD-dependent pathways, whereas BISC binding activates non-SMAD pathways [44, 53]. It is not known whether preferential signalling activation occurs in neural stem and progenitor cells. It should be noted that studies on interactions between ligand-receptor complexes and uncharacterised proteins are still informing the field on novel BMP signalling mechanisms. For example, recent proteomic analysis of novel regulators of BMP signalling has identified for the first time a non-SMAD protein (protein associated with SMAD1, or PAWS1) that can act as a substrate for BMPRIA phosphorylation. Subsequent interaction with SMAD1 leads to the upregulation of SMAD4-independent target genes, as well as possible novel interactions beyond the canonical BMP signalling pathway [74].

3. BMP4 Signalling in Neural Stem and Precursor Cells during Embryonic Development

Since their discovery as osteoinductive factors, BMPs have also been shown to play a crucial role in the development of the nervous system, specifically neuroectoderm induction,

neural crest cell specification, and CNS neural patterning [20, 64, 75]. BMP4 in particular has been shown to be critical during early murine embryonic development: *Bmp4* deletion in mice is lethal 6.5 to 9.5 days postcoitum [76]. Deletion of its cognate receptor BMPRIA is also embryonic-lethal in mice [77]. During early embryonic development, expression of BMPs is actively inhibited by secretion of noggin, chordin, and follistatin from embryonic organisers to allow neural induction to commence [78]. *In vitro* application of noggin to human embryonic stem cells activates microRNA-mediated degradation of SMAD4 transcripts. This mechanism putatively acts *in vivo* to block BMP4-SMAD signalling pathways during neural induction [79]. Repression or activation of BMP signalling, in conjunction with a corresponding gradient of Sonic Hedgehog (Shh) expression, actively specifies the ectoderm into neuronal or nonneuronal tissue, respectively. Additionally, other signalling pathways that generally antagonise BMP signalling, such as fibroblast growth factor (FGF) [19] and Insulin-like Growth Factor-1 (IGF1) [80], play a role in modulating the levels of active BMP signalling at this stage, by downregulating both BMP4 expression and phospho-SMAD activation by BMPRIA/B. This complementary morphogenic gradient of BMPs (including BMP4 and others including BMP2 and Growth Differentiation Factor 7 (GDF7)) and Shh signalling establishes the dorsoventral axis, with the area of intermediate signalling specifying the neural crest cells (NCCs) that eventually form the peripheral, sympathetic, and sensory nervous systems [81–83]. NCC specification occurs in conjunction with two pathways strongly associated with BMP: WNT/β-catenin [84, 85] and Notch signalling [86]. WNT/β-catenin is a particularly frequent collaborator with BMP4, with temporally and spatially similar actions in development and adulthood [87, 88].

Following neural induction, secretion of BMP4 from ectoderm and neural tube roofplate cells promotes subsequent neural patterning of several key CNS topographies, including forebrain, cerebellum, and dorsal spinal cord. Again, the dualistic relationship between BMP4 and Shh signalling from the notochord and floorplate is important for dorsoventral axis development in the spinal cord. Liem et al. showed that a dorsal cellular identity does not occur by default due to lack of the Shh ventralising signal. Rather, they showed that the dorsalising signal provided by BMP4 (and 7) to early neural tissue explants directly induces the expression of high levels of definitive dorsal cell markers MSX1, PAX3, DSL1, and SLUG in these cells [89].

Further to this finding, Wine-Lee et al. showed that ablation of BMP Type I receptors BMPRIA/B from the neural tube disrupts proper dorsal-ventral interneuron specification [90], with BMPRIA/B overexpression in the chick spinal cord causing dorsalisation to occur in ventral spinal cord regions [91]. Interactions of BMP4 with WNT/β-catenin are crucially important at this stage. Shortly following neural crest formation, contemporaneous WNT/β-catenin signalling downstream of BMP4 coordinates transcriptional control of the neurogenin-1 (Ngn1) and Olig3 neuron-specific TFs [92, 93], which are crucial for dorsal interneuron specification. Ille et al. have shown that this involves a balance of the proliferation-inducing WNT effect against the differentiation-promoting BMP4 effect on NSCs. This balance may be required to maintain a population of cycling, dorsal interneuron progenitors during spinal cord development [16]. In addition to spinal cord patterning, BMP4 has also been implicated in proper forebrain development [94], as well as early postnatal cerebellar cell differentiation [95].

As well as these early effects in neural induction, neural crest specification, and dorsoventral patterning, BMPs have a significant, temporally dependent influence on both neuronal and glial differentiation of embryonic NSCs and NPCs. Following gastrulation, BMP4 signalling specifies NSCs and NPCs towards neuronal lineage commitment in both the CNS and PNS [64]. In the CNS, Li et al. showed that treatment of embryonic day 13 (E13) rat neocortical neuroepithelial cells with BMP4 *in vitro* significantly increased expression of neural markers MAP-2 and TUJ1 and resulted in longer neurite outgrowth. This was mediated through BMPRIA, as truncated forms of the receptor significantly reduced this effect [96]. The capacity of BMPRIA mutant neurites to respond to brain-derived neurotrophic factor (BDNF) was preserved, suggesting that this was not a blockage of non-specific differentiation factors. Further investigation using bioinformatic techniques would be helpful to rule out the possibility of the truncated receptor blocking other signalling pathways beyond BMP signalling.

The neurogenic effect of BMP4 during forebrain cortical neurogenesis has been associated with the SMAD-independent MAPK/extracellular signal-related kinase (ERK) pathway. Moon et al. used primary cerebral cortical stem cells from E13.5 rats to demonstrate that BMP4 exposure promotes expression of TUJ1 through MAPK/ERK activation. This was further linked to signal crosstalk between BMP4 and WNT/β-catenin, with a WNT/β-catenin signalling activator increasing subsequent downstream BMP4 mRNA transcription. Increased BMP4 levels promote Ras-mediated ERK signalling cascade activation. This occurs synergistically with suppression of epidermal growth factor receptor (EGFR) signalling by BMP4, simultaneously arresting the mitotic effect of EGF [21] on NSCs and allowing ERK to activate the TUJ1 promoter to induce neuronal differentiation [97]. Here, the complexity of neurogenic signalling regulation is clearly displayed, with BMP4 a key mediator in this major cell signalling network.

4. BMP4 Promotes Astrocytic Differentiation through Multiple Mechanisms

As neurogenesis nears completion in late embryonic/early postnatal development, the neurogenic effect of BMP4 is subdued and its dual function as a promoter of astrogliogenesis and inhibitor of oligodendrogliogenesis becomes more pronounced. Several studies from the laboratory of Jack Kessler in the mid-to-late 1990s revealed that BMP4 (as well as related members BMP2 and BMP7) promotes astroglial phenotypes in embryonic neural progenitor cells at late embryonic/perinatal stages. Gross et al. cultured mouse embryonic

(E17) multipotent nestin+ neural progenitors from the subventricular zone with exogenous BMPs, including BMP4, and found that BMP treatment significantly increased GFAP+ cells at several time points. They showed that these cells expressed the relevant BMPRIA/B/II receptors to mediate BMP signalling, but no measurements of downstream signalling molecules (e.g., phospho-SMAD1/SMAD5/SMAD8) were attempted [98]. Subsequent studies showed dose-dependent effects of BMP4 on embryonic stem cells *in vitro*, with different concentrations potentially activating different signalling responses and outcomes [99].

The signalling mechanisms by which BMPs promote astrogliogenesis were subsequently revealed by Nakashima et al. to occur through SMAD1 activation and subsequent association with signal transducers and activators of transcription (STATs). STATs are cytoplasmic transcription factors that have a crucial role in relaying signals from the cell membrane to the nucleus. Activated SMAD1/STAT3 form complexes with p300 and CBP (CREB-binding protein), which are multifunctional coactivators that facilitate binding of SMAD1/STAT3 to astrocytic promoters [100]. Rajan et al. further demonstrated that BMP4-mediated astrocytic differentiation also occurs through the interactions of FKBP12/rapamycin associated protein (FRAP) with BMPRIA. FRAP is activated by the serine-threonine kinase FKBP12, which is normally tethered to the inactive BMPRIA receptor. Upon BMP4-induced conformational changes to BMPRIA, FKBP12 is released and associates with FRAP to activate STAT3 [52]. STAT3 then associates with p300/CBP to activate astrocytic gene promoters as described above.

Prior to this, Bonni et al. and others had shown that ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF) can promote astrogliogenic specification from NPCs [101, 102]. These pathways synergise with BMP4 signalling-regulated STAT3 activation to promote astrogliogenesis through further activation of STATs via Janus kinases (JaK) [103, 104]. These pathways are not redundant: LIF signalling appears to be important for production of GFAP+ astrocyte progenitors, with BMP4-induced astrocytes producing a more mature, lineage restricted astrocyte morphology [105].

In addition to these mechanisms, p57kip2 is an important upstream promoter of the BMP4-mediated astrogliogenesis and regulates expression of BMP4 antagonists. Short-hairpin RNA suppression of p57kip2 abrogated the typical increase in GFAP+ cells generated from SVZ and SGZ NSCs by exogenous BMP4 *in vitro*. They further showed that noggin and chordin expression was increased upon p57kip2 suppression, suggesting possible regulation of expression of these BMP4 antagonists by p57kip2 [106].

5. The Specific BMP4 Effect on Embryonic NSCs Is Temporally Controlled

Given the dual role of BMP4 as both a neurogenic (early embryonic) and astrogliogenic (late embryonic/early postnatal) factor in NSCs, precise temporal control of BMP4 activity by extrinsic factors is crucial. For example, Ngn1 is a critical regulator of neurogenesis [107] with high levels of protein and mRNA transcript expression during neurogenesis (~E12.5 to E15.5) but reduced levels during gliogenesis (~P0–P4) [108]. Sun et al. showed that Ngn1 suppresses BMP4-induced astrogliogenesis in E13.5 cortical NSC cultures by sequestering SMAD1/STAT complexes and blocking their interaction with coactivators p300/CBP. This disrupts the activation of astrocytic promoters such as GFAP by these TF complexes. As neurogenesis nears completion, downregulation of Ngn1 expression by mature neuronal cells releases these astrogliogenic transcriptional promoters from sequestration [109]. Further investigation of this effect by Zhao et al. suggests that Ngn1 also increases transcription of microRNA miR-9 to downregulate JaK-STAT1-mediated astrogliogenesis in embryonic stem cell cultures [110]. Thus, the specific effect exerted by BMP4 on NSCs may depend on levels of temporally dependent external regulators, such as Ngn1.

Evidence from chick embryo studies suggests that differential expression of the Type I receptors may also play a part in regulating the dual neurogenic-astrogliogenic effect of BMP4. Using chick explant cultures, Agius et al. observed that NPCs migrated from the neuroepithelium in the mantle layer from E5 (corresponding to a period of neurogenesis in the developing chick embryo) and that GFAP+ astrocytes were not generated in the dorsal neuroepithelium. They demonstrated *in vitro* that these dorsally derived progenitors were amenable to astroglial lineage commitment, but that increased BMP4-SMAD signalling through BMPRIB in the dorsal-most regions of the neuroepithelium promoted neuronal specification. BMP4 treatment, presumably acting through BMPRIB, completely prevented astrocyte development from more ventrally derived chick spinal cord explants at E5. However, at E6, when BMPRIA is significantly upregulated, astrocyte development was permitted. Noggin treatment at E5 permitted copious GFAP+ expression from dorsal neuroepithelial neural progenitors, suggesting this restriction was an effect of blocking dorsal BMP4-BMPRIB signalling. Thus, the astrogliogenic effect may be due to sudden upregulation of BMPRIA, when at E5 the neurogenic effect of BMP4 is mediated through BMPRIB [111]. This meshes well with evidence describing a direct role of BMPRIA in promoting astrogliogenesis in a SMAD-independent manner through FRAP activation of STATs [52]. Replicating these findings in transgenic mouse models allowing selective ablation of these receptors is crucial to further clarify specific temporal functions of BMPRIA/B during neuronal development.

6. BMP4 Is Critical for Suppression of Oligodendrogliogenesis

Perhaps the most well-characterised effect of BMP4 is its inhibitory effect on the myelin-forming oligodendrocyte lineage cells *in vitro* and *in vivo* [98, 112–115]. OPCs are specified from NSCs during development and adulthood and can differentiate into the myelin-forming oligodendrocytes of the CNS [116]. OPCs can also form astrocytes and, in special cases, neurons, leading some to consider the OPC to be more aptly described as an adult NSC [117]. The exact region OPCs are originally derived from was a contentious

topic for many years, but recent evidence has determined that embryonic stage OPCs are firstly specified in the ventral ventricular zone of the spinal cord [118]. Specification of these ventrally derived oligodendrocyte-lineage cells as OPCs has been shown to be influenced by Shh signalling from the floorplate and notochord [17, 119].

OPCs also arise from dorsal sources at a later stage in development and are influenced by a Shh-independent pathway [120]. BMP4 has also been shown to inhibit the generation of these dorsally derived OPCs [121]. Developmentally, OPCs are generally characterised by the expression of several markers including the key basic helix-loop-helix (bHLH) transcription factor Olig2, a critical factor that promotes oligodendrocyte lineage commitment [122], and others such as platelet-derived growth factor receptor-α (PDGFRα) [123], chondroitin sulphate proteoglycan NG2 [124], and the monoclonal antibody O4 [125].

Several groups have shown that excess exogenous BMP4 during development reduces subsequent oligodendrogliogenesis in both the mouse [112] and chick embryo [113]. Mekki-Dauriac et al. also showed that disruption of endogenous BMP4 signalling by transplanting noggin-overexpressing cells produced early dorsal oligodendrocyte production [113]. The effect of BMP4 on OPCs has been shown to be dose-dependent. Grinspan et al. exposed OPCs and "pre-OPCs" (Nestin−/Olig2+ cells lacking classical OPC marker expression) to increasing concentrations of BMP4, with diminishing effects on maturation as the progenitor cells progress through the oligodendrocyte lineage [114].

Given that global genetic knockout of BMP4 and its receptors is embryonic-lethal, conditional genetic ablation driven by expression of temporal markers offers a more nuanced approach to understanding BMP4 signalling in embryonic development. Genetic manipulation of the BMPRI receptors has provided interesting and somewhat counterintuitive insights into the role of BMP4 receptors in specifying OPCs from NSCs during development. Two studies in particular have looked at disruption of BMP4 signalling through deletion of the BMPRIA/B receptors. See et al. used Cre-*loxP*-mediated transgenic excision of the *Bmpr1a* gene from cells expressing BRN4, a broad neural TF activated in early embryogenesis. This was crossed with a conventional *Bmpr1b* KO mouse to generate *Bmpr1a*-*Bmpr1b* double KO mice. This modification leads to several developmental defects in mice at P0. While numbers of astrocytes in the spinal cord are decreased at P0 compared to controls, disrupted BMP4 signalling through BMPRIA/B does not appear to affect total numbers of OPCs. Intriguingly, the numbers of mature oligodendrocytes expressing common myelin proteins including myelin basic protein (MBP) were reduced at P0. This suggests that some basal level of embryonic BMP signalling through BMPRIA/B is required for prenatal oligodendrocyte maturation [126]. The second study by Samanta et al. deleted BMPRIA only from NPCs expressing Olig1 in the neural tube from E13.5. This did not affect subsequent OPC numbers at birth or P20 [127]. However, at P20, there was an increase in mature oligodendrocytes in the BMPRIA KO group. This study did not discount the possibility of increased compensatory signalling through BMPRIB, as phospho-SMAD1, phospho-SMAD5, and phospho-SMAD8 were still detected.

A further study utilising the *Bmpr1a* conditional KO system examined the role of deleting BMPRIA in *Emx-1* expressing NSCs of the murine telencephalon. It was found that subsequent astrocytes derived from these NSCs aberrantly expressed vascular endothelial growth factor (VEGF) at P10, leading to the disruption of cerebrovascular angiogenesis as well as impaired blood-brain barrier formation [128]. Interestingly, while previous studies using Olig1-*Cre*-driven *Bmpr1a* deletion showed increases in mature O4+ oligodendrocytes at P20, no differences in O4+ cells were observed at P20 in this study. In addition, compared to the earlier study deleting both *Bmpr1a* and *Bmpr1b* from BRN4-expressing cells in which GFAP+ astrocytes are reduced, no such decreases were observed here. These observations can be attributed to the different regional and temporal expression profiles of the Cre promoter driving the deletion of *Bmpr1a*, respectively.

In summary, embryonic studies utilising broad overexpression or inhibition of BMP4 during, or immediately prior to, gliogenesis demonstrate that this decreases or increases subsequent oligodendrogliogenesis, respectively. However, blocking BMP4-SMAD signalling through deletion of BMPRIA/B from early embryonic embryogenesis (prior to OPC specification) reduces numbers of mature oligodendrocytes at P0. Importantly, this is not due to reduction in the numbers of OPCs specified, as no changes in OPC number were detected. Additionally, a second line of inquiry found that reduction, but not complete suppression, of BMP4 signalling through BMPRIA deletion at E13.5 has no effect on OPC numbers at P0. However, by this stage, reduced BMP signalling increases mature oligodendrocyte number by P20. The reasons for this remain unclear. Conclusions from both studies and others suggest that BMP signalling through BMPRIA/BMPRIB does not play a role in specification of OPCs from NSCs but has a strong negative effect on subsequent OPC differentiation [129]. It is likely from the current data that signalling through particular combinations of receptors (e.g., BMPRIA-BMPRII versus BMPRIB-BMPRII complexes) could have unique effects on oligodendrogliogenesis from NSCs and NPCs. Moreover, as described above, the specific regional and temporal expression of particular BMP receptors during development must be considered. Further research using inducible, cell-specific genetic knockouts and pharmacological inhibition of individual BMP receptors could potentially elucidate these mechanisms.

The mechanisms by which BMP4 is thought to modulate oligodendroglial lineage commitment are thought to involve the basic helix-loop-helix transcription factors named "inhibitors of differentiation" or IDs, which are known to be a key downstream target of the BMP/SMAD signalling pathway [130]. Overexpression of ID4 in OPC cultures promotes astrogliogenesis and mimics the effect of BMP4 [131]. Samanta and Kessler cultured neural progenitor cells with BMP4 and a microarray analysis showed that, within the culture, the ID family of transcription factors was significantly upregulated, particularly ID4. Both ID2 and ID4 were then used in a lentivirus overexpression assay using cultured neural

progenitor cells. The ID4 group showed a marked decrease in the number of oligodendrocytes, while the number of astrocytes increased 2.5-fold. Mechanistically, coimmunoprecipitation studies showed that the ID proteins inhibited differentiation by complexing with Olig1/2 and preventing them from entering the nucleus. Immunohistochemical analysis showed that, in the absence of BMP4, the Olig transcription factors were localised predominantly in the nucleus. However, in the BMP4 treated group, they were found to be colocalised in the cytoplasm with ID proteins [132].

A recent RNA sequencing (RNA-Seq) transcriptome database constructed by Zhang et al. examined whole cell gene transcription in multiple CNS cell types in postnatal mice [133]. Interestingly, *Bmp4* shows a tenfold increase in transcription by OPCs compared to astrocytes, neurons, microglia, and endothelial cells at P7. Furthermore, newly formed oligodendrocytes that are not expressing myelin proteins such as MBP have a further approximate fourfold increase in *Bmp4* transcription over OPCs, but this response is downregulated upon maturation to myelinating oligodendrocytes (Figure 2). The obvious question arises: why is *Bmp4* transcription upregulated in OPCs, whose differentiation is significantly impaired by BMP4? One explanation is that increased local expression of BMP4 antagonists balances out this increased BMP4 expression by OPCs. Kondo and Raff observed increased noggin mRNA expression in both P6 optic nerve OPCs and astrocytes, but not oligodendrocytes [134]. Furthermore, experiments showing downregulation of BMP4 by WNT/β-catenin regulator Transcription Factor 7-like 2 (TCF7L) suggest that BMP4 expression by OPCs could be antagonised during development in a posttranscriptional manner to allow for oligodendrocyte differentiation [135]. This type of posttranscriptional regulation of BMP4 by specific glial progenitors requires further exploration.

However, as mentioned above, BMP4 can be expressed as a localised form tethered to the ECM as well as a secreted form. To date, no study has examined the exact isoform of BMP4 being expressed by OPCs. Another conjectural explanation for increased BMP4 expression in OPCs could be that local, ECM-tethered BMP4 acts as a spatial configuring mechanism during OPC differentiation to correctly space developing oligodendrocytes in the CNS. Oligodendrocytes can myelinate up to 50 individual axons in the CNS [136], and proper regional distribution of oligodendrocytes is likely crucial to maintaining appropriate and coordinated myelination. Perhaps BMP4 has a pragmatic role, preventing the differentiation of nearby OPCs and allowing newly developing oligodendrocyte progenitors to "stake out" a place nearby an unmyelinated neuron for subsequent myelination? Whether differential expression of localised and secreted forms of BMP4 in OPCs has functional relevance is an open and intriguing question.

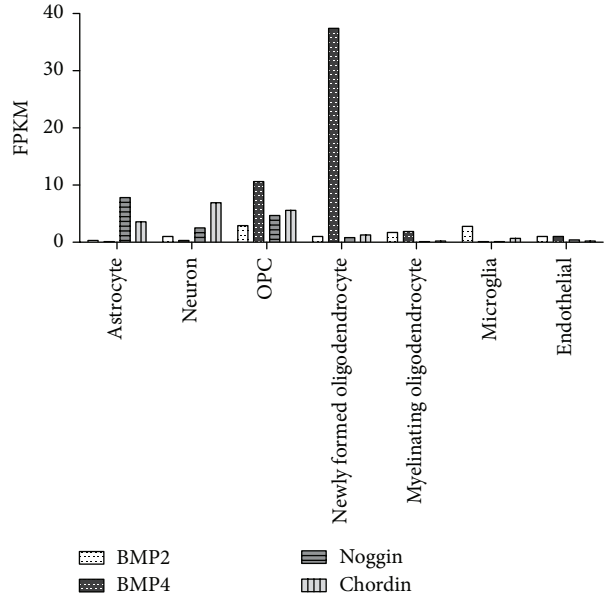

Figure 2: RNA-Seq transcriptome analysis showing increased transcription of *Bmp4* transcripts in OPCs and newly formed oligodendrocytes in postnatal mice compared to other CNS cells. This is unique to *Bmp4*; closely related BMP2 does not show similar levels of increased transcription in oligodendroglial lineage cells compared to other CNS cells. Additionally, increased *Bmp4* transcription does not appear to be counteracted by concomitant transcriptional increases expression of BMP inhibitors, such as noggin and chordin. The functional relevance of increased *Bmp4* transcription by OPCs and immature oligodendrocytes remains to be clarified (FPKM = fragments per kilobase of transcript per million mapped reads.).

7. BMP4 Signalling in SVZ NSCs and Neural/Glial Progenitors during Adulthood

BMP4 continues to regulate NSC differentiation into neurons, astrocytes, and oligodendrocytes in the adult CNS. Given its role as a developmental regulator of NSC differentiation, it forms a crucial part of a larger signalling network maintaining an undifferentiated pool of NSCs and neural progenitors in the SVZ [10]. The cellular componentry of the SVZ consists of ependymal cells, and three classes of progenitor cells known as Type A, B, and C cells (see Figure 3). SVZ ependymal cells are nondividing support cells that facilitate cerebrospinal fluid circulation to the area [137] and can contribute to neurogenesis during stroke [138]. Type A cells are defined as chains of migrating neuroblasts and are generated from nearby highly proliferative Type C cells, which are known as transient amplifying progenitors (TAPs) or intermediate precursor cells (IPCs). Type B cells (or NSCs) are slow cycling progenitors with characteristics of astrocytes, but retaining stem-cell properties. These cells are further subdivided into B1 cells, which are located near the ependymal layer, and B2 cells, which associate closely with the adjacent striatal parenchyma [139].

BMP4 and its associated canonical receptors are expressed in both NSCs (B cells) and TAPs (C cells) in the adult SVZ [55, 140, 141]. SVZ ependymal cells also express noggin, which regulates levels of BMP4 signalling and modulates its neurogenic-gliogenic effects [55]. Colak et al. showed that deletion of SMAD1, a key downstream mediator of BMP4 signalling, severely impairs neurogenesis in the murine SVZ

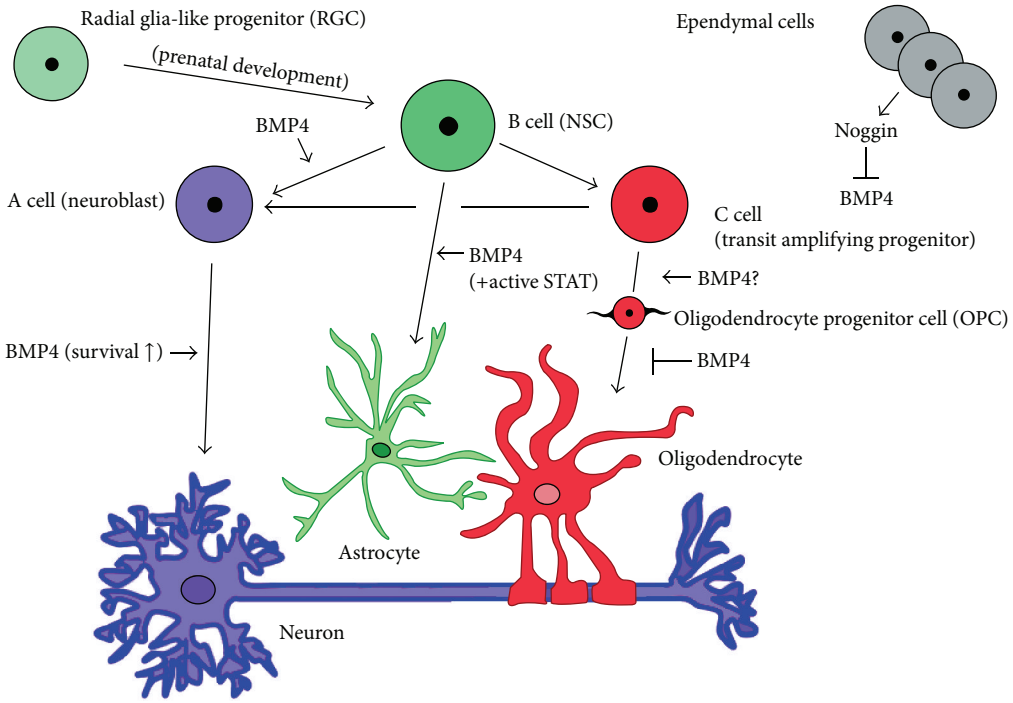

Figure 3: Simplified diagram of adult SVZ illustrating BMP4 involvement in NSC development. Adult NSCs (B cells) are specified from radial glia-like cells during prenatal development. Neuroblasts (A cells) and transient amplifying progenitors (B cells) are derived from NSCs and generate neurons and glia. Ependymal cells provide support by regulating CSF circulation and secrete BMP4 inhibiting noggin to modulate BMP signalling in the SVZ. BMP4 signalling through SMAD4 is important for neural specification of neuroblasts but does not influence further neuroblastic differentiation. It does appear to have a prosurvival effect on neuroblasts committed to the neuronal lineage. BMP4 signalling can promote astrogliogenesis from adult NSCs, but only with concomitant STAT-signalling, typically seen in CNS injury models. Recent evidence has shown that adult astrogliogenesis can occur from nestin+ SVZ NPCs, but the role of BMP4 in this process was not investigated. The role of BMP4 in OPC specification during development and adulthood is not completely resolved, but most data suggest that it does not play a significant role. However, there is a very clear inhibitory BMP4 effect on OPC progression towards an oligodendrocyte lineage during development, adulthood and CNS injury.

and acts early in the specification of NSCs to TAPs, which sequentially generate neuroblasts. Exogenous noggin infusion to the mouse SVZ promoted oligodendrogenesis over neurogenesis from TAPs. Phosphorylated SMAD1, SMAD5, and SMAD8 were also detected in SVZ GFAP+ cells and TAPs, but not in doublecortin (DCX)+ neuroblasts. The study did not specifically implicate BMP4 as a regulator of this effect but did note its increased expression and the presence of its canonical receptors and activated downstream SMADs. Interestingly, this study did note that BMP signalling in the SVZ does not promote astrogliogenesis. The authors speculated that, due to the lack of STAT expression in the SVZ [142], the induction of astrocytes via the BMP-dependent SMAD1/STAT interactions does not occur; thus, the neurogenic effect of BMP4 signalling predominates [143]. A recent study by Sohn et al. has demonstrated that corpus callosum and rostral migratory stream astrocytes are generated from SVZ nestin+ NPCs in mice; however, the role of BMP4/STAT signalling in this process has not yet been investigated [144]. Endogenous noggin expression likely allows for tight regulation of BMP concentrations in the SVZ to maintain progenitor pools. A similar result was also found in a study using chordin to modulate BMP4 levels and maintain progenitor cell plasticity in the SVZ [145].

8. BMP Signalling in Neural Stem and Precursor Cells Following CNS Injury

Given the role of the BMP4 in regulating maintenance and differentiation of NSCs during embryogenesis and adulthood, they represent a clear factor of interest in manipulation of endogenous and exogenous NSCs for therapeutic applications. The role of BMPs in CNS injury was comprehensively reviewed in a recent article by Grinspan [24]; this section will further examine key studies implicating BMP4 in the specification of NSCs and NPCs in CNS disease models.

Several CNS injuries have been shown to exhibit increased BMP4-SMAD signalling in neural stem cells and endogenous glial progenitors [146]. Given the well-characterised inhibitory effect of BMP4 on oligodendrocyte production, it has been extensively studied in the context of demyelinating disease [24, 129]. However, it has also been implicated in several other neurodegenerative and acute injuries of the CNS. BMP4 increases reactive astrogliosis *in vivo* [147] and is commonly upregulated by several cell types as a response to CNS injury [24]. It is unknown exactly what regulates this injury-induced upregulation of BMP4. Oxidative stress has been implicated in an intrauterine growth retardation

(IUGR) model [148], but whether this is a common mechanism amongst other CNS injuries is currently unclear.

9. CNS Demyelination

BMP4 was first implicated in demyelinating disease through mRNA upregulation in demyelinated Multiple Sclerosis (MS) brain lesions [149]. Neural progenitors with a bipolar morphology and expression of polysialylated neuronal cell adhesion molecule (PSA-NCAM) have been identified as early oligodendroglial progenitors migrating from the SVZ during a demyelinating event [150]. Several experimental models of induced demyelination have demonstrated upregulated BMP4 signalling in both NSCs and OPCs. Increased generation of OPCs from NSCs has been shown to occur during focal demyelination localised near the SVZ [11, 151]. Interestingly, there is compelling evidence that pedigree matters during remyelination in the CNS. Xing et al. used genetic fate-mapping strategies during cuprizone-induced focal demyelination in the mouse corpus callosum to investigate the relative activities of NSC-derived OPCs and OPCs that migrate and differentiate from the brain parenchyma. From this, they showed that NSC-derived OPCs contribute to more extensive remyelination (measured by myelinated axon diameter) in the mouse corpus callosum after 6 weeks compared to parenchymal-derived OPCs [152]. As discussed above, BMP4 suppresses both NSC-derived oligodendrogliogenesis in the SVZ and adult OPC differentiation; thus, it may affect cell-mediated remyelination after demyelination at multiple levels in the CNS.

Ethidium bromide-induced demyelination causes a significant upregulation of BMP4 in mice. Zhao et al. observed that BMP4 mRNA was significantly upregulated in OPCs, whereas the expression of other BMPs, as well as noggin, did not change significantly [153]. The increased expression of BMP4 in OPCs upon commencement of remyelination (an endogenous repair response by local and migratory OPCs to a demyelinating insult) did not act in an autocrine manner as OPC differentiation during remyelination was not impeded. These data corroborate with subsequent RNA-Seq analysis of upregulated BMP4 transcription by postnatal OPCs [133] and further the intrigue of BMP4 expression in OPCs during adulthood and injury.

The effect of BMP4 in regulating NSC- and OPC-mediated CNS remyelination has also been investigated in two studies. Cate et al. showed that cuprizone-induced demyelination causes a significant upregulation of BMP4, its receptors BMPRIA, BMPRIB, and BMPRII, and phosphorylated SMAD1, SMAD5, and SMAD8 in the mouse SVZ [154]. Interestingly, in a follow-up study, BMP4 infusion during demyelination increased the numbers of proliferating OPCs [155]. However, increased generation of OPCs did not lead to increased numbers of oligodendrocytes, as has been shown in many studies assessing the differentiation block of OPCs in chronic demyelinating diseases [156]. Both studies showed that blocking BMP4 signalling via noggin infusion into the demyelinated areas of the mouse brain increased remyelination of damaged myelin sheaths.

As described above, during development, BMP4 is a crucial part of a complex signalling network involving WNT/β-catenin, FGF, Shh, and other major signalling pathways. As such, any attempt to modulate BMP4 signalling to ameliorate damage during and enhance repair after CNS injury must take into consideration possible crosstalk and regulation of associated pathways. For example, the inhibitory action of dysregulated WNT/β-catenin signalling on OPC differentiation has been demonstrated in demyelinating disease [157]. Feigenson et al. used *in vitro* OPC cultures to demonstrate that WNT/β-catenin signalling operates upstream of BMP4 signalling to mediate this effect. Both BMP4 and WNT/β-catenin signalling component Wnt3a inhibits oligodendrocyte differentiation in OPC cultures. Blocking of BMP4 via noggin application negated its astrogliogenic effect in OPC cultures despite the continued presence of Wnt3a, whereas inhibiting Wnt3a while retaining exogenous BMP4 treatment did not prevent increased astrogliogenesis [158]. They also demonstrated that Wnt3a does not promote astrogliogenesis from early postnatal OPC cultures derived from BMPRIA/B knockout mice. This relationship was further confirmed by a separate group *in vivo* using genetic knockout studies [135]. Genetic knockout of WNT/β-catenin effector TCF7l2, previously thought to suppress oligodendroglial differentiation by activation of WNT/β-catenin signalling, revealed that it has a dual role in inhibiting BMP4-mediated SMAD activation in OPCs and early oligodendrocytes. Whether this factor is relevant for NSC patterning and differentiation during embryonic development remains uncertain.

In addition to this, Wu et al. used an epigenetic approach to identify downstream histone deacetylation as a key transcriptional process regulated by BMP4 during CNS demyelination. BMP4 infusion during cuprizone-induced demyelination led to a significant increase in proliferating astrocyte numbers with elevated levels of acetylated histone H3 compared to vehicle-infused mice after 6 weeks. This was coupled with a decrease in mature oligodendrocytes and a transcriptional increase in downstream effectors for Notch and WNT/β-catenin such as *Hey1* and *Hes*. This agreed with their findings *in vitro* that BMP4 acts to suppress Shh-mediated histone deacetylation in OPCs. They postulated that, in OPCs, Shh-mediated histone deacetylases (HDACs) compacts chromatin and blocks access to promoters of astrocytic differentiation gene networks that are activated by convergence of BMP4, WNT/β-catenin, and Notch signalling [22]. Further investigation into common transcriptional elements between these related pathways is crucial for identifying optimal therapeutic targets for NSC/NPC-based regenerative therapies.

10. Spinal Cord and Other CNS Injuries and Disorders

BMP4 also has a defined role in regulating NPC differentiation in spinal cord injury (SCI). Wang et al. showed that conditioned media from astrocytes derived from the injured spinal cord of the mouse inhibited differentiation of OPCs into mature oligodendrocytes. Subsequent protein expression analysis indicated that BMP4 was upregulated in

cultures of reactive astrocytes isolated from the injured spinal cord. Xiao et al. observed that BMP4 and phospho-SMAD1, phospho-SMAD5, and phospho-SMAD8 were upregulated in most neural cell types, including nestin+ NSCs, in response to induced SCI in mice. Predictably, *in vitro* spinal cord-derived NSCs pretreated with exogenous noggin prevented astrogliogenesis from subsequent BMP4 exposure. However, *in vivo* noggin application failed to completely suppress elevated GFAP+ expression in the injured spinal cord. The researchers attributed this to the continued activity of BMP4-independent promoters of astrogliogenesis including the CNTF/LIF-mediated JaK-STAT pathway [159].

Investigations into the formation of the astrocytic glial scar characteristic of spinal cord lesions have revealed intriguing functions for individual BMP Type I receptors. Sahni et al. conditionally deleted *Bmpr1a* and *Bmpr1b* from GFAP-expressing astrocytes prior to induction on SCI [160]. Injured wildtype animals displayed increased astrogliosis, increased phospho-SMAD1/SMAD5/SMAD8 expression by reactive astrocytes, as well as increased *Bmpr1a* transcript production. Injured *Bmpr1a* KO animals had reduced astrocytic hypertrophy compared to wildtype mice, leading to decreased astrogliosis around injured SC lesions, with increased immune cell infiltration as an indirect result. Surprisingly, *Bmpr1b* KO mice displayed an opposite reaction to SCI compared to the *Bmpr1a* mouse, with increased astrogliosis and accelerated wound closure, presumably from increased signalling through BMPRIA. Long-term, increased signalling through BMPRIB was found to attenuate glial scar progression and slow wound closure, leading to a poorer functional outcome compared to *Bmpr1a* KO mice. From this, it was suggested that signalling levels through the two receptors play opposing roles in modulating levels of astrocyte reactivity and subsequent glial scarring. While no differences in phosphorylated STAT3 or SMAD levels were observed in the *Bmpr1a* KO, differences in expression of microRNA-21 between *Bmpr1a* and *Bmpr1b* KO were suggested as a possible regulator of GFAP expression during SCI. This was further corroborated in a miRNA-21 overexpression system in mice [161].

Further work by North et al. showed that possible interactions of BMPRIB with β1-integrin at the cell membrane may alter the levels of downstream signalling activated by the receptor. β1-integrin is an ECM-interacting protein, a group of proteins that play an important role in stem cell maintenance. It was initially shown that β1-integrin expression was upregulated by ependymal zone cells in mice following SCI. Deleting β1-integrin in ependymal zone cell cultures, which generate nearly half of newly differentiated astrocytes following SCI, led to significant increases in GFAP expression and astrocytic differentiation compared to wildtype cells. Protein analysis of isolated lipid raft fractions from β1-integrin KO cultures identified an increased presence of BMPRIB. Subsequent disruption of lipid raft formation with a lipid raft inhibitor decreased both phospho-SMAD signalling and GFAP expression in these cultures. The researchers suggested that β1-integrin prevents the localisation of BMPRIB into lipid rafts, blocking further downstream signalling [162].

Experiments by Sandner et al. utilised bone marrow stromal cells (BMSCs) cotransplanted with NPCs to enhance repair after SCI in rats. The group had previously demonstrated that coculturing of BMSCs with hippocampal NPCs enhances the differentiation of the NPCs into mature oligodendrocytes via unknown secreted factors [163]. In this study, they demonstrated a similar effect *in vitro* using SVZ-derived NPCs. However, in the rat injured spinal cord, cotransplantation of BMSCs with SVZ-derived NPCs did not lead to enhanced oligodendrocyte numbers compared to animals receiving only NPCs. This differentiation block was linked to increased expression of BMPs 2 and 4 around the site of injury. Returning to *in vitro* culture assays, they showed that concurrent BMP2/4 treatment was sufficient to block the positive effect of BMSCs on oligodendrocyte differentiation from SVZ-derived NPCs. *In vitro*, they demonstrated that coculturing of NPCs with BMSCs overexpressing noggin via lentiviral gene transfer blocked the inhibitory effect of BMP4 [164]. Cotransplantation of noggin-overexpressing BMSCs combined with SVZ derived NPCs was not attempted but would be an interesting follow-up investigation. This study highlights a key challenge facing NSC transplantation for therapeutic applications: identifying and modulating critical intrinsic factors in the disease environment that may compromise the desired differentiation of newly transplanted cells.

Elevated BMP4 levels have also been observed in other CNS injuries and disorders including Alzheimer's disease [165] and intraventricular haemorrhage (IVH) in premature infants [166]. The latter study found BMP4 levels particularly elevated in the SVZ and in OPCs, suggesting a possible role in the hypomyelination displayed by IVH patients.

Clearly, the well-characterised astrogliogenic and anti-oligodendrogliogenic effect of BMP4 on glial progenitors can be problematic in CNS injuries, especially white matter injuries such as demyelination and SCI. As such, therapeutic targeting of BMP4 in CNS injury may be beneficial. However, the viability of BMP4-based therapeutics will depend on future research efforts to identify key components mediating the signalling pathway network. This will allow therapeutic approaches to effectively modulate specific undesirable BMP4 signalling outcomes without disrupting any of its potentially beneficial effects during the disease course.

11. Concluding Remarks and Future Perspectives

For a single protein, BMP4 has extensive influence on a multitude of CNS developmental and postnatal processes, as well as after CNS injury. BMP4 plays a part in the differentiation of NSCs into all three major classes of CNS cells: firstly neurons, then astrocytes, and all the while repressing oligodendrocyte lineage commitment throughout development and adulthood. Many questions have been addressed, but many remain: Does signalling through different receptor combinations produce different context-specific effects in NSCs? Is there extensive posttranscriptional regulation of BMP4 in NSCs and NPCs, adding further variation on an already dazzlingly complex regulatory network? To what extent does signal crosstalk between BMP4 and other signalling pathways

regulate remyelination in the demyelinated CNS? Clearly, the BMP4 signalling pathway has extraordinary breadth of activity in regulating NSC and NPC biology during development, adulthood, and disease. Fully expounding its intricacies and relationships with other signalling pathways will be beneficial for further therapeutic application of NSCs and NPCs.

Abbreviations

NSC: Neural stem cell
NPC: Neural progenitor cell
BMP: Bone morphogenetic protein
SVZ: Subventricular zone
SGZ: Subgranular zone
OPC: Oligodendrocyte progenitor cell
GFAP: Glial fibrillary acidic protein
MH: Mad homology
Shh: Sonic hedgehog
FGF: Fibroblast growth factor
MAPK: Mitogen-activated protein kinase
TGF-β: Transforming growth factor β
EGFR: Epidermal growth factor receptor
ERK: Extracellular signal-related kinase
BMPR: Bone morphogenetic protein receptor
ECM: Extracellular matrix
bHLH: Basic helix-loop-helix
RGMa: Repulsive guidance molecule A
PAWS1: Protein associated with SMAD1
SMURF: SMAD Ubiquitin Regulatory Factor (SMAD specific E3 ubiquitin protein ligase)
PDGFRα: Platelet-derived growth factor receptor-α
TAPs: Transient amplifying progenitors
IPCs: Intermediate precursor cells.

Competing Interests

The authors declare that they have no competing interests.

References

[1] S. Temple, "The development of neural stem cells," *Nature*, vol. 414, no. 6859, pp. 112–117, 2001.

[2] A. M. Bond, G.-L. Ming, and H. Song, "Adult mammalian neural stem cells and neurogenesis: five decades later," *Cell Stem Cell*, vol. 17, no. 4, pp. 385–395, 2015.

[3] J. Altman and G. D. Das, "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats," *Journal of Comparative Neurology*, vol. 124, no. 3, pp. 319–335, 1965.

[4] J. G. Emsley, B. D. Mitchell, G. Kempermann, and J. D. Macklis, "Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells," *Progress in Neurobiology*, vol. 75, no. 5, pp. 321–341, 2005.

[5] F. Doetsch, I. Caille, D. A. Lim, J. M. Garcia-Verdugo, and A. Alvarez-Buylla, "Subventricular zone astrocytes are neural stem cells in the adult mammalian brain," *Cell*, vol. 97, no. 6, pp. 703–716, 1999.

[6] D. C. Lagace, M. C. Whitman, M. A. Noonan et al., "Dynamic contribution of nestin-expressing stem cells to adult neurogenesis," *Journal of Neuroscience*, vol. 27, no. 46, pp. 12623–12629, 2007.

[7] C. Giachino, O. Basak, S. Lugert et al., "Molecular diversity subdivides the adult forebrain neural stem cell population," *STEM CELLS*, vol. 32, no. 1, pp. 70–84, 2014.

[8] F. T. Merkle, A. D. Tramontin, J. M. García-Verdugo, and A. Alvarez-Buylla, "Radial glia give rise to adult neural stem cells in the subventricular zone," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 50, pp. 17528–17532, 2004.

[9] L. C. Fuentealba, S. B. Rompani, J. I. Parraguez et al., "Embryonic origin of postnatal neural stem cells," *Cell*, vol. 161, no. 7, pp. 1644–1655, 2015.

[10] R. A. Ihrie and A. Álvarez-Buylla, "Lake-front property: a unique germinal niche by the lateral ventricles of the adult brain," *Neuron*, vol. 70, no. 4, pp. 674–686, 2011.

[11] B. Nait-Oumesmar, L. Decker, F. Lachapelle, V. Avellana-Adalid, C. Bachelin, and A. Baron-Van Evercooren, "Progenitor cells of the adult mouse subventricular zone proliferate, migrate and differentiate into oligodendrocytes after demyelination," *European Journal of Neuroscience*, vol. 11, no. 12, pp. 4357–4366, 1999.

[12] B. Menn, J. M. Garcia-Verdugo, C. Yaschine, O. Gonzalez-Perez, D. Rowitch, and A. Alvarez-Buylla, "Origin of oligodendrocytes in the subventricular zone of the adult brain," *The Journal of Neuroscience*, vol. 26, no. 30, pp. 7907–7918, 2006.

[13] N. D. Bull and P. F. Bartlett, "The adult mouse hippocampal progenitor is neurogenic but not a stem cell," *The Journal of Neuroscience*, vol. 25, no. 47, pp. 10815–10821, 2005.

[14] G. Kempermann, "The pessimist's and optimist's views of adult neurogenesis," *Cell*, vol. 145, no. 7, pp. 1009–1011, 2011.

[15] J. M. Encinas and G. Enikolopov, "Identifying and quantitating neural stem and progenitor cells in the adult brain," *Methods in Cell Biology*, vol. 85, pp. 243–272, 2008.

[16] F. Ille, S. Atanasoski, S. Falk et al., "Wnt/BMP signal integration regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord," *Developmental Biology*, vol. 304, no. 1, pp. 394–408, 2007.

[17] N. P. Pringle, W.-P. Yu, S. Guthrie et al., "Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog," *Developmental Biology*, vol. 177, no. 1, pp. 30–42, 1996.

[18] Q. R. Lu, D.-I. Yuk, J. A. Alberta et al., "Sonic Hedgehog-regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system," *Neuron*, vol. 25, no. 2, pp. 317–329, 2000.

[19] S. I. Wilson, E. Graziano, R. Harland, T. M. Jessell, and T. Edlund, "An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo," *Current Biology*, vol. 10, no. 8, pp. 421–429, 2000.

[20] A. M. Bond, O. G. Bhalala, and J. A. Kessler, "The dynamic role of bone morphogenetic proteins in neural stem cell fate and maturation," *Developmental Neurobiology*, vol. 72, no. 7, pp. 1068–1084, 2012.

[21] L. Lillien and H. Raphael, "BMP and FGF regulate the development of EGF-responsive neural progenitor cells," *Development*, vol. 127, no. 22, pp. 4993–5005, 2000.

[22] M. Wu, M. Hernandez, S. Shen et al., "Differential modulation of the oligodendrocyte transcriptome by sonic hedgehog and

bone morphogenetic protein 4 via opposing effects on histone acetylation," *The Journal of Neuroscience*, vol. 32, no. 19, pp. 6651–6664, 2012.

[23] A. C. Varga and J. L. Wrana, "The disparate role of BMP in stem cell biology," *Oncogene*, vol. 24, no. 37, pp. 5713–5721, 2005.

[24] J. B. Grinspan, "Bone morphogenetic proteins: inhibitors of myelination in development and disease," *Vitamins & Hormones*, vol. 99, pp. 195–222, 2015.

[25] S. Scarfì, "Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair," *World Journal of Stem Cells*, vol. 8, no. 1, pp. 1–12, 2016.

[26] M. R. Urist, "Bone: formation by autoinduction," *Science*, vol. 150, no. 3698, pp. 893–899, 1965.

[27] M. R. Urist, A. Mikulski, and A. Lietze, "Solubilized and insolubilized bone morphogenetic protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 76, no. 4, pp. 1828–1832, 1979.

[28] S. S. Murray, E. J. B. Murray, J. C. Wang, and M. E. L. Duarte, "The history and histology ofbone morphogenetic protein," *Histology and Histopathology*, 2016.

[29] B. Bragdon, O. Moseychuk, S. Saldanha, D. King, J. Julian, and A. Nohe, "Bone morphogenetic proteins: a critical review," *Cellular Signalling*, vol. 23, no. 4, pp. 609–620, 2011.

[30] A. Guzman, M. Zelman-Femiak, J. H. Boergermann et al., "SMAD versus non-SMAD signaling is determined by lateral mobility of bone morphogenetic protein (BMP) receptors," *The Journal of Biological Chemistry*, vol. 287, no. 47, pp. 39492–39504, 2012.

[31] J. M. Wozney, V. Rosen, A. J. Celeste et al., "Novel regulators of bone formation: molecular clones and activities," *Science*, vol. 242, no. 4885, pp. 1528–1534, 1988.

[32] UniProt Consortium, "UniProt: a hub for protein information," *Nucleic Acids Research*, vol. 43, no. 1, pp. D204–D212, 2015.

[33] Y.-T. Xiao, L.-X. Xiang, and J.-Z. Shao, "Bone morphogenetic protein," *Biochemical and Biophysical Research Communications*, vol. 362, no. 3, pp. 550–553, 2007.

[34] S. M. Nelsen and J. L. Christian, "Site-specific cleavage of BMP4 by furin, PC6, and PC7," *The Journal of Biological Chemistry*, vol. 284, no. 40, pp. 27157–27166, 2009.

[35] C. Degnin, F. Jean, G. Thomas, and J. L. Christian, "Cleavages within the prodomain direct intracellular trafficking and degradation of mature bone morphogenetic protein-4," *Molecular Biology of the Cell*, vol. 15, no. 11, pp. 5012–5020, 2004.

[36] N. N. Nahar, L. R. Missana, R. Garimella, S. E. Tague, and H. C. Anderson, "Matrix vesicles are carriers of bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), and noncollagenous matrix proteins," *Journal of Bone and Mineral Metabolism*, vol. 26, no. 5, pp. 514–519, 2008.

[37] B. L. M. Hogan, "Bone morphogenetic proteins: multifunctional regulators of vertebrate development," *Genes and Development*, vol. 10, no. 13, pp. 1580–1594, 1996.

[38] J. Massagué, L. Attisano, and J. L. Wrana, "The TGF-β family and its composite receptors," *Trends in Cell Biology*, vol. 4, no. 5, pp. 172–178, 1994.

[39] K. Miyazono, Y. Kamiya, and M. Morikawa, "Bone morphogenetic protein receptors and signal transduction," *Journal of Biochemistry*, vol. 147, no. 1, pp. 35–51, 2010.

[40] A. Nohe, E. Keating, P. Knaus, and N. O. Petersen, "Signal transduction of bone morphogenetic protein receptors," *Cellular Signalling*, vol. 16, no. 3, pp. 291–299, 2004.

[41] L. Kan, J. A. Kitterman, D. Procissi et al., "CNS demyelination in fibrodysplasia ossificans progressiva," *Journal of Neurology*, vol. 259, no. 12, pp. 2644–2655, 2012.

[42] K. Heinecke, A. Seher, W. Schmitz, T. D. Mueller, W. Sebald, and J. Nickel, "Receptor oligomerization and beyond: a case study in bone morphogenetic proteins," *BMC Biology*, vol. 7, article 1741, 2009.

[43] D. Yadin, P. Knaus, and T. D. Mueller, "Structural insights into BMP receptors: specificity, activation and inhibition," *Cytokine & Growth Factor Reviews*, vol. 27, pp. 13–34, 2016.

[44] S. Hassel, S. Schmitt, A. Hartung et al., "Initiation of Smad-dependent and Smad-independent signaling via distinct BMP-receptor complexes," *The Journal of Bone & Joint Surgery—American Volume*, vol. 85, supplement 3, pp. 44–51, 2003.

[45] A. Hartung, K. Bitton-Worms, M. M. Rechtman et al., "Different routes of bone morphogenic protein (BMP) receptor endocytosis influence BMP signaling," *Molecular and Cellular Biology*, vol. 26, no. 20, pp. 7791–7805, 2006.

[46] K. Miyazono, S. Maeda, and T. Imamura, "BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk," *Cytokine and Growth Factor Reviews*, vol. 16, no. 3, pp. 251–263, 2005.

[47] P. ten Dijke, H. Yamashita, T. K. Sampath et al., "Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4," *The Journal of Biological Chemistry*, vol. 269, no. 25, pp. 16985–16988, 1994.

[48] T. Natsume, S. Tomita, S. Iemura, N. Kinto, A. Yamaguchi, and N. Ueno, "Interaction between soluble type I receptor for bone morphogenetic protein and bone morphogenetic protein-4," *The Journal of Biological Chemistry*, vol. 272, no. 17, pp. 11535–11540, 1997.

[49] C. Sieber, J. Kopf, C. Hiepen, and P. Knaus, "Recent advances in BMP receptor signaling," *Cytokine and Growth Factor Reviews*, vol. 20, no. 5-6, pp. 343–355, 2009.

[50] E. Gazzerro and C. Minetti, "Potential drug targets within bone morphogenetic protein signaling pathways," *Current Opinion in Pharmacology*, vol. 7, no. 3, pp. 325–333, 2007.

[51] S. Itoh, F. Itoh, M.-J. Goumans, and P. T. Dijke, "Signaling of transforming growth factor-β family members through Smad proteins," *European Journal of Biochemistry*, vol. 267, no. 24, pp. 6954–6967, 2000.

[52] P. Rajan, D. M. Panchision, L. F. Newell, and R. D. G. McKay, "BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells," *Journal of Cell Biology*, vol. 161, no. 5, pp. 911–921, 2003.

[53] A. Nohe, S. Hassel, M. Ehrlich et al., "The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways," *The Journal of Biological Chemistry*, vol. 277, no. 7, pp. 5330–5338, 2002.

[54] R. Derynck and Y. E. Zhang, "Smad-dependent and Smad-independent pathways in TGF-β family signalling," *Nature*, vol. 425, no. 6958, pp. 577–584, 2003.

[55] D. A. Lim, A. D. Tramontin, J. M. Trevejo, D. G. Herrera, J. M. García-Verdugo, and A. Alvarez-Buylla, "Noggin antagonizes BMP signaling to create a niche for adult neurogenesis," *Neuron*, vol. 28, no. 3, pp. 713–726, 2000.

[56] Y. Sasai, B. Lu, H. Steinbeisser, and E. M. de Robertis, "Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus," *Nature*, vol. 376, no. 6538, pp. 333–336, 1995.

[57] Y. Geng, Y. Dong, M. Yu et al., "Follistatin-like 1 (Fstl1) is a bone morphogenetic protein (BMP) 4 signaling antagonist in controlling mouse lung development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 17, pp. 7058–7063, 2011.

[58] W.-T. Hung, F.-J. Wu, C.-J. Wang, and C.-W. Luo, "DAN (NBL1) specifically antagonizes BMP2 and BMP4 and modulates the actions of GDF9, BMP2, and BMP4 in the rat ovary1," *Biology of Reproduction*, vol. 86, no. 5, article 158, 2012.

[59] J. Sun, F.-F. Zhuang, J. E. Mullersman et al., "BMP4 activation and secretion are negatively regulated by an intracellular Gremlin-BMP4 interaction," *Journal of Biological Chemistry*, vol. 281, no. 39, pp. 29349–29356, 2006.

[60] B. Mulloy and C. C. Rider, "The bone morphogenetic proteins and their antagonists," *Vitamins & Hormones*, vol. 99, pp. 63–90, 2015.

[61] D. Onitchtchouck, Y. G. Chen, R. Dosch et al., "Silencing of TGF-β signaling by the pseudoreceptor BAMBI," *Nature*, vol. 405, no. 6752, pp. 757–763, 1999.

[62] T. A. Samad, A. Rebbapragada, E. Bell et al., "DRAGON, a bone morphogenetic protein co-receptor," *The Journal of Biological Chemistry*, vol. 280, no. 14, pp. 14122–14129, 2005.

[63] J. L. Babitt, Y. Zhang, T. A. Samad et al., "Repulsive guidance molecule (RGMa), a DRAGON homologue, is a bone morphogenetic protein co-receptor," *The Journal of Biological Chemistry*, vol. 280, no. 33, pp. 29820–29827, 2005.

[64] S. V. Hegarty, G. W. O'Keeffe, and A. M. Sullivan, "BMP-Smad 1/5/8 signalling in the development of the nervous system," *Progress in Neurobiology*, vol. 109, pp. 28–41, 2013.

[65] H. Hayashi, S. Abdollah, Y. Qiu et al., "The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling," *Cell*, vol. 89, no. 7, pp. 1165–1173, 1997.

[66] A. Hata, G. Lagna, J. Massagué, and A. Hemmati-Brivanlou, "Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor," *Genes and Development*, vol. 12, no. 2, pp. 186–197, 1998.

[67] H. Zhu, P. Kavsak, S. Abdollah, J. L. Wrana, and G. H. Thomsen, "A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation," *Nature*, vol. 400, no. 6745, pp. 687–693, 1999.

[68] P. Kavsak, R. K. Rasmussen, C. G. Causing et al., "Smad7 binds to smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation," *Molecular Cell*, vol. 6, no. 6, pp. 1365–1375, 2000.

[69] S. C. Little and M. C. Mullins, "Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis," *Nature Cell Biology*, vol. 11, no. 5, pp. 637–643, 2009.

[70] J. T. Koh, Z. Zhao, Z. Wang, I. S. Lewis, P. H. Krebsbach, and R. T. Franceschi, "Combinatorial gene therapy with BMP2/7 enhances cranial bone regeneration," *Journal of Dental Research*, vol. 87, no. 9, pp. 845–849, 2008.

[71] D. I. Israel, J. Nove, K. M. Kerns et al., "Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo," *Growth Factors*, vol. 13, no. 3-4, pp. 291–300, 1996.

[72] A. Aono, M. Hazama, K. Notoya et al., "Potent ectopic bone-inducing activity of bone morphogenetic protein- 4-7 heterodimer," *Biochemical and Biophysical Research Communications*, vol. 210, no. 3, pp. 670–677, 1995.

[73] L. Attisano and J. L. Wrana, "Signal transduction by the TGF-β superfamily," *Science*, vol. 296, no. 5573, pp. 1646–1647, 2002.

[74] J. Vogt, K. S. Dingwell, L. Herhaus et al., "Protein associated with SMAD1 (PAWS1/FAM83G) is a substrate for type I bone morphogenetic protein receptors and modulates bone morphogenetic protein signalling," *Open Biology*, vol. 4, no. 2, Article ID 130210, 2014.

[75] M. F. Mehler, P. C. Mabie, D. Zhang, and J. A. Kessler, "Bone morphogenetic proteins in the nervous system," *Trends in Neurosciences*, vol. 20, no. 7, pp. 309–317, 1997.

[76] G. Winnier, M. Blessing, P. A. Labosky, and B. L. M. Hogan, "Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse," *Genes and Development*, vol. 9, no. 17, pp. 2105–2116, 1995.

[77] Y. Mishina, A. Suzuki, N. Ueno, and R. R. Behringer, "Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis," *Genes & Development*, vol. 9, no. 24, pp. 3027–3037, 1995.

[78] G. H. Thomsen, "Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning," *Trends in Genetics*, vol. 13, no. 6, pp. 209–211, 1997.

[79] C. Boissart, X. Nissan, K. Giraud-Triboult, M. Peschanski, and A. Benchoua, "miR-125 potentiates early neural specification of human embryonic stem cells," *Development*, vol. 139, no. 7, pp. 1247–1257, 2012.

[80] E. M. Pera, O. Wessely, S.-Y. Li, and E. M. De Robertis, "Neural and head induction by insulin-like growth factor signals," *Developmental Cell*, vol. 1, no. 5, pp. 655–665, 2001.

[81] R. Morgan and M. G. Sargent, "The role in neural patterning of translation initiation factor eIF4AII; induction of neural fold genes," *Development*, vol. 124, no. 14, pp. 2751–2760, 1997.

[82] X. Huang and J.-P. Saint-Jeannet, "Induction of the neural crest and the opportunities of life on the edge," *Developmental Biology*, vol. 275, no. 1, pp. 1–11, 2004.

[83] D. Sela-Donenfeld and C. Kalcheim, "Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube," *Development*, vol. 126, no. 21, pp. 4749–4762, 1999.

[84] C. LaBonne and M. Bronner-Fraser, "Neural crest induction in Xenopus: evidence for a two-signal model," *Development*, vol. 125, no. 13, pp. 2403–2414, 1998.

[85] J.-P. Saint-Jeannet, X. He, H. E. Varmus, and I. B. Dawid, "Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 25, pp. 13713–13718, 1997.

[86] C. R. Coffman, P. Skoglund, W. A. Harris, and C. R. Kintner, "Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus embryos," *Cell*, vol. 73, no. 4, pp. 659–671, 1993.

[87] N. Itasaki and S. Hoppler, "Crosstalk between Wnt and bone morphogenic protein signaling: a turbulent relationship," *Developmental Dynamics*, vol. 239, no. 1, pp. 16–33, 2010.

[88] F. Ulloa and E. Martí, "Wnt won the war: antagonistic role of Wnt over Shh controls dorso-ventral patterning of the vertebrate neural tube," *Developmental Dynamics*, vol. 239, no. 1, pp. 69–76, 2010.

[89] K. F. J. Liem, G. Tremml, H. Roelink, and T. M. Jessell, "Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm," *Cell*, vol. 82, no. 6, pp. 969–979, 1995.

[90] L. Wine-Lee, K. J. Ahn, R. D. Richardson, Y. Mishina, K. M. Lyons, and E. B. Crenshaw III, "Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord," *Development*, vol. 131, no. 21, pp. 5393–5403, 2004.

[91] J. R. Timmer, C. Wang, and L. Niswander, "BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors," *Development*, vol. 129, no. 10, pp. 2459–2472, 2002.

[92] C. Marcelle, M. R. Stark, and M. Bronner-Fraser, "Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite," *Development*, vol. 124, no. 20, pp. 3955–3963, 1997.

[93] D. Zechner, T. Müller, H. Wende et al., "Bmp and Wnt/β-catenin signals control expression of the transcription factor Olig3 and the specification of spinal cord neurons," *Developmental Biology*, vol. 303, no. 1, pp. 181–190, 2007.

[94] R. M. Anderson, A. R. Lawrence, R. W. Stottmann, D. Bachiller, and J. Klingensmith, "Chordin and noggin promote organizing centers of forebrain development in the mouse," *Development*, vol. 129, no. 21, pp. 4975–4987, 2002.

[95] C. Angley, M. Kumar, K. J. Dinsio, A. K. Hall, and R. E. Siegel, "Signaling by bone morphogenetic proteins and Smad1 modulates the postnatal differentiation of cerebellar cells," *The Journal of Neuroscience*, vol. 23, no. 1, pp. 260–268, 2003.

[96] W. Li, C. A. Cogswell, and J. J. LoTurco, "Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP," *Journal of Neuroscience*, vol. 18, no. 21, pp. 8853–8862, 1998.

[97] B.-S. Moon, J.-Y. Yoon, M.-Y. Kim, S.-H. Lee, T. Choi, and K.-Y. Choi, "Bone morphogenetic protein 4 stimulates neuronal differentiation of neuronal stem cells through the ERK pathway," *Experimental and Molecular Medicine*, vol. 41, no. 2, pp. 116–125, 2009.

[98] R. E. Gross, M. F. Mehler, P. C. Mabie, Z. Zang, L. Santschi, and J. A. Kessler, "Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells," *Neuron*, vol. 17, no. 4, pp. 595–606, 1996.

[99] M. F. Mehler, P. C. Mabie, G. Zhu, S. Gokhan, and J. A. Kessler, "Developmental changes in progenitor cell responsiveness to gone morphogenetic; Proteins differentially modulate progressive CNS lineage fate," *Developmental Neuroscience*, vol. 22, no. 1-2, pp. 74–85, 2000.

[100] K. Nakashima, M. Yanagisawa, H. Arakawa et al., "Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300," *Science*, vol. 284, no. 5413, pp. 479–482, 1999.

[101] A. Bonni, Y. Sun, M. Nadal-Vicens et al., "Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway," *Science*, vol. 278, no. 5337, pp. 477–483, 1997.

[102] P. Rajan and R. D. G. McKay, "Multiple routes to astrocytic differentiation in the CNS," *The Journal of Neuroscience*, vol. 18, no. 10, pp. 3620–3629, 1998.

[103] M. Namihira and K. Nakashima, "Mechanisms of astrocytogenesis in the mammalian brain," *Current Opinion in Neurobiology*, vol. 23, no. 6, pp. 921–927, 2013.

[104] R. Kanski, M. E. van Strien, P. van Tijn, and E. M. Hol, "A star is born: new insights into the mechanism of astrogenesis," *Cellular and Molecular Life Sciences*, vol. 71, no. 3, pp. 433–447, 2014.

[105] M. A. Bonaguidi, T. McGuire, M. Hu, L. Kan, J. Samantha, and J. A. Kessler, "LIF and BMP signaling generate separate and discrete types of GFAP-expressing cells," *Development*, vol. 132, no. 24, pp. 5503–5514, 2005.

[106] J. J. Jadasz, F. J. Rivera, A. Taubert et al., "p57kip2 regulates glial fate decision in adult neural stem cells," *Development*, vol. 139, no. 18, pp. 3306–3315, 2012.

[107] Q. Ma, C. Kintner, and D. J. Anderson, "Identification of neurogenin, a vertebrate neuronal determination gene," *Cell*, vol. 87, no. 1, pp. 43–52, 1996.

[108] F. He, W. Ge, K. Martinowich et al., "A positive autoregulatory loop of Jak-STAT signaling controls the onset of astrogliogenesis," *Nature Neuroscience*, vol. 8, no. 5, pp. 616–625, 2005.

[109] Y. Sun, M. Nadal-Vicens, S. Misono et al., "Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms," *Cell*, vol. 104, no. 3, pp. 365–376, 2001.

[110] J. Zhao, Q. Lin, K. J. Kim et al., "Ngn1 inhibits astrogliogenesis through induction of miR-9 during neuronal fate specification," *ELife*, vol. 4, Article ID e06885, 2015.

[111] E. Agius, Y. Decker, C. Soukkarieh, C. Soula, and P. Cochard, "Role of BMPs in controlling the spatial and temporal origin of GFAP astrocytes in the embryonic spinal cord," *Developmental Biology*, vol. 344, no. 2, pp. 611–620, 2010.

[112] W. A. Gomes, M. F. Mehler, and J. A. Kessler, "Transgenic overexpression of BMP4 increases astroglial and decreases oligodendroglial lineage commitment," *Developmental Biology*, vol. 255, no. 1, pp. 164–177, 2003.

[113] S. Mekki-Dauriac, E. Agius, P. Kan, and P. Cochard, "Bone morphogenetic proteins negatively control oligodendrocyte precursor specification in the chick spinal cord," *Development*, vol. 129, no. 22, pp. 5117–5130, 2002.

[114] J. B. Grinspan, E. Edell, D. F. Carpio et al., "Stage-specific effects of bone morphogenetic proteins on the oligodendrocyte lineage," *Journal of Neurobiology*, vol. 43, no. 1, pp. 1–17, 2000.

[115] J. See, X. Zhang, N. Eraydin et al., "Oligodendrocyte maturation is inhibited by bone morphogenetic protein," *Molecular and Cellular Neuroscience*, vol. 26, no. 4, pp. 481–492, 2004.

[116] R. H. Miller, "Regulation of oligodendrocyte development in the vertebrate CNS," *Progress in Neurobiology*, vol. 67, no. 6, pp. 451–467, 2002.

[117] A. H. Crawford, J. H. Stockley, R. B. Tripathi, W. D. Richardson, and R. J. M. Franklin, "Oligodendrocyte progenitors: adult stem cells of the central nervous system?" *Experimental Neurology*, vol. 260, pp. 50–55, 2014.

[118] W. D. Richardson, N. Kessaris, and N. Pringle, "Oligodendrocyte wars," *Nature Reviews Neuroscience*, vol. 7, no. 1, pp. 11–18, 2006.

[119] D. M. Orentas and R. H. Miller, "The origin of spinal cord oligodendrocytes is dependent on local influences from the notochord," *Developmental Biology*, vol. 177, no. 1, pp. 43–53, 1996.

[120] J. Cai, Y. Qi, X. Hu et al., "Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling," *Neuron*, vol. 45, no. 1, pp. 41–53, 2005.

[121] A. Vallstedt, J. M. Klos, and J. Ericson, "Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain," *Neuron*, vol. 45, no. 1, pp. 55–67, 2005.

[122] Q. Zhou, G. Choi, and D. J. Anderson, "The bHLH transcription factor Olig2 Promotes oligodendrocyte differentiation in collaboration with Nkx2.2," *Neuron*, vol. 31, no. 5, pp. 791–807, 2001.

[123] N. P. Pringle and W. D. Richardson, "A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage," *Development*, vol. 117, no. 2, pp. 525–533, 1993.

[124] M. R. L. Dawson, J. M. Levine, and R. Reynolds, "NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors?" *Journal of Neuroscience Research*, vol. 61, no. 5, pp. 471–479, 2000.

[125] I. Sommer and M. Schachner, "Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system," *Developmental Biology*, vol. 83, no. 2, pp. 311–327, 1981.

[126] J. See, P. Mamontov, K. Ahn, L. Wine-Lee, E. B. Crenshaw III, and J. B. Grinspan, "BMP signaling mutant mice exhibit glial cell maturation defects," *Molecular and Cellular Neuroscience*, vol. 35, no. 1, pp. 171–182, 2007.

[127] J. Samanta, G. M. Burke, T. McGuire et al., "BMPR1a signaling determines numbers of oligodendrocytes and calbindin-expressing interneurons in the cortex," *Journal of Neuroscience*, vol. 27, no. 28, pp. 7397–7407, 2007.

[128] R. Araya, M. Kudo, M. Kawano et al., "BMP signaling through BMPRIA in astrocytes is essential for proper cerebral angiogenesis and formation of the blood-brain-barrier," *Molecular and Cellular Neuroscience*, vol. 38, no. 3, pp. 417–430, 2008.

[129] J. M. See and J. B. Grinspan, "Sending mixed signals: bone morphogenetic protein in myelination and demyelination," *Journal of Neuropathology and Experimental Neurology*, vol. 68, no. 6, pp. 595–604, 2009.

[130] A. Hollnagel, V. Oehlmann, J. Heymer, U. Rüther, and A. Nordheim, "Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells," *The Journal of Biological Chemistry*, vol. 274, no. 28, pp. 19838–19845, 1999.

[131] T. Kondo and M. Raff, "The Id4 HLH protein and the timing of oligodendrocyte differentiation," *The EMBO Journal*, vol. 19, no. 9, pp. 1998–2007, 2000.

[132] J. Samanta and J. A. Kessler, "Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation," *Development*, vol. 131, no. 17, pp. 4131–4142, 2004.

[133] Y. Zhang, K. Chen, S. A. Sloan et al., "An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex," *The Journal of Neuroscience*, vol. 34, no. 36, pp. 11929–11947, 2014.

[134] T. Kondo and M. C. Raff, "A role for Noggin in the development of oligodendrocyte precursor cells," *Developmental Biology*, vol. 267, no. 1, pp. 242–251, 2004.

[135] E. Hammond, J. Lang, Y. Maeda et al., "The Wnt effector transcription factor 7-like 2 positively regulates oligodendrocyte differentiation in a manner independent of Wnt/β-catenin signaling," *The Journal of Neuroscience*, vol. 35, no. 12, pp. 5007–5022, 2015.

[136] E. R. Kandel, J. H. Schwartz, and T. M. Jessell, *Principles of Neural Science*, McGraw-Hill, 2000.

[137] K. Sawamoto, H. Wichterle, O. Gonzalez-Perez et al., "New neurons follow the flow of cerebrospinal fluid in the adult brain," *Science*, vol. 311, no. 5761, pp. 629–632, 2006.

[138] M. Carlén, K. Meletis, C. Göritz et al., "Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke," *Nature Neuroscience*, vol. 12, no. 3, pp. 259–267, 2009.

[139] F. Doetsch, J. M. García-Verdugo, and A. Alvarez-Buylla, "Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain," *The Journal of Neuroscience*, vol. 17, no. 13, pp. 5046–5061, 1997.

[140] P. Peretto, D. Cummings, C. Modena et al., "BMP mRNA and protein expression in the developing mouse olfactory system," *Journal of Comparative Neurology*, vol. 451, no. 3, pp. 267–278, 2002.

[141] P. Peretto, C. Dati, S. De Marchis et al., "Expression of the secreted factors noggin and bone morphogenetic proteins in the subependymal layer and olfactory bulb of the adult mouse brain," *Neuroscience*, vol. 128, no. 4, pp. 685–696, 2004.

[142] S. Bauer and P. H. Patterson, "Leukemia inhibitory factor promotes neural stem cell self-renewal in the adult brain," *The Journal of Neuroscience*, vol. 26, no. 46, pp. 12089–12099, 2006.

[143] D. Colak, T. Mori, M. S. Brill et al., "Adult neurogenesis requires Smad4-mediated bone morphogenic protein signaling in stem cells," *The Journal of Neuroscience*, vol. 28, no. 2, pp. 434–446, 2008.

[144] J. Sohn, L. Orosco, F. Guo et al., "The subventricular zone continues to generate corpus callosum and rostral migratory stream astroglia in normal adult mice," *The Journal of Neuroscience*, vol. 35, no. 9, pp. 3756–3763, 2015.

[145] B. Jablonska, A. Aguirre, M. Raymond et al., "Chordin-induced lineage plasticity of adult SVZ neuroblasts after demyelination," *Nature Neuroscience*, vol. 13, no. 5, pp. 541–550, 2010.

[146] J. K. Sabo, T. J. Kilpatrick, and H. S. Cate, "Effects of bone morphogenic proteins on neural precursor cells and regulation during central nervous system injury," *NeuroSignals*, vol. 17, no. 4, pp. 255–264, 2009.

[147] M. L. Fuller, A. K. DeChant, B. Rothstein et al., "Bone morphogenetic proteins promote gliosis in demyelinating spinal cord lesions," *Annals of Neurology*, vol. 62, no. 3, pp. 288–300, 2007.

[148] M. V. Reid, K. A. Murray, E. D. Marsh, J. A. Golden, R. A. Simmons, and J. B. Grinspan, "Delayed myelination in an intrauterine growth retardation model is mediated by oxidative stress upregulating bone morphogenetic protein 4," *Journal of Neuropathology and Experimental Neurology*, vol. 71, no. 7, pp. 640–653, 2012.

[149] M. Deininger, R. Meyermann, and H. Schluesener, "Detection of two transforming growth factor-β-related morphogens, bone morphogenetic proteins-4 and-5, in RNA of multiple sclerosis and Creutzfeldt-Jakob disease lesions," *Acta Neuropathologica*, vol. 90, no. 1, pp. 76–79, 1995.

[150] B. Nait-Oumesmar, N. Picard-Riera, C. Kerninon et al., "Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 11, pp. 4694–4699, 2007.

[151] J. K. Huang, S. P. J. Fancy, C. Zhao, D. H. Rowitch, C. Ffrench-Constant, and R. J. M. Franklin, "Myelin regeneration in multiple sclerosis: targeting endogenous stem cells," *Neurotherapeutics*, vol. 8, no. 4, pp. 650–658, 2011.

[152] Y. L. Xing, P. T. Röth, J. A. S. Stratton et al., "Adult neural precursor cells from the subventricular zone contribute significantly to oligodendrocyte regeneration and remyelination," *Journal of Neuroscience*, vol. 34, no. 42, pp. 14128–14146, 2014.

[153] C. Zhao, S. P. J. Fancy, L. Magy, J. E. Urwin, and R. J. M. Franklin, "Stem cells, progenitors and myelin repair," *Journal of Anatomy*, vol. 207, no. 3, pp. 251–258, 2005.

[154] H. S. Cate, J. K. Sabo, D. Merlo et al., "Modulation of bone morphogenic protein signalling alters numbers of astrocytes and oligodendroglia in the subventricular zone during cuprizone-induced demyelination," *Journal of Neurochemistry*, vol. 115, no. 1, pp. 11–22, 2010.

[155] J. K. Sabo, T. D. Aumann, D. Merlo, T. J. Kilpatrick, and H. S. Cate, "Remyelination is altered by bone morphogenic protein signaling in demyelinated lesions," *The Journal of Neuroscience*, vol. 31, no. 12, pp. 4504–4510, 2011.

[156] T. Kuhlmann, V. Miron, Q. Cui, C. Wegner, J. Antel, and W. Brück, "Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis," *Brain*, vol. 131, pp. 1749–1758, 2008.

[157] S. P. J. Fancy, S. E. Baranzini, C. Zhao et al., "Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS," *Genes & Development*, vol. 23, no. 13, pp. 1571–1585, 2009.

[158] K. Feigenson, M. Reid, J. See, E. B. Crenshaw III, and J. B. Grinspan, "Canonical Wnt signalling requires the BMP pathway to inhibit oligodendrocyte maturation," *ASN neuro*, vol. 3, no. 3, Article ID e00061, 2011.

[159] Q. Xiao, Y. Du, W. Wu, and H. K. Yip, "Bone morphogenetic proteins mediate cellular response and, together with Noggin, regulate astrocyte differentiation after spinal cord injury," *Experimental Neurology*, vol. 221, no. 2, pp. 353–366, 2010.

[160] V. Sahni, A. Mukhopadhyay, V. Tysseling et al., "BMPR1a and BMPR1b signaling exert opposing effects on gliosis after spinal cord injury," *The Journal of Neuroscience*, vol. 30, no. 5, pp. 1839–1855, 2010.

[161] O. G. Bhalala, L. Pan, V. Sahni et al., "microRNA-21 regulates astrocytic response following spinal cord injury," *Journal of Neuroscience*, vol. 32, no. 50, pp. 17935–17947, 2012.

[162] H. A. North, L. Pan, T. L. McGuire, S. Brooker, and J. A. Kessler, "β1-integrin alters ependymal stem cell BMP receptor localization and attenuates astrogliosis after spinal cord injury," *Journal of Neuroscience*, vol. 35, no. 9, pp. 3725–3733, 2015.

[163] C. Steffenhagen, F.-X. Dechant, E. Oberbauer et al., "Mesenchymal stem cells prime proliferating adult neural progenitors toward an oligodendrocyte fate," *Stem Cells and Development*, vol. 21, no. 11, pp. 1838–1851, 2012.

[164] B. Sandner, F. J. Rivera, M. Caioni et al., "Bone morphogenetic proteins prevent bone marrow stromal cell-mediated oligodendroglial differentiation of transplanted adult neural progenitor cells in the injured spinal cord," *Stem Cell Research*, vol. 11, no. 2, pp. 758–771, 2013.

[165] D. Li, J. Tang, H. Xu, X. Fan, Y. Bai, and L. Yang, "Decreased hippocampal cell proliferation correlates with increased expression of BMP4 in the APPswe/PS1ΔE9 mouse model of Alzheimer's disease," *Hippocampus*, vol. 18, no. 7, pp. 692–698, 2008.

[166] K. Dummula, G. Vinukonda, P. Chu et al., "Bone morphogenetic protein inhibition promotes neurological recovery after intraventricular hemorrhage," *The Journal of Neuroscience*, vol. 31, no. 34, pp. 12068–12082, 2011.

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Research Article

GDNF Enhances Therapeutic Efficiency of Neural Stem Cells-Based Therapy in Chronic Experimental Allergic Encephalomyelitis in Rat

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Multiple sclerosis (MS) is an autoimmune disease in the CNS. The current immunomodulating drugs for MS do not effectively prevent the progressive neurological decline. Neural stem cells (NSCs) transplantation has been proven to promote repair and functional recovery of experimental allergic encephalomyelitis (EAE) animal model for MS, and glial cell line-derived neurotrophic factor (GDNF) has also been found to have capability of promoting axonal regeneration and remyelination of regenerating axons. In the present study, to assess whether GDNF would enhance therapeutic effect of NSCs for EAE, GDNF gene-modified NSCs (GDNF/NSCs) and native NSCs were transplanted into each lateral ventricle of rats at 10 days and rats were sacrificed at 60 days after EAE immunization. We found that NSCs significantly reduced the clinical signs, and GDNF gene-modification further promoted functional recovery. GDNF/NSCs more profoundly suppressed brain inflammation and improved density of myelin compared with NSCs. The survival of GDNF/NSCs was significantly higher than that of transplanted NSCs. Transplanted GDNF/NSCs, in contrast to NSCs, differentiated into more neurons and oligodendrocytes. Moreover, the mRNA expression of oligodendrocyte lineage cells in rats with GDNF/NSCs was significantly increased compared to rats with NSCs. These results suggest that GDNF enhances therapeutic efficiency of NSCs-based therapy for EAE.

1. Introduction

Experimental allergic encephalomyelitis (EAE), most commonly used animal model of multiple sclerosis (MS), is an autoimmune disease in the central nervous system (CNS) induced and mediated by myelin-reactive T cells responses against myelin antigens [1]. In EAE and MS, autoreactive T cells migrate from peripheral tissues into the CNS where they are reactivated, thereby triggering an inflammatory cascade that results in extensive loss of myelin and myelinating cells (oligodendrocytes) as well as damage to axons and neurons [2, 3]. Since neural stem cells (NSCs) have abilities to differentiate into various neural cell types [4] and have myelinogenic potential [5] in the injured areas, they may be a potential cell type of replacement therapy for EAE. However, lower survival rate [6] and low levels of differentiation of oligodendrocytes [7] and neurons [8] after transplantation limit the therapeutic efficacy of NSCs.

Glial cell line-derived neurotrophic factor (GDNF), a potent neurotrophic factor, has been demonstrated to have neuroprotection against a variety of neuronal insults [9, 10]. Moreover, GDNF can promote axon regeneration and myelination after spinal cord injury [11–13]. Our previous studies have shown that GDNF gene-modified NSCs provided more efficient neuroprotection for rats subjected to stroke than native NSCs [14]. Therefore, here, we speculate that GDNF may enhance therapeutic efficiency of NSCs therapy for EAE. The purpose of this study is to investigate whether GDNF gene-modified NSCs provide a more efficacious treatment for EAE than NSCs alone.

2. Materials and Methods

2.1. Culture and Infection of NSCs. The culture and infection of NSCs were performed as described in previous study [14]. Briefly, the cell suspensions from the cerebral hemispheres of newborn Wistar rats (inbred strain, Animal House Center, Southwest Medical University, Sichuan, China) were placed in 25 mL flask at a density of 1×10^5 cells/mL in serum-free DMEM/F12 medium supplemented with 20 ng/mL of epidermal growth factor (EGF), 20 ng/mL of basic fibroblast growth factor (bFGF), and 1% N2 supplement (all from Gibco, USA). After 5–7 days of culture, neurospheres were formed. To obtain GDNF gene-modified NSCs (GDNF/NSCs), neurospheres were infected using GDNF recombinant adenovirus (pAdEasy-1-pAdTrack CMV-GDNF) for 2 days. Adeasy-1 plasmid contains the gene for green fluorescent protein and the titers of viral were 1×10^9 PFU/mL. The expression of GDNF in NSCs was examined by a reverse transcriptase-PCR assay 2 days after infection. The primer sequences are as follows: sense, 5′-CCGAAGATTATCCTGACC-3′, and antisense, 5′-GTAGCCCAAACCCAAGT-3′, and the product length is 242 bp fragment. For labeling the grafted cells *in vivo*, NSCs and GDNF/NSCs were pretreated for 3 days before grafting with 10 μM of 5-bromo-2′-deoxyuridine (BrdU, Sigma, USA).

2.2. Neural Differentiation of Neurospheres In Vitro. To induced NSCs and GDNF/NSCs differentiation, neurospheres were adhered to coverslips precoated with poly-L-lysine (10 μg/mL, Sigma) in six-well plates in DMEM/F12 medium in the absence of bFGF, EGF, and N2 supplement but with 10% fetal bovine serum (FBS) for 7 days. The cultures were fixed with 4% paraformaldehyde for 30 min and incubated with neuronal specific markers microtubule-associated protein 2 (MAP2, 1 : 100, rabbit, Abcam, UK), astrocytes specific markers glial fibrillary acidic protein (GFAP, 1 : 100, rabbit, Abcam), and oligodendrocyte specific marker galactocerebroside (GalC, 1 : 50, rabbit, Chemicon, USA) overnight at 4°C, followed by appropriate biotinylated secondary antibodies (1 : 100, Wuhan Boster Biological Technology, China) and horseradish peroxidase- (HRP-) streptavidin (Boster) for 30 min at 37°C. Immunoreactivity was visualized with diaminobenzidine (DAB, Boster). Counterstaining by hematoxylin was also performed. A negative control was performed using the same procedures without primary antibody. The ratio of $MAP2^+$, $GalC^+$, or $GFAP^+$ cells to total cells was quantified in 5 regions in 3 coverslips per group.

2.3. EAE Induction and NSCs Transplantation. All animal use and relevant experiments were approved by the Chinese Academy of Sciences, PR China. EAE is induced in female Wistar rats by guinea pig spinal cord homogenate emulsified with complete Freund's adjuvant (CFA, Sigma). Briefly, Wistar rats (6–8 weeks old, female, Southwest Medical University) were injected in four footpads with 100 mg of guinea pig spinal cord tissue in 0.4 mL of PBS emulsified with equal volume of CFA supplemented with 6 mg/mL of *Mycobacterium tuberculosis* H37Ra (Shijiazhuang Weitian Scientific Instruments Equipment Co., Ltd., China). On the day of immunization and on day 2, 300 ng of bordetella pertussis toxin (Weitian) in 0.1 mL PBS was injected subcutaneously. Rats were daily monitored for the severity of clinical disease. After immunization, the following scale for clinical symptoms was utilized: 0, no clinical symptom, 1, limpness in tail, 2, hind-leg ataxia, 3, hind-leg paralysis, 4, paraplegia, and 5, moribund or dead. At ten days after EAE induction, rats were anesthetized with intraperitoneal injection of pentobarbital sodium (30 mg/kg) and then were fixed in a stereotactic device (Angle Two™ Stereotaxic Instrument w/Rat Atlas Product: # 464601, USA). Quantities of 5×10^5 NSCs or GDNF/NSCs in a volume of 10 μL were injected once into each lateral ventricle (bregma as origin, AP = −0.8 to 1.0 mm, L or R = −1.8 to 2.0 mm, and V = −4.0 to 5.0 mm). The control group experienced the same injection with 10 μL saline into each lateral ventricle.

2.4. Histological and Immunohistochemical Assessment. Animals were sacrificed with a lethal dose of pentobarbital sodium on day 60 after EAE induction. Fifteen rats (5 per group) were perfused by 4% paraformaldehyde for histopathological analysis. Histopathology and immunohistochemistry were assessed in 6 μm thick paraffin sections at various levels (for bregma −0.2 to −1.6 mm). Hematoxylin-eosin (HE) staining was used to evaluate inflammatory infiltration. 10 nuclei or more gathered in cerebrum white matter or surrounded by a blood vessel were considered as an infiltration lesion. To estimate the degree of inflammation, the number of infiltrates and the number of cells per infiltrate were counted. Luxol fast blue (LFB, Sigma) staining was used to visualize myelin sheath and the measurement parameter was the integrated optical density (IOD).

BrdU immunohistochemistry staining was used to identify grafted cells in brain. Sections were incubated with mouse anti-BrdU (1 : 200, Abcam, UK) at 4°C overnight; then goat anti-mouse IgG (1 : 100, Wuhan Boster Biological Technology, China) was added at 37°C for 30 min. After alkaline phosphatase- (AP-) streptavidin (Boster) was incubated at 37°C for 30 min, 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitroblue Tetrazolium Chloride (BCIP/NBT, Boster) was used as a chromogen for 10 min. For double-labeling experiments, MAP2, GFAP, and GalC were used to identify differentiated cells from grafted cells. Sections were incubated again with rabbit anti-MAP2 (1 : 100), GFAP (1 : 100), and GalC (1 : 50) at 4°C overnight, and goat anti-rabbit IgG (1 : 100, Boster) was then incubated for 30 min. Horseradish peroxidase- (HRP-) streptavidin (Boster) was incubated for 30 min, followed by diaminobenzidine (DAB, Boster) or 3-amino-9-ethylcarbazole (AEC, Boster) for 10 min as a chromogen. Immunohistochemistry controls were routinely performed with incubations in which primary antibodies were omitted.

Myelin sheath was assessed as IOD in corpus callosum in 3 sections per animal by ImageJ 1.44p software. The infiltrates in the corpus striatum were counted in sections per rats. And 3 fields/striatum were imaged, so 6 fields in bilateral striatum were imaged and counted. Immunopositive cells were counted in 6 fields situated within bilaterally striatum in 3

sections per animal. Measurement was made in a predefined field (0.6 mm × 0.6 mm) by image-pro plus 6.0 software.

2.5. Reverse Transcription- (RT-) PCR Analysis. For RT-PCR experiments, fifteen rats (5 per group) were analyzed. Total RNA was prepared from brain tissue at 0.2–1.6 mm behind bregma using TRIZOL reagent (Invitrogen, USA) following the manufacturer's instructions. Two μg of RNA was reversely transcribed into cDNA using a Takara RNA PCR kit (Dalian Takara Biotechnology, China). The reaction conditions were 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. Oligonucleotides used as specific primers were as follows: platelet-derived growth factor α receptor (PDGFαR), sense, 5′-CCAAATACTCCGACATCC-3′, antisense, 5′-CCAGAGCAGAACGCCATA-3′, 404 bp fragment; GalC, sense, 5′-CGGTGCCCTTGTTGTTGTG-3′, antisense, 5′-TGCCGTCTGTTGTTTGTCC-3′, 252 bp fragment; myelin basic protein (MBP), sense, 5′-TTCTTTAGCGGTGAC-AGGG-3′, antisense, 5′-GGAGCCGTAGTGGGTAGTT-3′, 156 bp fragment; and GAPDH, sense, 5′-ACCACAGTC-CATGCCATCAC-3′, antisense, 5′-TCCACCACCCTG-TTGCTGTA-3′, 450 bp fragment. cDNA was amplified by 30 cycles and the parameters of amplification were as follows: denaturing for 30 sec, 94°C; annealing for 30 sec, PDGFαR at 57°C, GalC at 59°C, MBP at 55°C, and GAPDH at 57°C; extension for 4 min, at 72°C. GAPDH was used as an endogenous control. PCR products were electrophoresed on agarose gels and stained by ethidium bromide.

2.6. Statistical Analysis. All measured values were expressed as mean ± SD, and statistical analysis was performed using SPSS software, version 17.0. For the clinical scores, the multiple comparisons were done using two-way repeated measures ANOVA followed by Tukey's posttest for multiple pairwise examinations. For the other histological, immunostaining, and PCR analyses, multiple comparisons were done using one-way ANOVA followed by Tukey's *post hoc* test for multiple pairwise examinations. Difference was considered significant at $P < 0.05$.

3. Results

3.1. GDNF Promoted Neuronal Differentiation of NSCs In Vitro. After 5–7 days of culture, neurospheres appeared (Figure 1(a)), which expressed the neuroepithelial cells specific markers nestin (Figure 1(b)). After they were infected by GDNF recombinant adenovirus for 2 days, the neurospheres displayed green fluorescence under the fluorescent microscope (Figure 1(c)) and strongly expressed GDNF mRNA (Figure 1(d)). To observe the differentiation, neurospheres were plated in coverslips with medium containing 10% FBS without bFGF, EGF, and N2 supplement for 7 days. The cell specific antibodies including MAP2, GFAP, and GalC were used to label differentiated cells. Compared to NSCs, the ratio of MAP2-positive neurons from GDNF/NSCs was significantly increased ($P < 0.05$), while the ratio of GFAP-positive astrocytes from GDNF/NSCs was significantly decreased ($P < 0.05$). Although the increased ratio of GalC-positive oligodendrocytes in GDNF/NSCs group was observed compared to NSCs, it did not reach the significant difference ($P > 0.05$) (Figures 1(e)–1(k)).

3.2. GDNF Accelerated the Recovery of NSCs on Clinical Symptoms. To evaluate the effects of NSCs on EAE rats, NSCs were transplanted into lateral ventricles before the onset of the disease (on day 10 after EAE induction). Disabilities appeared at 12 days after the initial EAE induction in the control rats, whereas cells transplantation delayed onset time, starting 1-2 days later. As shown in Figure 2, the rats receiving NSCs and those receiving GDNF/NSCs had reduced clinical symptoms compared to the control rats ($P < 0.05$). Moreover, the GDNF/NSCs rats had improved clinical outcomes relative to the NSCs group ($P < 0.05$). Although the control rats showed improved clinical scores over time, there were apparent deficits at the end of the follow-up period (60 days after EAE induction). The rats that received NSCs recovered their normal gait at 50 days after EAE induction, whereas the rats receiving GDNF/NSCs recovered at 45 days after EAE induction.

3.3. Attenuated Inflammation and More Myelin Sheath in GDNF/NSCs Compared to NSCs. Inflammatory infiltrates and density of myelin sheath in brain of EAE rats were evaluated at 60 days after EAE induction. The majority of infiltrates were located in the corpus striatum adjacent to fibers, and fewer appeared in the cortex. The infiltrates in the corpus striatum were counted, and the results showed that the rats with saline showed more infiltrates than the rats with transplantation ($P < 0.05$) (Figures 3(a)–3(d)); moreover, fewer cells per infiltration were observed in rats with GDNF/NSCs compared with rats with NSCs ($P < 0.05$) (Figure 3(e)). The saline-injected rats showed sparse and thin myelin sheath (Figure 3(f)). In addition, myelin was observed by LFB staining. In our setting, phagocytic cells were also labeled by LFB staining. More myelin sheath indicated by stronger color intensity than control rats was observed in NSC transplantation, in particular GDNF/NSC transplantation ($P < 0.05$), although it may contain phagocytic cells (Figures 3(g)–3(i)).

3.4. GDNF Enhanced Survival and Neuron and Oligodendrocyte Differentiation of Transplanted NSCs. The transplanted cells were detected by BrdU-immunostaining in rats at 60 days after EAE induction. The $BrdU^+$ cells located predominantly in the inflamed areas (Figures 4(a)–4(f)), and the density of $BrdU^+$ cells per mm^2 in rats with GDNF/NSCs was significantly increased compared to rats with NSCs ($P < 0.05$) (Figure 4(g)). The $MAP2^+/BrdU^+$ cells and $GalC^+/BrdU^+$ cells in rats with GDNF/NSCs were apparently higher compared to rats with NSCs ($P < 0.05$); in contrast, the $GFAP^+/BrdU^+$ cells in rats with GDNF/NSCs were decreased compared to rats with NSCs ($P < 0.05$) (Figure 4(h)), which suggested that GDNF/NSCs differentiated into more neurons and oligodendrocytes but fewer astrocytes compared to NSCs in the EAE-induced rats.

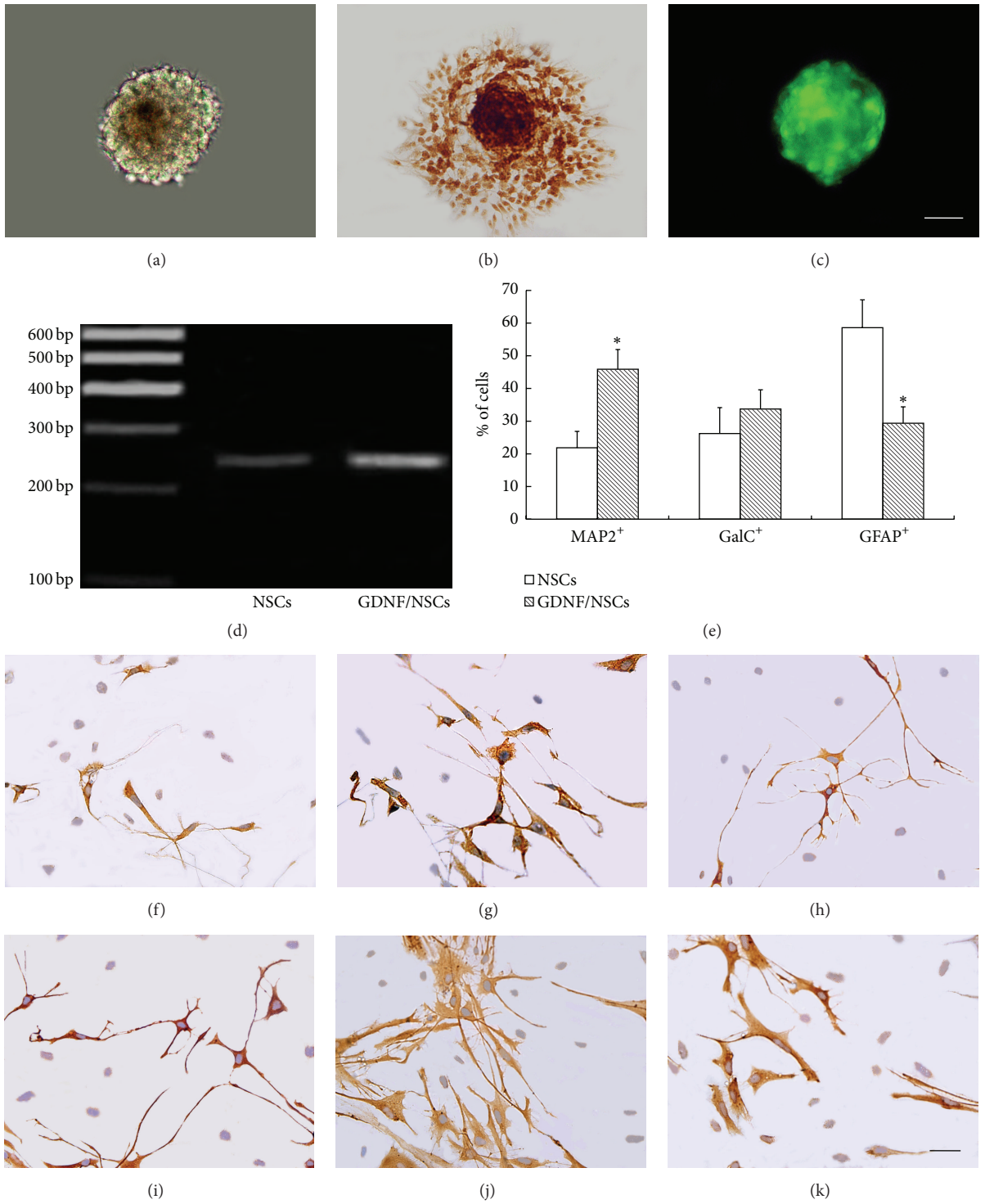

Figure 1: The generation and characterization of the neurosphere *in vitro*. (a) Cultured neurospheres derived from cerebral tissue of newborn rats at culture of 7 d. (b) Immunocytochemical staining of nestin in neurospheres. (c) Neurospheres infected by GDNF recombinant adenovirus for 2 days showed green fluorescence under fluorescence microscope. (d) RT-PCR showed higher level of GDNF mRNA in the GDNF/NSCs than NSCs. (e) Quantitative analysis of the percentages of NSCs and GDNF/NSCs differentiated into each type of neural cells (mean ± SD, $n = 5$). *Compared with NSCs group, $P < 0.05$. (f–k) Immunostaining showed, respectively, MAP2, GalC, and GFAP-positive cells from neurospheres. (f) MAP2$^+$ cells from NSCs; (g) MAP2$^+$ cells from GDNF/NSCs; (h) GalC$^+$ cells from NSCs; (i) GalC$^+$ cells from GDNF/NSCs; (j) GFAP$^+$ cells from NSCs; (k) GFAP$^+$ cells from GDNF/NSCs; (a–c) bar = 50 μm; (f–k) bar = 75 μm.

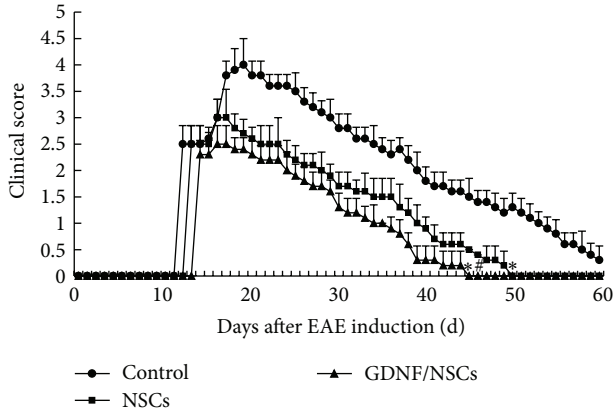

Figure 2: The improvement of clinical symptoms after transplantation (mean ± SD, $n = 5$). *Comparison with control group, $P < 0.05$; #comparison with NSCs group, $P < 0.05$.

3.5. GDNF Increased the Expression of mRNA for Oligodendrocyte Lineage Cells. The PDGFαR, the marker of oligodendrocyte progenitor cells, GalC, the marker of immature and mature oligodendrocytes, and MBP, the markers of myelinating oligodendrocytes, were used to evaluate oligodendrocyte lineage cells after transplantation. NSCs and GDNF/NSCs transplantation significantly increased mRNA expressions of PDGFαR, GalC, and MBP compared with control group ($P < 0.05$), and GDNF/NSCs transplantation appeared more efficient than NSCs ($P < 0.05$) (Figures 5(a) and 5(b)). These results showed that oligodendrogenesis and remyelination are significantly increased following NSCs transplantation, especially GDNF/NSCs transplantation.

4. Discussion

Our study showed that grafting NSCs and GDNF/NSCs into rats subjected to EAE significantly improved function compared with control group, and GDNF/NSCs group showed better efficacy. More myelin sheath and fewer inflammatory infiltration were observed in GDNF/NSCs group. More $BrdU^+$ GDNF/NSCs were observed compared to $BrdU^+$ NSCs, and more MAP2 and GalC-positive cells from GDNF/NSCs were detected compared with transplanted NSCs. Moreover, the increased expression of mRNA for PDGFαR, GalC, and MBP in brains was observed in GDNF/NSCs group compared to NSCs group.

Previous studies have reported that NSCs transplantation could reduce EAE-induced inflammation of CNS and promote clinical improvement [15–17]. When injecting subcutaneously, NSCs migrated into lymph nodes of EAE mice where they hampered the activation of myeloid dendritic cells and steadily restrained the expansion of encephalitogenic T cells, therefore reducing immune cell mobilization from the periphery [17, 18]. Transplanted systemically, NSCs entered perivascular CNS areas and subsequently induced apoptosis of blood-borne CNS-infiltrating encephalitogenic T cells [7, 15, 19]. Previous studies have shown that NSCs transplantation ameliorated the clinical symptoms and reduced tissue injury after EAE, which was related to the reduction in the number of perivascular infiltrates and of brain encephalitogenic T cells [20]. In this study, we showed a more significant reduction in the amount and size of inflammatory cell infiltration in GDNF/NSCs group compared with NSCs group. These results indicate that GDNF/NSCs more effectively reduce infiltrating inflammatory cells, which may result in greater therapeutic efficacy than NSCs on EAE, whereas the limitation in the present study is that control NSCs should be infected by empty virus vector expressing GFP alone because dividing cells may dilute out the BrdU label.

An inflammatory environment inevitably results in axonal degeneration and demyelination, which is correlated with chronic disability and brain atrophy in advanced MS [21]. Previous studies have suggested that transplanted NSCs generated new neurons and oligodendrocyte lineage cells to replace lost or degenerative cells, markedly promoting axonal regeneration and decreasing the extent of demyelination [16, 22]. The present results showed that more new neurons and oligodendrocytes were generated from transplanted GDNF/NSCs than that from transplanted NSCs, at least partially providing the possibility of remyelination and may result in better clinical recovery. Moreover, under pathological environments in the CNS, transplanted NSCs differentiate into astrocytes, which potentially cause reactive gliosis [23, 24] and hamper endogenous axonal remyelination [7, 13]. Here we showed that GDNF/NSCs generated less astrocytes than NSCs in EAE brain, which may be conducive to remyelination.

PDGFαR, GalC, and MBP, the markers of oligodendrocyte lineage cells, were strongly diminished at the chronic phase of EAE [21, 25, 26]. In this study, cells transplantation increased the expression of mRNA for PDGFαR, GalC, and MBP in the lesion areas in the brain, with higher expressions in the GDNF/NSCs group compared with the NSCs group. This suggests that a large number of oligodendrocyte lineage cells are generated after grafting, especially GDNF/NSCs. Therefore, rats with GDNF/NSCs grafting may possess more extent of remyelination than rats with NSCs for more myelin

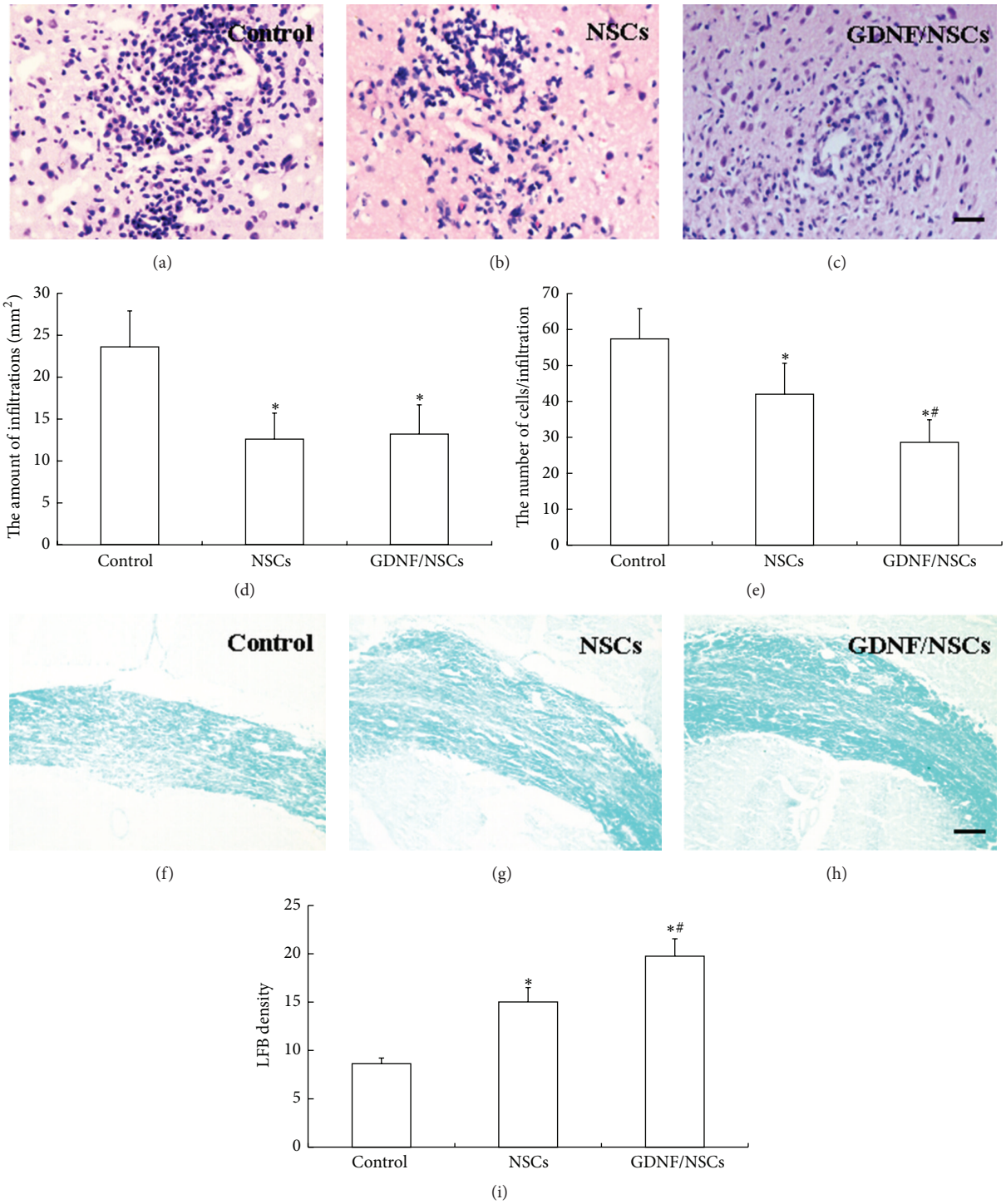

Figure 3: Attenuated inflammation and more myelin sheath in GDNF/NSCs compared to NSCs in EAE rats. (a–c) HE staining showed inflammatory infiltrates of the cerebral parenchyma in control, NSCs, and GDNF/NSCs groups, respectively. The control group showed much more infiltration with inflammatory cells. The transplanted groups displayed a reduction in the amount and size of the inflammatory infiltration, especially for the GDNF/NSC transplanted group. (d) Quantitative analysis of the amount of infiltration per mm^2 (mean ± SD, $n = 5$). *Compared with control group, $P < 0.05$. (e) Quantitative analysis of the number of cells per infiltration (mean ± SD, $n = 5$). *Compared with control group, $P < 0.05$. #Compared with NSCs group, $P < 0.05$. (f–h) LFB staining showed myelin sheath of the corpus callosum in control, NSCs, and GDNF/NSCs groups, respectively. Control group showed marked sparser and thinner myelin sheath compared with transplanted groups. In particular, GDNF/NSCs groups displayed more myelinated fibers than NSCs group. (i) Quantitative analysis of LFB density (mean ± SD, $n = 5$). *Compared with control group, $P < 0.05$; #compared with NSCs group, $P < 0.05$. Bar = 100 μm.

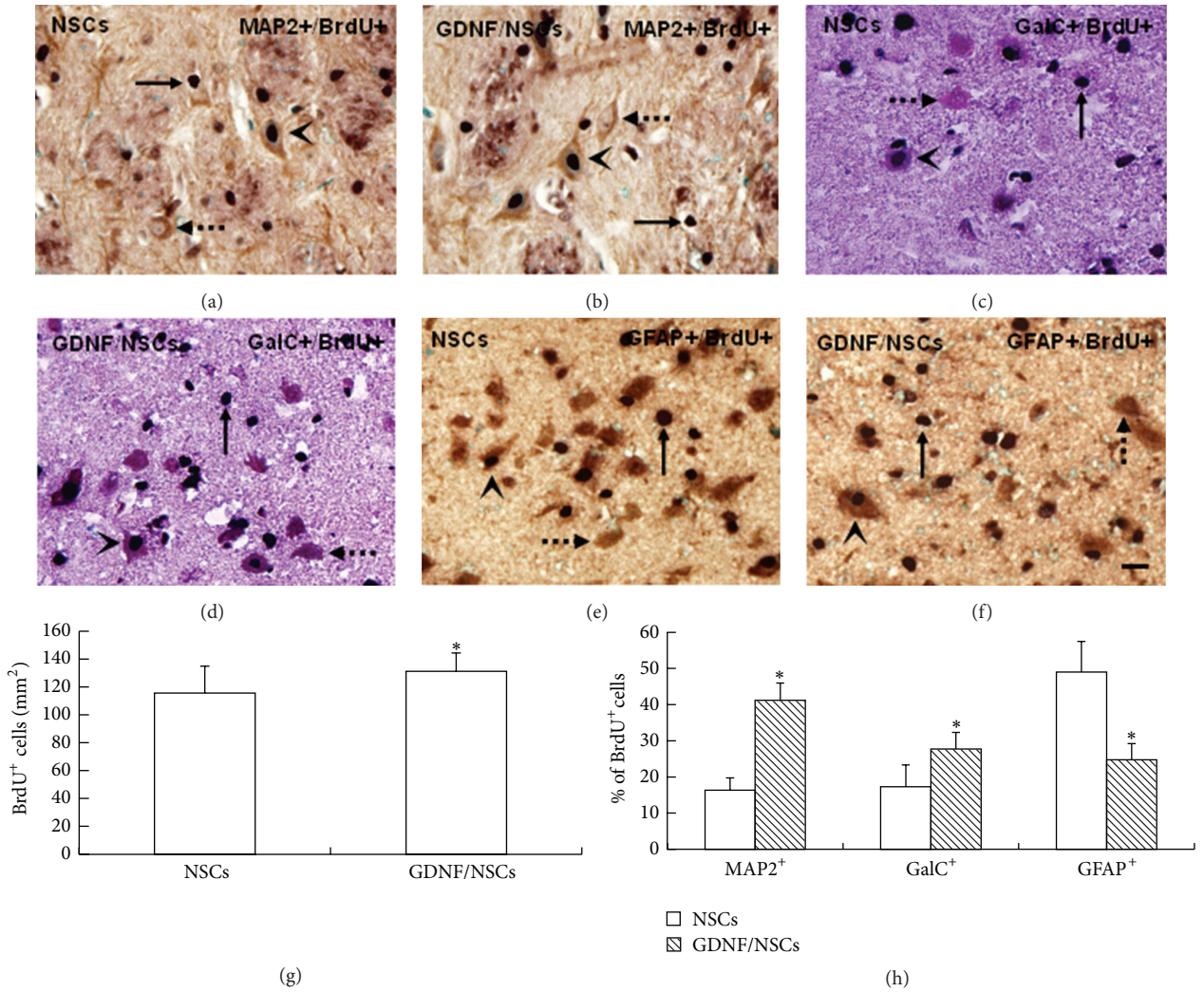

Figure 4: Double-immunohistochemical staining showed differentiation of transplanted NSCs or GDNF/NSCs in the corpus striatum. (a, b) $BrdU^+$ cells (solid arrow), $MAP2^+$ cells (dotted arrow), and $MAP2^+/BrdU^+$ cells (arrowhead) in NSCs group and GDNF/NSCs group, respectively. (c, d) $BrdU^+$ positive cells (solid arrow), $GalC^+$ cells (dotted arrow), and $GalC^+/BrdU^+$ cells (arrowhead) in NSCs group and GDNF/NSCs group, respectively. (e, f) $BrdU^+$ cells (solid arrow), $GFAP^+$ cells (dotted arrow), and $GFAP^+/BrdU^+$ cells (arrowhead) in NSCs group and GDNF/NSCs group, respectively. (g) Quantitative analysis of $BrdU^+$ cells in NSCs and GDNF/NSCs groups (mean ± SD, $n = 5$). *Compared with NSCs group, $P < 0.05$. (h) Quantitative analysis of the ratio of transplanted NSCs and GDNF/NSCs differentiated into each type of neural cells (mean ± SD, $n = 5$). *Compared with NSCs group, $P < 0.05$. Bar = 25 μm.

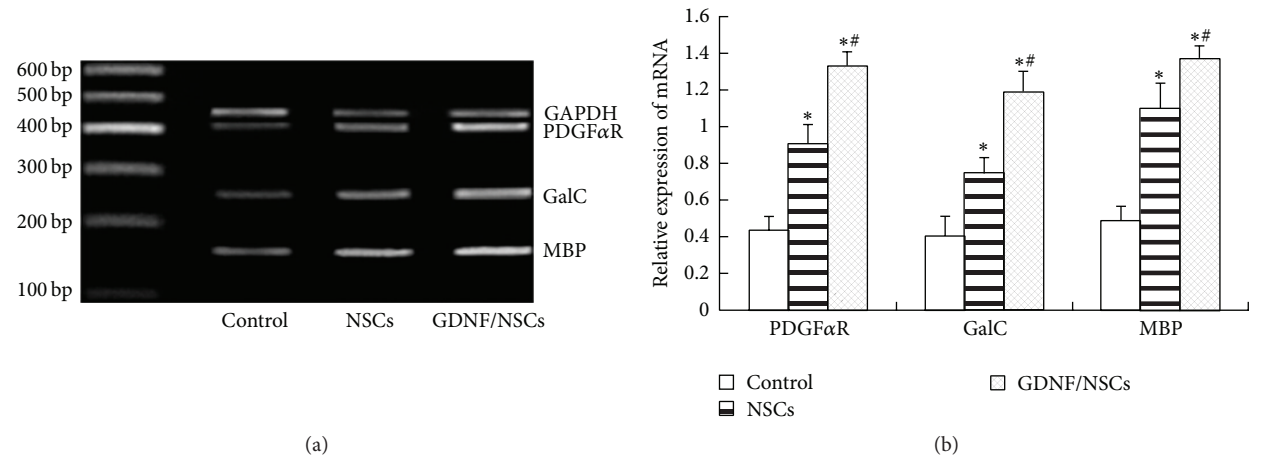

Figure 5: GDNF increased expression of mRNA for oligodendrocyte lineage cells. (a) mRNA expression of PDGFαR, GalC, and MBP in the cerebral lesion areas in each group. (b) Relative expression of PDGFαR, GalC, and MBP mRNA following transplantation (PDGFαR/GAPDH, GalC/GAPDH, and MBP/GAPDH) (mean ± SD, $n = 5$). *Compared with control group, $P < 0.05$; #compared with NSCs group, $P < 0.05$.

sheath by LFB staining was observed although the possibility that phagocytic cells were contained in these regions could not be excluded.

In summary, we showed here that GDNF/NSCs grafting significantly promotes functional recovery in EAE rats, reducing brain inflammatory infiltration, improving density of myelin, increasing repopulation of neurons and oligodendrocyte lineage cells, and decreasing the astrocyte differentiation of NSCs. These effects suggest that GDNF augments therapeutic efficiency of NSCs-based therapy on chronic EAE, providing a more promising approach to therapy EAE.

Competing Interests

The authors declare no competing interests.

Acknowledgments

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References

[1] J. M. Fletcher, S. J. Lalor, C. M. Sweeney, N. Tubridy, and K. H. G. Mills, "T cells in multiple sclerosis and experimental autoimmune encephalomyelitis," *Clinical and Experimental Immunology*, vol. 162, no. 1, pp. 1–11, 2010.

[2] G. C. Furtado, M. C. G. Marcondes, J.-A. Latkowski, J. Tsai, A. Wensky, and J. J. Lafaille, "Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis," *The Journal of Immunology*, vol. 181, no. 7, pp. 4648–4655, 2008.

[3] J. Yang, Y. Yan, C.-G. Ma et al., "Accelerated and enhanced effect of CCR5-transduced bone marrow neural stem cells on autoimmune encephalomyelitis," *Acta Neuropathologica*, vol. 124, no. 4, pp. 491–503, 2012.

[4] J. Yang, Y. Yan, Y. Xia et al., "Neurotrophin 3 transduction augments remyelinating and immunomodulatory capacity of neural stem cells," *Molecular Therapy*, vol. 22, no. 2, pp. 440–450, 2014.

[5] S. Grade, L. Bernardino, and J. O. Malva, "Oligodendrogenesis from neural stem cells: perspectives for remyelinating strategies," *International Journal of Developmental Neuroscience*, vol. 31, no. 7, pp. 692–700, 2013.

[6] F. Sher, S. Amor, W. Gerritsen et al., "Intraventricularly injected Olig2-NSCs attenuate established relapsing-remitting EAE in mice," *Cell Transplantation*, vol. 21, no. 9, pp. 1883–1897, 2012.

[7] M. Aharonowiz, O. Einstein, N. Fainstein, H. Lassmann, B. Reubinoff, and T. Ben-Hur, "Neuroprotective effect of transplanted human embryonic stem cell-derived neural precursors in an animal model of multiple sclerosis," *PLoS ONE*, vol. 3, no. 9, Article ID e3145, 2008.

[8] M. E. Cohen, N. Muja, N. Fainstein, J. W. M. Bulte, and T. Ben-Hur, "Conserved fate and function of ferumoxides-labeled neural precursor cells in vitro and in vivo," *Journal of Neuroscience Research*, vol. 88, no. 5, pp. 936–944, 2010.

[9] L.-F. H. Lin, D. H. Doherty, J. D. Lile, S. Bektesh, and F. Collins, "GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons," *Science*, vol. 260, no. 5111, pp. 1130–1132, 1993.

[10] C. E. Henderson, H. S. Phillips, R. A. Pollock et al., "GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle," *Science*, vol. 266, no. 5187, pp. 1062–1064, 1994.

[11] M. S. Ramer, J. V. Priestley, and S. B. McMahon, "Functional regeneration of sensory axons into the adult spinal cord," *Nature*, vol. 403, no. 6767, pp. 312–316, 2000.

[12] A. Blesch and M. H. Tuszynski, "Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination," *Journal of Comparative Neurology*, vol. 467, no. 3, pp. 403–417, 2003.

[13] L. Zhang, Z. Ma, G. M. Smith et al., "GDNF-enhanced axonal regeneration and myelination following spinal cord injury is mediated by primary effects on neurons," *GLIA*, vol. 57, no. 11, pp. 1178–1191, 2009.

[14] B. Chen, X.-Q. Gao, C.-X. Yang et al., "Neuroprotective effect of grafting GDNF gene-modified neural stem cells on cerebral ischemia in rats," *Brain Research*, vol. 1284, pp. 1–11, 2009.

[15] S. Pluchino, A. Quattrini, E. Brambilla et al., "Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis," *Nature*, vol. 422, no. 6933, pp. 688–694, 2003.

[16] S. Pluchino, L. Zanotti, B. Rossi et al., "Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism," *Nature*, vol. 436, no. 7048, pp. 266–271, 2005.

[17] S. Pluchino, L. Zanotti, E. Brambilla et al., "Immune regulatory neural stem/precursor cells protect from central nervous system autoimmunity by restraining dendritic cell function," *PLoS ONE*, vol. 4, no. 6, Article ID e5959, 2009.

[18] R. Brambilla, P. D. Morton, J. J. Ashbaugh, S. Karmally, K. L. Lambertsen, and J. R. Bethea, "Astrocytes play a key role in EAE pathophysiology by orchestrating in the CNS the inflammatory response of resident and peripheral immune cells and by suppressing remyelination," *Glia*, vol. 62, no. 3, pp. 452–467, 2014.

[19] C. Hackett, J. Knight, and Y. Mao-Draayer, "Transplantation of Fas-deficient or wild-type neural stem/progenitor cells (NPCs) is equally efficient in treating experimental autoimmune encephalomyelitis (EAE)," *American Journal of Translational Research*, vol. 6, no. 2, pp. 119–128, 2014.

[20] O. Einstein, N. Grigoriadis, R. Mizrachi-Kol et al., "Transplanted neural precursor cells reduce brain inflammation to attenuate chronic experimental autoimmune encephalomyelitis," *Experimental Neurology*, vol. 198, no. 2, pp. 275–284, 2006.

[21] L. Calza, M. Fernandez, A. Giuliani, L. Aloe, and L. Giardino, "Thyroid hormone activates oligodendrocyte precursors and increases a myelin-forming protein and NGF content in the spinal cord during experimental allergic encephalomyelitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 5, pp. 3258–3263, 2002.

[22] C. M. Rice, K. Kemp, A. Wilkins, and N. J. Scolding, "Cell therapy for multiple sclerosis: an evolving concept with implications for other neurodegenerative diseases," *The Lancet*, vol. 382, no. 9899, pp. 1204–1213, 2013.

[23] S. Obayashi, H. Tabunoki, S. U. Kim, and J.-I. Satoh, "Gene expression profiling of human neural progenitor cells following the serum-induced astrocyte differentiation," *Cellular and Molecular Neurobiology*, vol. 29, no. 3, pp. 423–438, 2009.

[24] Z. Gao, Q. Wen, Y. Xia et al., "Osthole augments therapeutic efficiency of neural stem cells-based therapy in experimental autoimmune encephalomyelitis," *Journal of Pharmacological Sciences*, vol. 124, no. 1, pp. 54–65, 2014.

[25] M. Fernandez, A. Giuliani, S. Pirondi et al., "Thyroid hormone administration enhances remyelination in chronic demyelinating inflammatory disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 46, pp. 16363–16368, 2004.

[26] F. Girolamo, G. Ferrara, M. Strippoli et al., "Cerebral cortex demyelination and oligodendrocyte precursor response to experimental autoimmune encephalomyelitis," *Neurobiology of Disease*, vol. 43, no. 3, pp. 678–689, 2011.

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Research Article

Establishment of Human Neural Progenitor Cells from Human Induced Pluripotent Stem Cells with Diverse Tissue Origins

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Human neural progenitor cells (hNPCs) have previously been generated from limited numbers of human induced pluripotent stem cell (hiPSC) clones. Here, 21 hiPSC clones derived from human dermal fibroblasts, cord blood cells, and peripheral blood mononuclear cells were differentiated using two neural induction methods, an embryoid body (EB) formation-based method and an EB formation method using dual SMAD inhibitors (dSMADi). Our results showed that expandable hNPCs could be generated from hiPSC clones with diverse somatic tissue origins. The established hNPCs exhibited a mid/hindbrain-type neural identity and uniform expression of neural progenitor genes.

1. Introduction

Human neural progenitor cells (hNPCs), which are present in fetal and adult neural tissues, have the potential to be therapeutically beneficial in the treatment of neuronal diseases such as spinal cord injury or stroke; however, it is technically difficult to obtain hNPCs from human neural tissues. The development of human embryonic stem cells (hESCs) [1] and human induced pluripotent stem cells (hiPSCs) [2, 3] has greatly improved the prospects of regenerative medicine. We are now able to obtain unlimited hiPSCs from every somatic tissue source [4]. However, hiPSC clones exhibit variable differentiation propensities [5], similar to hESCs [6].

Many protocols have been reported for the neural induction of hESCs/hiPSCs. Transplantable neural precursors were first derived from hESCs, which were subjected to spontaneous embryoid body (EB) formation, followed by neural rosette selection [7]. EB-mediated neural rosette formation is used not only for establishing rosette-stage neural stem cells (R-NSCs) from hESCs [8], but also for generating long-term self-renewing neuroepithelial-like stem (lt-NES) cells from hESCs/hiPSCs [9, 10]. However, the neural induction efficiency of these methods depends on the innate differentiation propensity of the hESC/hiPSC clones [11]. Using a strategy based on the neural default model, inhibitors of the bone morphogenic protein (BMP) signaling pathway, such as Noggin or the small molecule Dorsomorphin, have been used to direct the differentiation of hESCs/hiPSCs toward the neural lineage [12]. In addition, Lefty-A or the small molecule SB431542 can be used to inhibit Nodal, a member

of the transforming growth factor (TGF) β family which contributes to endodermal or mesodermal fate selection, promoting neural induction of hESCs. The combination of a BMP antagonist and a TGFβ/Activin/Nodal inhibitor has been used to accelerate the neural induction of hESCs/hiPSCs [11, 13–15]. To establish a reproducible EB-based method, Eiraku et al. subjected fully dissociated hESCs/hiPSCs to SFEBq (serum-free culture of EB-like aggregates) in the presence of a Rho-associated protein kinase (ROCK) inhibitor, resulting in the formation of uniformly sized EBs [16]. It has also been shown that neural precursor cells can be derived from hESCs at physiological oxygen levels (3 to 5%) [17].

Neural induction using these methods has been successful for only a limited number of hESC/hiPSC clones. Koyanagi-Aoi et al. recently reported on the SFEBq-mediated induction of dopaminergic neurons from hESCs and hiPSCs derived from various somatic tissues [18]. The aim of the current study was to determine whether hNPCs can be derived from any hiPSC clone regardless of its somatic tissue origin. We evaluated 21 hiPSC clones derived from human dermal fibroblasts (HDFs, 13 clones), cord blood (CB) cells (3 clones), and peripheral blood mononuclear (PBMN) cells (5 clones) using an EB formation-based method (EBFM) and an EB formation method that includes dual SMAD inhibitors (dSMADi). Although there is consensus that SMAD inhibition is necessary for neural induction as mentioned above, there are many variations among methods. Therefore, we performed the dSMADi method, in which the conditions are easily controlled, using two types of media and two different oxygen levels. Thus, the previously reported neural induction method was expanded into four conditions. Our data indicate that dual SMAD inhibition can be used to generate mid/hindbrain-type hNPCs from hiPSCs regardless of their somatic tissue origin. These expandable hNPCs may be a useful cell source for regenerative medicine research and the treatment of neuronal diseases.

2. Materials and Methods

2.1. Culture of hiPSCs. This study was conducted in accordance with the principles of the Helsinki Declaration, and the use of hiPSC clones was approved by the ethics committee of Osaka National Hospital (number 110) and CiRA, Kyoto University. All hiPSC clones (Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7235757) were cultured at the CiRA on mitomycin C-treated SNL feeder cells in primate ES cell medium (ReproCELL) until ~50% confluent and then transported to our laboratory at Osaka National Hospital. The hiPSC clones were cultured for two days before neural induction.

2.2. Neural Induction of hiPSCs. Two neural induction methods were used in this study: EBFM [19, 20] and an EB formation method using dual SMAD inhibitors (dSMADi) [14, 16]. Each of the hiPSC clones was simultaneously subjected to neural induction using the two methods.

For EBFM, the hiPSCs were treated with 10 μM Y-27632 (ROCK inhibitor) for 1 h at 37°C and then detached using 1 mg/mL collagenase IV (Life Technologies) and floated onto gelatin-coated dishes to remove the SNL feeder cells. After 30 min, floating EBs were transferred to Petri dishes containing DMEM/F12 (D6421, Sigma) with 20% knockout serum replacement (KSR, Life Technologies), 0.1 mM nonessential amino acids (NEAA, Life Technologies), 2 mM L-glutamine (Life Technologies), 0.1 mM 2-Mercaptoethanol (2-ME, Life Technologies), antibiotic-antimycotic (Life Technologies), and 10 μM Y-27632 (day 0). The next day (day 1), the medium was replaced with 5% KSR-containing medium and cultured further for 30 days with a medium change every two days.

For dSMADi, hiPSCs were treated with 10 μM Y-27632 for 1 h at 37°C and then dissociated with Trypsin/EDTA to generate single-cell suspensions and suspended in two types of medium: KSR-based medium [DMEM/F12 (D6421) with 20% KSR, 0.1 mM 2-ME, 10 μM SB-431542 (SB, Sigma), and 2 μM Dorsomorphin (DSM, Wako)] and B27N2-based medium [DMEM/F12 (D8062) with 15 mM HEPES, 5% B27, 5% N2 supplement (N2, Life Technologies), 10 μM SB, 2 μM DSM, and 10 ng/mL bFGF]. Both media were supplemented with 30 μM Y-27632 for the first 3 days. Completely dissociated cells were then seeded into ultralow attachment 96-well plates (PrimeSurface® 96-well, Sumitomo Bakelite) at 9,000 cells/well, centrifuged at 700 rpm for 3 min (quick-aggregation), and cultured in a 5% CO_2 incubator with 5 or 20% O_2. Thus, the dSMADi neural induction was conducted using four conditions: KSR/20% O_2, KSR/5% O_2, B27N2/20% O_2, and B27N2/5% O_2. The cells were cultured for 14 days with daily replacement of half the spent medium with fresh medium. On day 14, the aggregates were dissociated mechanically and cultured on Petri dishes in a 5% CO_2 incubator with 20% O_2 to generate the first passage of hNPCs. Neurospheres were generated from the second passage of NPCs by completely dissociating the cells with Accutase™ (Innovative Cell Technologies) and then cultured on nontreated flasks. If the hNPCs attached to the culture vessels at early passages, we used ultralow attachment dishes (PrimeSurface, Sumitomo Bakelite) to establish the hNPCs as neurospheres.

2.3. Maintenance of hNPCs. The hNPCs were seeded at 1 $\times$ 10^5 cells/mL and cultured as floating neurospheres in hNPC medium [DMEM/F12 (D8062) with 15 mM HEPES, 2% B27, 20 ng/mL EGF (PeproTech), 20 ng/mL FGF2 (PeproTech), 10 ng/mL leukemia inhibitory factor (Millipore), and 5 μg/mL heparin (Sigma-Aldrich)].

2.4. Quantitative Reverse Transcription-Polymerase Chain Reaction (Quantitative RT-PCR). Total RNA was extracted using the RNeasy MinElute Cleaning Kit (Qiagen), and the cDNAs were synthesized using the PrimeScript® RT Master Mix (Takara Bio) according to the manufacturer's specifications. Quantitative PCR analysis was performed using gene-specific primers (Table S4), the Power SYBR® Green PCR Master Mix, and the 7300 Real-Time PCR System (Applied Biosystems). Gene expression levels were expressed as delta Ct values normalized to *GAPDH* [23].

2.5. Measurement of Neural Aggregate Size. Phase-contrast images of eight wells per condition for each clone were captured (one representative image per condition is shown in Figure S3). The projected areas of the neural aggregates were measured using ImageJ [24]. The aggregate size was calculated as a sphere volume using the circular diameter determined from the projected area [25].

2.6. In Vitro Neuronal Differentiation. To avoid disturbing the naturally formed niche, the neurospheres were not dissociated. The intact neurospheres were transferred to vessels coated with Growth Factor Reduced Matrigel™ (diluted to 1 : 30, BD Biosciences) and cultured in Neurobasal Medium (Life Technologies) containing 2% B27 and 1% L-glutamine for 2 weeks [22].

2.7. Immunocytochemical Staining. Cells were fixed in 4% paraformaldehyde and washed with PBS. The fixed samples were then blocked with 10% normal goat serum and incubated with anti-βIII tubulin antibody (clone TuJ1, Babco) overnight at 4°C. The samples were then incubated with AlexaFluor-488-conjugated goat anti-mouse IgG (Molecular Probes, Life Technologies) for 1 h at room temperature. The stained samples were examined with a confocal laser-scanning microscope. All staining procedures were performed with matched-isotype controls [22].

2.8. Neurite Analysis. βIII tubulin-positive neurites in four regions were detected by appropriate thresholding and then skeletonized using ImageJ [24]. Total neurite length was determined by counting the positive pixels [26].

2.9. Statistical Analysis. Significant differences in gene expression levels obtained by quantitative RT-PCR were analyzed using the Steel-Dwass nonparametric multiple comparison test or Welch's t-test. Significant differences in neural aggregate size were also analyzed by the Steel-Dwass comparison test. Significant differences in total neurite length were analyzed by Dunnett's test. See figure legends for details.

3. Results and Discussion

There are many variations among neural induction methods, although there is consensus about the necessity of SMAD inhibition. To determine whether hiPSC clones derived from different somatic tissues could differentiate into hNPCs without specific neural induction methods (Figure 1(a)), we examined 21 hiPSC clones established in CiRA (Table S1). These hiPSC clones were derived from three different tissues: HDFs, CB cells, and PBMN cells (Table S1).

All of the hiPSCs exhibited a typical undifferentiated hESC-like morphology (Figure S1A). Quantitative RT-PCR showed that the clones expressed uniformly high levels of the pluripotency marker genes *Oct4*, *NANOG*, and *LIN28A* but very low levels of the differentiation marker genes *SOX17* (endoderm), *T* (mesoderm), and *SOX1* and *PAX6* (both neural) just prior to neural induction (Figure 1(b) and Figure S1B).

Two predominant methods for inducing the neural differentiation of hESCs/hiPSCs are the EB formation-based method (EBFM) and EB formation with dual SMAD inhibitors (dSMADi). We controlled the aggregate size in the dSMADi method using a quick-aggregation procedure [16] and examined four additional conditions using this method by assessing combinations of two culture media and two different oxygen levels. In the EBFM approach, we used a low concentration (5%) of knockout serum replacement (KSR) [19, 20] to limit the amount of BMP-like activity [27], which opposes neural induction and is present in the KSR.

We subjected the 21 hiPSC clones to the five different neural induction procedures (Figure 1(a)). To assess the neural induction efficiency using dSMADi, we compared gene expression levels among the hiPSCs, day 30 EBFM-derived EBs, and day 14 dSMADi-derived aggregates, by quantitative RT-PCR (Figure 1(b), Figure S1B, Figure S1C, and Figure S2). Bivariate box plots showed that the pluripotency marker genes, *Oct4* and *NANOG,* were strongly and uniformly downregulated in day 14 dSMADi-derived aggregates but not in day 30 EBFM-derived EBs, which exhibited more variable expression among the clones (Figure 1(b) and Figure S1C). Interestingly, dSMADi treatment also resulted in the slight downregulation of another pluripotency marker gene, *LIN28A* (Figure 1(b)).

Day 30 EBs did not uniformly express the endoderm marker gene *SOX17*, mesoderm marker gene *T*, or neural marker genes *SOX1* and *PAX6* and were classified as nonneural, neural, or three germ layer-containing EBs (Figure S1D). In contrast, almost all of the day 14 dSMADi-derived aggregates exhibited the upregulation of both neural marker genes but not nonneural marker genes (Figure 1(b)). These findings indicated that while EBFM-derived EBs exhibited cell lineage variability (only 6 clones differentiated toward specifically neural lineage), dSMADi-derived aggregates exhibited less clonal variation, and almost all of them underwent neural lineage induction regardless of somatic tissue origin and the differentiation protocols (over 90% of clones differentiated toward neural lineage). Hereafter, we will refer to day 14 dSMADi aggregates as neural aggregates.

We further evaluated the effects of the four dSMADi conditions by comparing the gene expression levels of the neural aggregates derived in two types of media (KSR or B27N2) and two different oxygen levels (20% or 5%) (Figure 2(a)). Although the expression levels of the pluripotency marker gene *Oct4* and neural marker gene *SOX1* were similar in aggregates cultured in the four conditions, those of *NANOG* in aggregates cultured in KSR/5% O_2 and of *PAX6* in aggregates cultured in KSR/20% O_2 and KSR/5% O_2 were significantly higher than in the B27N2-based conditions (Figure 2(a)). The oxygen level alone did not significantly impact the expression levels of these genes. Although we used a quick-aggregation procedure in the dSMADi experiments to eliminate the size and shape variability observed in EBFM-derived EBs (Figure S1A), we noticed obvious differences in neural aggregate size among the four conditions (Figure S3).

To examine the effects of the culture conditions in detail, we measured the size of the neural aggregates on day 7 and day 14 (Figure 2(b)). The day 14 neural aggregates cultured in

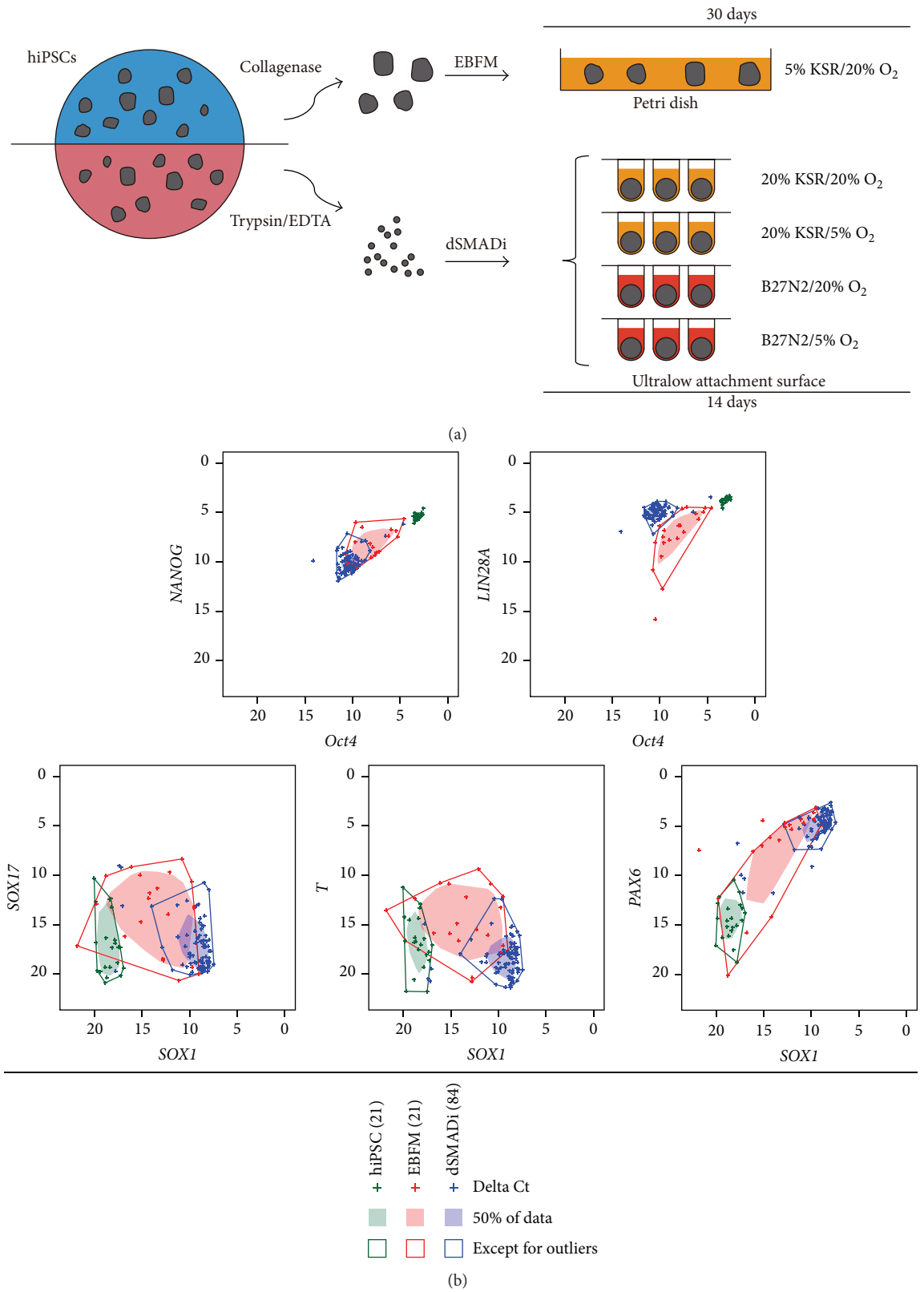


Figure 1: dSMADi improves the neural induction efficiency of hiPSCs regardless of their somatic tissue origin. (a) Schematic drawing of the neural induction methods used in this study. (b) Bivariate box plots displaying gene expression levels in hiPSCs, day 30 EBFM-derived EBs, and day 14 dSMADi-derived aggregates. Quantitative RT-PCR-generated delta Ct values for the pluripotency marker genes (*Oct4*, *NANOG*, and *LIN28A*), endoderm marker gene (*SOX17*), mesoderm marker gene (*T*), and neural marker genes (*SOX1* and *PAX6*) are shown. Green: hiPSCs; red: EBFM; blue: dSMADi. The numbers of clones analyzed are indicated in parentheses and "+" symbols represent each of the delta Ct values. Filled-in regions contain the 50% of the data points, and data points outside of the surrounding lines represent outliers. See also Figures S1 and S2.

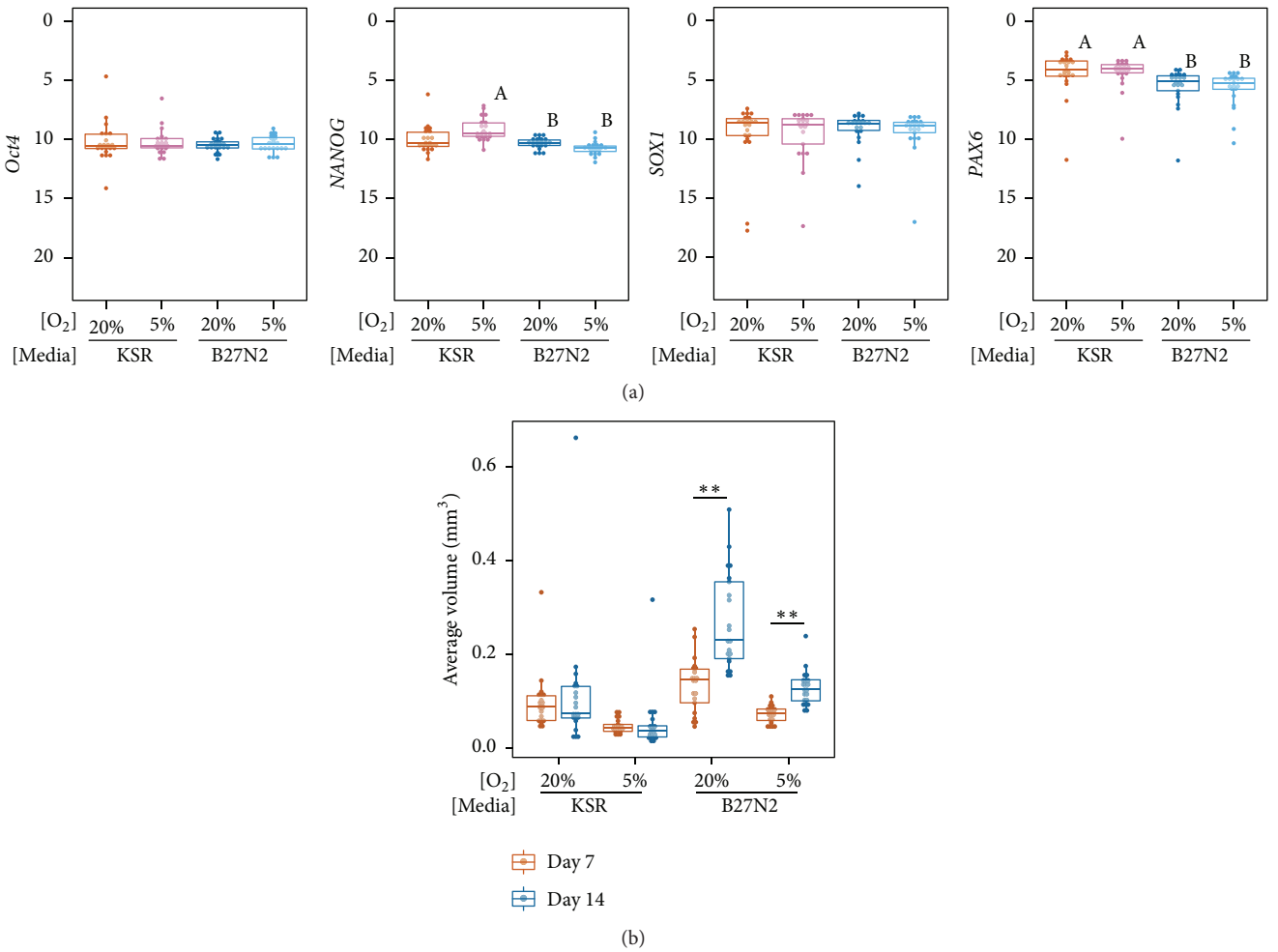


Figure 2: Effects of media and oxygen level on neural aggregate gene expression and size. (a) Dot plots and box plots showing for the gene expression levels of pluripotency markers (*Oct4* and *NANOG*) and neural markers (*SOX1* and *PAX6*) in day 14 aggregates cultured in the various conditions. Twenty-one clones were analyzed in each condition. Statistical significance was determined by the Steel-Dwass test. Statistically significant differences ($p < 0.01$) were found between samples "A" and "B". See also Figure S2. (b) Dot plot and box plot showing the size of day 7 and day 14 neural aggregates. Statistical analysis was performed with the Steel-Dwass test ($^{**}P < 0.01$). All p values are shown in Table S2.

B27N2-based conditions were significantly larger than those cultured in KSR-based conditions at each oxygen level, and they were also significantly larger when cultured in 20% O_2 than in 5% O_2, in both types of media (Figure 2(b) and Table S2). Notably, neural aggregates did not grow in the KSR-based conditions over the induction period regardless of the oxygen level. In contrast, B27N2, which contains basic fibroblast growth factor, supported the growth of the neural aggregates (Figure 2(b)). These findings indicated that although the four dSMADi conditions efficiently promoted neural lineage induction, the culture medium influenced both gene expression and aggregate size, whereas the oxygen level primarily affected aggregate size.

Next, we investigated whether the neural aggregates exhibited a forebrain-type property, as previously observed for lt-NES cells and R-NSCs [8–10]. Because the expression of *PAX6* was higher in KSR-derived aggregates than in B27N2-derived aggregates, we first compared the expression levels of the forebrain marker genes, *FOXG1* and *OTX1*, in day 14 neural aggregates cultured in all four conditions. Although there were no significant differences in *FOXG1* and *OTX1* expression levels among aggregates cultured in the different conditions (Figure 3(a)), the *FOXG1* expression was more variable among the clones compared to the *OTX1* expression. Therefore, we compared the *FOXG1* expression among aggregates derived from somatic tissues of different origins (Figure 3(b)). Notably, the *FOXG1* expression was significantly lower in the clones derived from PBMN cells than in those derived from HDFs and CB cells (Figure 3(b)), although the HDF- and CB cell-derived clones exhibited variable *FOXG1* expression. Given that ES cells generate anterior forebrain-like neural precursor cells in the absence of external signals [28], the variability of *FOXG1* expression in the HDF- and CB-derived clones might reflect variable Wnt activation in the hiPSCs [29].

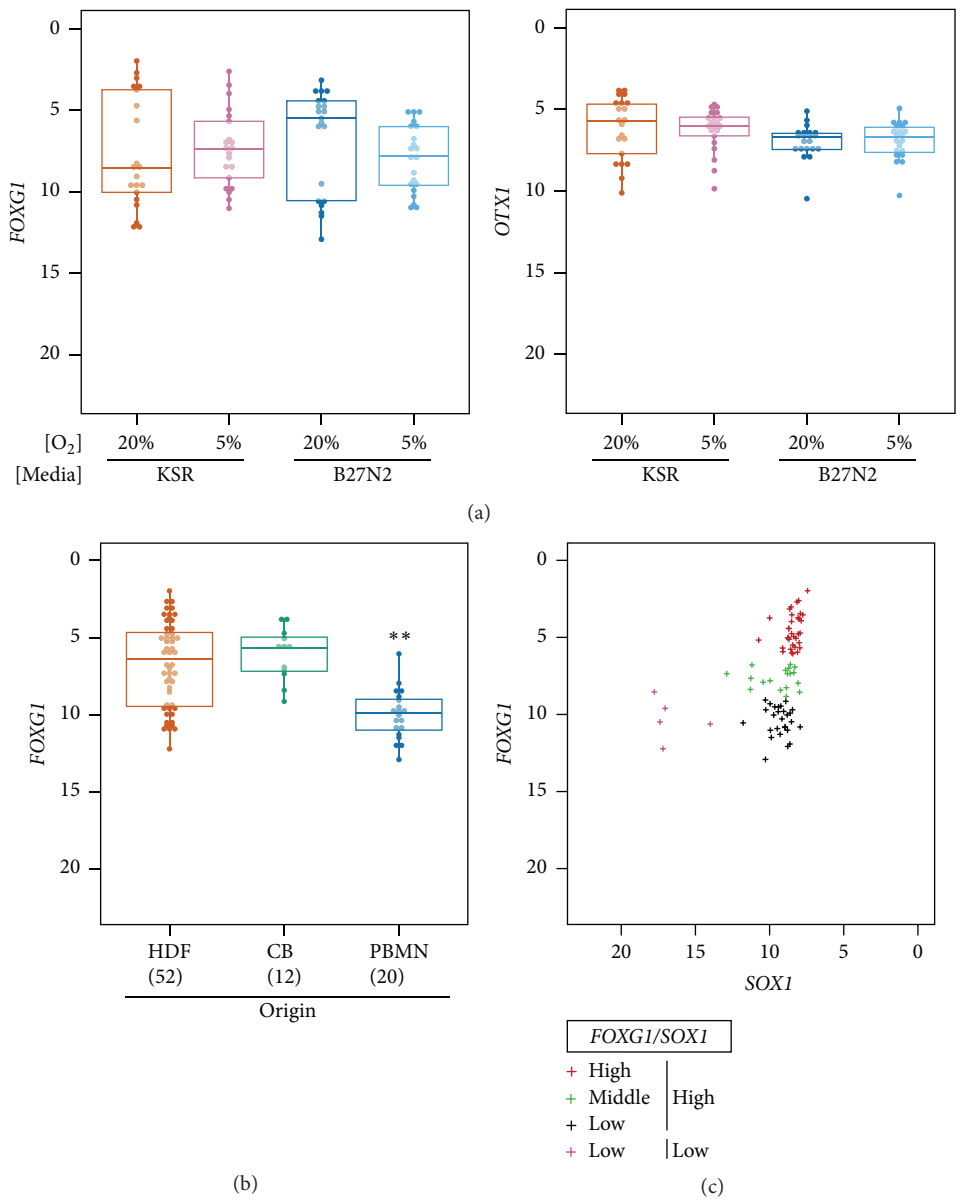
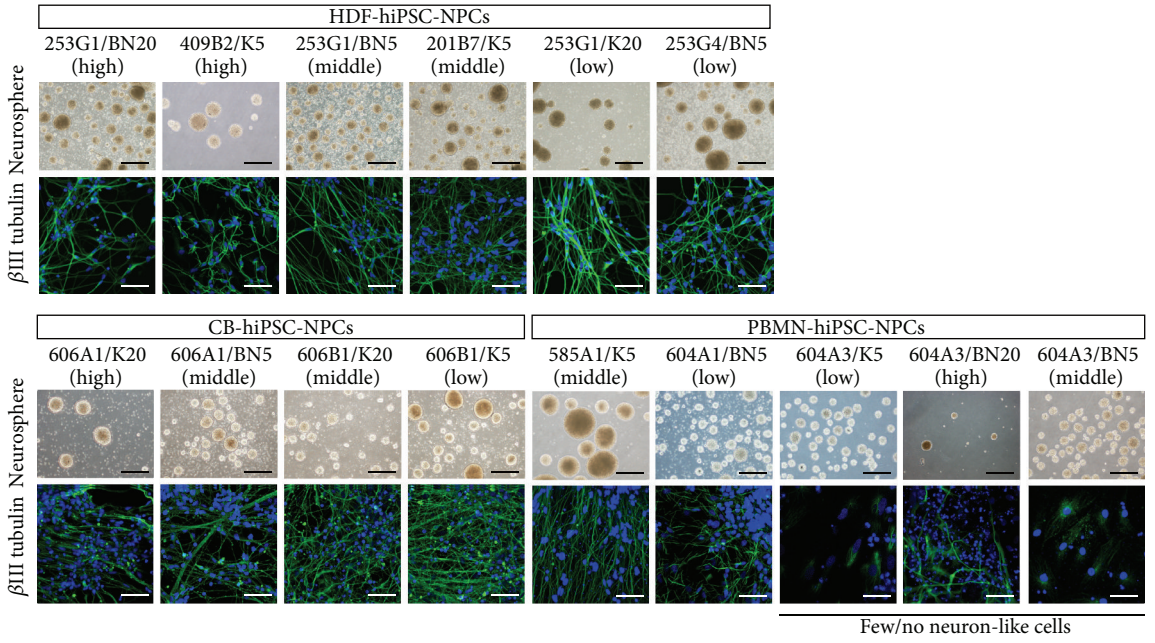
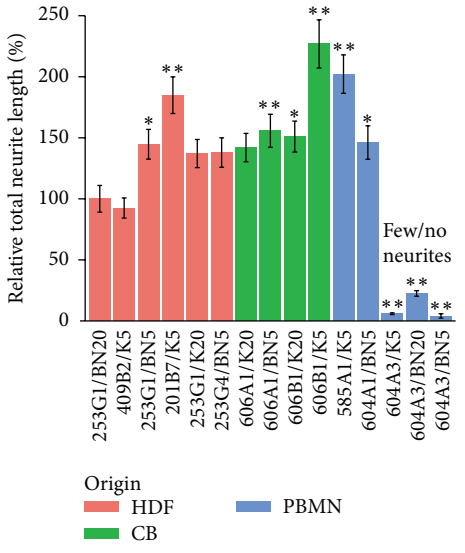


Figure 3: PBMN-derived neural aggregates exhibit low *FOXG1* expression levels. (a) Dot plots and box plots showing *FOXG1* and *OTX1* expression in day 14 neural aggregates cultured in the various conditions. (b) Dot plots and box plots showing the *FOXG1* expression levels according to tissue origin. The number of clones analyzed is indicated in parentheses. Statistical analysis was performed by the Steel-Dwass test. $^{**}p < 0.01$. (c) Clustering of clones, based on the expression of *FOXG1* and *SOX1*, using the *K*-medoids method. The delta Ct values are indicated by "+" symbols. Four clusters were identified: *FOXG1*-high/*SOX1*-high (red), *FOXG1*-middle/*SOX1*-high (green), *FOXG1*-low/*SOX1*-high (black), and *FOXG1*-low/*SOX1*-low (pink). See also Table S3.

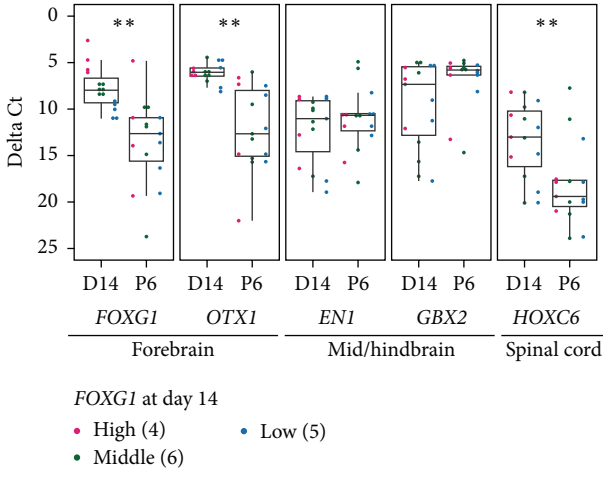
However, the mechanisms regulating differential *FOXG1* expression between CB- and PBMN-derived clones are unclear because both hiPSC clones were derived from mesodermal tissues, cultured for over 20 passages, and were considered to have lost their somatic tissue-specific epigenetic memory [30–32]. Given that all of the PBMN cells were $\alpha\beta$T cells in this study, their genomes were modified due to T-cell receptor (TCR) rearrangement, in contrast to the HDF and CB cells. The genes encoding T-cell receptor alpha (*TRA*) and T-cell receptor beta (*TRB*) are located at chromosomal regions 14q11.2 and 7q34, respectively. *FOXG1* is located at chromosomal region 14q13. Although TRA and *FOXG1* are separated by about 6 million bases, some epigenetic modifications might still occur in the regulatory region of *FOXG1*. *FOXG1* is critical for normal corticogenesis [33]; therefore, PBMN-derived hiPSCs might not be a good source for analyzing normal cortical development or disease modeling. Further studies are required to elucidate whether inhibition of Wnt signaling improves the induction of anterior neural progenitors from PBMN-derived clones. On the other hand, we hypothesized that neural aggregates with the potential for producing uniform hNPCs could be



(a)

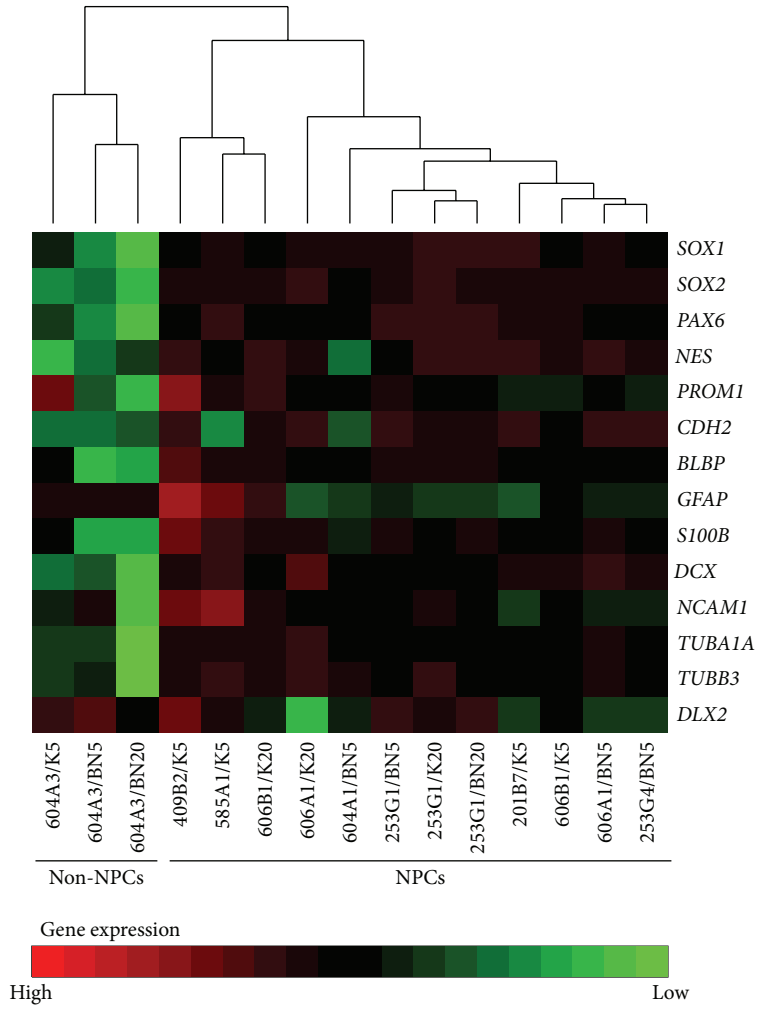


(b)



(c)

Figure 4: Continued.



(d)

Figure 4: Established hNPCs express regional markers indicative of a mid/hindbrain-type property. (a) Morphologies of the expanded neurospheres at approximately passage 6 (upper panel). βIII tubulin-positive neurons after 14 days of neuronal differentiation. Green: βIII tubulin; blue: nucleus (lower panel). Scale bars: 500 μm (black) and 50 μm (white). (b) Neurite analysis after 14 days of neuronal differentiation. Relative total neurite length is shown as the mean ± SD. Statistical analysis was performed using Dunnett's test. $^{*}p < 0.05$ and $^{**}p < 0.01$. (c) Dot plots and box plots showing the expression of forebrain markers (*FOXG1* and *OTX1*), mid/hindbrain markers (*EN1* and *GBX2*), and a spinal cord marker (*HOXC6*) in day 14 (D14) neural aggregates and neurospheres at approximately passage 6 (P6). The numbers of clones analyzed are shown in parentheses. Statistical analysis was performed using Welch's two-sample t-test. $^{**}p < 0.01$. (d) Hierarchical clustering of clones based on neural progenitor marker expression. Red: high gene expression; green: low gene expression.

identified by screening for *FOXG1* expression. To evaluate this possibility, we grouped all of the neural aggregates into four categories (*FOXG1*-high/*SOX1*-high, *FOXG1*-middle/*SOX1*-high, *FOXG1*-low/*SOX1*-high, and *FOXG1*-low/*SOX1*-low) by clustering based on *FOXG1* and *SOX1* expression levels (Figure 3(c) and Table S3). We then selected neural aggregates from each category, except for the *SOX1*-low, nonneural lineage category, for further expansion. Notably, these neural aggregates included clones derived from each of the three different tissue types (Table S3).

To facilitate the expansion of homogeneous populations of hNPCs, we cultured them as floating neurospheres [22] rather than as adherent cells like lt-NES cells. Expandable neurospheres were established at approximately passage 6 regardless of the *FOXG1* expression level in day 14 neural aggregates or the somatic tissue origin (Figure 4(a)). To confirm that the neurospheres were hNPCs, neuronal differentiation was induced using the serum-free neuronal differentiation protocol. All of the HDF- and CB cell-derived neurospheres differentiated into βIII tubulin-positive neurons with long neurites, whereas only two of the five PBMN cell-derived neurospheres underwent neuronal differentiation (Figures 4(a) and 4(b)). Notably, all of the clones that failed to differentiate were derived from the hiPSC clone 604A3 (Figures 4(a) and 4(b)). These results also indicated that hNPC establishment was independent of the *FOXG1*

expression level in day 14 neural aggregates and of the hiPSC somatic tissue origin. *FOXG1* expression decreases with increasing passage of lt-NES cells [9, 10] and neurospheres [22]. Thus, we hypothesized that *FOXG1* expression in day 14 neural aggregates may decrease over time, resulting in the formation of mid/hindbrain-type progenitors. To assess this supposition, we compared the expression of regional identity marker genes in day 14 neural aggregates and passage 6 neurospheres (Figure 4(c)). We found that the neural aggregates expressed variable levels of not only the forebrain markers, *FOXG1* and *OTX1*, but also each marker gene for the mid/hindbrain (*EN1*/*GBX2*) and spinal cord (*HOXC6*) (Figure 4(c)). These broad expression patterns of regional marker genes were consistent with a previous report on the activity of endogenous Wnt signaling [34]. In contrast, passage 6 neurospheres expressed higher and less variable levels of the mid/hindbrain markers and reduced levels of the forebrain and spinal cord markers (Figure 4(c)). Thus, while the day 14 neural aggregates exhibited upregulated forebrain marker expression, established hNPCs displayed a mid/hindbrain-like regional property, consistent with previous findings [22].

Finally, we compared the expression of neural progenitor genes among the established hNPCs (Figure 4(d)). We found that hNPCs and 604A3-derived non-hNPCs were separately clustered and that the hNPCs exhibited similar neural progenitor gene expression patterns regardless of the dSMADi conditions and their somatic tissue origin. All of the PBMN clones met our criteria of high *SOX1* and *PAX6* expressions on day 14 (Figure 3(c)). All three of the 604A3 clones had almost the same properties on day 14 but differed from the other clones at passage 6 (Figure 4(d)). We could not eliminate the possibility that the day 14 neural aggregates contained nonneural cells, because gene expression analysis was performed on the bulk population, not at the single-cell level. Therefore, we applied the neurosphere culture method to select a homogeneous population and we were successful in almost every case. However, given that at least neural crest cells can grow in our neurosphere conditions [35], some cell population that was preferentially induced in the 604A3 clone might have been selected and expanded in the neurosphere culture process.

4. Conclusions

We conclude that neural lineage cells can be derived from most hiPSC clones, regardless of their somatic tissue origin, using dual SMAD inhibition. We found that PBMN cell-derived hiPSC clones did not exhibit increased expression of the forebrain marker gene, *FOXG1*, but generated hNPCs with neuronal differentiation ability as efficiently as HDF- and CB-derived hiPSC clones. Moreover, neural aggregates at the early neural induction stage exhibited variable neural regional marker gene expression patterns and gave rise to hNPCs that uniformly exhibited a mid/hindbrain-type property and expressed similar levels of neural progenitor genes. These findings suggest that the hNPCs described here may be a useful cell source for basic and pharmaceutical research aimed at developing regenerative therapies for treating various neuronal diseases.

Competing Interests

The authors declare that there are no competimg interests regarding the publication of this paper.

Authors' Contributions

Yonehiro Kanemura conceived the study and wrote the paper with Hayato Fukusumi and finally approved it. Yonehiro Kanemura, Hayato Fukusumi, Tomoko Shofuda, and Yohei Bamba designed experiments. Hayato Fukusumi, Tomoko Shofuda, Atsuyo Yamamoto, Daisuke Kanematsu, and Yukako Handa collected and analyzed data. Keisuke Okita established and distributed hiPSC clones. Masaya Nakamura, Shinya Yamanaka, and Hideyuki Okano provided critical reading and scientific discussions.

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References

[1] J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro et al., "Embryonic stem cell lines derived from human blastocysts," *Science*, vol. 282, no. 5391, pp. 1145–1147, 1998.

[2] K. Takahashi, K. Tanabe, M. Ohnuki et al., "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872, 2007.

[3] J. Yu, M. A. Vodyanik, K. Smuga-Otto et al., "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, no. 5858, pp. 1917–1920, 2007.

[4] K. Okita, T. Yamakawa, Y. Matsumura et al., "An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells," *STEM CELLS*, vol. 31, no. 3, pp. 458–466, 2013.

[5] A. Nasu, M. Ikeya, T. Yamamoto et al., "Genetically matched human iPS cells reveal that propensity for cartilage and bone differentiation differs with clones, not cell type of origin," *PLoS ONE*, vol. 8, no. 1, Article ID e53771, 2013.

[6] K. Osafune, L. Caron, M. Borowiak et al., "Marked differences in differentiation propensity among human embryonic stem cell lines," *Nature Biotechnology*, vol. 26, no. 3, pp. 313–315, 2008.

[7] S.-C. Zhang, M. Wernig, I. D. Duncan, O. Brüstle, and J. A. Thomson, "In vitro differentiation of transplantable neural precursors from human embryonic stem cells," *Nature Biotechnology*, vol. 19, no. 12, pp. 1129–1133, 2001.

[8] Y. Elkabetz, G. Panagiotakos, G. Al Shamy, N. D. Socci, V. Tabar, and L. Studer, "Human ES cell-derived neural rosettes reveal

a functionally distinct early neural stem cell stage," *Genes and Development*, vol. 22, no. 2, pp. 152–165, 2008.

[9] A. Falk, P. Koch, J. Kesavan et al., "Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons," *PLoS ONE*, vol. 7, no. 1, Article ID e29597, 2012.

[10] P. Koch, T. Opitz, J. A. Steinbeck, J. Ladewig, and O. Brüstle, "A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 9, pp. 3225–3230, 2009.

[11] D.-S. Kim, J. S. Lee, J. W. Leem et al., "Robust enhancement of neural differentiation from human ES and iPS cells regardless of their innate difference in differentiation propensity," *Stem Cell Reviews and Reports*, vol. 6, no. 2, pp. 270–281, 2010.

[12] T. Wada, M. Honda, I. Minami et al., "Highly efficient differentiation and enrichment of spinal motor neurons derived from human and monkey embryonic stem cells," *PLoS ONE*, vol. 4, no. 8, Article ID e6722, 2009.

[13] S. M. Chambers, C. A. Fasano, E. P. Papapetrou, M. Tomishima, M. Sadelain, and L. Studer, "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling," *Nature Biotechnology*, vol. 27, no. 3, pp. 275–280, 2009.

[14] A. Morizane, D. Doi, T. Kikuchi, K. Nishimura, and J. Takahashi, "Small-molecule inhibitors of bone morphogenic protein and activin/nodal signals promote highly efficient neural induction from human pluripotent stem cells," *Journal of Neuroscience Research*, vol. 89, no. 2, pp. 117–126, 2011.

[15] K. Watanabe, M. Ueno, D. Kamiya et al., "A ROCK inhibitor permits survival of dissociated human embryonic stem cells," *Nature Biotechnology*, vol. 25, no. 6, pp. 681–686, 2007.

[16] M. Eiraku, K. Watanabe, M. Matsuo-Takasaki et al., "Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals," *Cell Stem Cell*, vol. 3, no. 5, pp. 519–532, 2008.

[17] S. R. Stacpoole, B. Bilican, D. J. Webber et al., "Efficient derivation of NPCs, spinal motor neurons and midbrain dopaminergic neurons from hESCs at 3% oxygen," *Nature Protocols*, vol. 6, no. 8, pp. 1229–1240, 2011.

[18] M. Koyanagi-Aoi, M. Ohnuki, K. Takahashi et al., "Differentiation-defective phenotypes revealed by large-scale analyses of human pluripotent stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20569–20574, 2013.

[19] Y. Imaizumi, Y. Okada, W. Akamatsu et al., "Mitochondrial dysfunction associated with increased oxidative stress and α-synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue," *Molecular Brain*, vol. 5, article 35, 2012.

[20] E. Ohta, T. Nihira, A. Uchino et al., "I2020T mutant LRRK2 iPSC-derived neurons in the Sagamihara family exhibit increased Tau phosphorylation through the AKT/GSK-3β signaling pathway," *Human Molecular Genetics*, vol. 24, no. 17, pp. 4879–4900, 2015.

[21] Y. Kanemura, H. Mori, S. Kobayashi et al., "Evaluation of in vitro proliferative activity of human fetal neural stem/progenitor cells using indirect measurements of viable cells based on cellular metabolic activity," *Journal of Neuroscience Research*, vol. 69, no. 6, pp. 869–879, 2002.

[22] T. Shofuda, H. Fukusumi, D. Kanematsu et al., "A method for efficiently generating neurospheres from human-induced pluripotent stem cells using microsphere arrays," *NeuroReport*, vol. 24, no. 2, pp. 84–90, 2013.

[23] O. Adewumi, B. Aflatoonian, L. Ahrlund-Richter et al., "Characterization of human embryonic stem cell lines by the International Stem Cell Initiative," *Nature Biotechnology*, vol. 25, no. 7, pp. 803–816, 2007.

[24] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH image to imageJ: 25 years of image analysis," *Nature Methods*, vol. 9, no. 7, pp. 671–675, 2012.

[25] H. Mori, K. Ninomiya, M. Kino-Oka et al., "Effect of neurosphere size on the growth rate of human neural stem/progenitor cells," *Journal of Neuroscience Research*, vol. 84, no. 8, pp. 1682–1691, 2006.

[26] L. Dehmelt, G. Poplawski, E. Hwang, and S. Halpain, "NeuriteQuant: an open source toolkit for high content screens of neuronal Morphogenesis," *BMC Neuroscience*, vol. 12, article 100, 2011.

[27] R.-H. Xu, R. M. Peck, D. S. Li, X. Feng, T. Ludwig, and J. A. Thomson, "Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells," *Nature Methods*, vol. 2, no. 3, pp. 185–190, 2005.

[28] K. Watanabe, D. Kamiya, A. Nishiyama et al., "Directed differentiation of telencephalic precursors from embryonic stem cells," *Nature Neuroscience*, vol. 8, no. 3, pp. 288–296, 2005.

[29] T. A. Blauwkamp, S. Nigam, R. Ardehali, I. L. Weissman, and R. Nusse, "Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors," *Nature Communications*, vol. 3, article 1070, 2012.

[30] K. Kim, A. Doi, B. Wen et al., "Epigenetic memory in induced pluripotent stem cells," *Nature*, vol. 467, no. 7313, pp. 285–290, 2010.

[31] R. Lister, M. Pelizzola, Y. S. Kida et al., "Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells," *Nature*, vol. 471, no. 7336, pp. 68–73, 2011.

[32] J. M. Polo, S. Liu, M. E. Figueroa et al., "Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells," *Nature Biotechnology*, vol. 28, no. 8, pp. 848–855, 2010.

[33] M. N. Manuel, B. Martynoga, M. D. Molinek et al., "The transcription factor Foxg1 regulates telencephalic progenitor proliferation cell autonomously, in part by controlling Pax6 expression levels," *Neural Development*, vol. 6, article 9, 2011.

[34] N. Moya, J. Cutts, T. Gaasterland, K. Willert, and D. A. Brafman, "Endogenous WNT signaling regulates hPSC-derived neural progenitor cell heterogeneity and specifies their regional identity," *Stem Cell Reports*, vol. 3, no. 6, pp. 1015–1028, 2014.

[35] G. Lee, S. M. Chambers, M. J. Tomishima, and L. Studer, "Derivation of neural crest cells from human pluripotent stem cells," *Nature Protocols*, vol. 5, no. 4, pp. 688–701, 2010.

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Review Article

A Common Language: How Neuroimmunological Cross Talk Regulates Adult Hippocampal Neurogenesis

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Immune regulation of the brain is generally studied in the context of injury or disease. Less is known about how the immune system regulates the brain during normal brain function. Recent work has redefined the field of neuroimmunology and, as long as their recruitment and activation are well regulated, immune cells are now known to have protective properties within the central nervous system in maintaining brain health. Adult neurogenesis, the process of new neuron generation in the adult brain, is highly plastic and regulated by diverse extrinsic and intrinsic cues. Emerging research has shown that immune cells and their secreted factors can influence adult neurogenesis, both under baseline conditions and during conditions known to change neurogenesis levels, such as aging and learning in an enriched environment. This review will discuss how, under nonpathological conditions, the immune system can interact with the neural stem cells to regulate adult neurogenesis with particular focus on the hippocampus—a region crucial for learning and memory.

1. Introduction

Adult neurogenesis occurs predominantly in two specialized stem cell niches, the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) below the ependymal lining of the lateral ventricles. In these neurogenic niches precursor cells reside in close proximity to the microvasculature, indicating a close connection between the nervous system and the circulation. In the hippocampus, the multistep process of neurogenesis is regulated on different levels by intrinsic and extrinsic factors that have either a positive or negative influence, or even both. Physical exercise and environmental enrichment, mainly increasing precursor cell proliferation and survival of the newborn cells, respectively, represent two of the most prominent positive external neurogenic stimuli [1–3]. Aging and stress on the other hand lead to a reduction in hippocampal neurogenesis [4, 5]. All of the above examples are linked to changes in the physiological environment and recent data, especially in the field of aging, have revealed how changes in the circulatory system can influence the brain [6–8].

The brain is generally considered an immunologically privileged organ and it has long been assumed that cross talk between the immune and nervous systems is unlikely due to their physical separation by the blood brain barrier (BBB). It seemed that the brain functioned optimally when no immune cells were present. Work over the past decade, however, has made major advances in the field of neuroimmunology. The recent rediscovery of the central nervous system (CNS) lymphatic system that drains immune cells from the cerebral spinal fluid (CSF) to the lymph nodes has further highlighted this route of communication [9, 10]. The brain can now be considered to some extent to have immune properties, as it is able to respond to injury, degeneration, or infection, albeit in a different way than the peripheral immune system. We are only beginning to understand the importance of the immune system in maintaining brain homeostasis under nonpathological conditions and the exact mechanisms of how neuroimmunological communication maintains the tight regulatory balance required for proper brain function have yet to be fully elucidated. In this review we will explore the close association between the hippocampal stem cell niche

and the microvasculature. We will outline the multitude of both brain resident and peripheral immune cells and discuss the potential significance of their cross talk with the neural stem cells, an interaction that is facilitated by a plethora of secreted immune molecules. In the context of aging, we will describe how the circulatory system can influence the brain and raise the interesting question of whether blood cells could provide a source of new neurons.

2. The Hippocampal Microvasculature Is a Gateway between the Circulatory System and the Nervous System

The BBB is formed by tight junctions between endothelial cells and is ensheathed by the endfeet of perivascular astrocytes and pericytes. Due to their physical separation by the BBB, cross talk between the immune and nervous systems was generally considered unlikely. However, despite its name, the BBB is dynamic and allows, when required, peripheral immune cells to enter the brain under both physiological and pathological conditions [11, 12]. Circulating immune cells and blood-borne factors travelling through the bloodstream into the brain might be more involved in the regulation of neurogenesis than long thought. The finding that nestin-GFP-expressing hippocampal precursor cells directly contact the niche-resident endothelial cells via vascular endfeet [13, 14] supports this hypothesis.

The hippocampus is a highly vascularized area in the brain and proliferating adult hippocampal precursor cells reside in close proximity to blood vessels and dividing endothelial cells [15]. This suggests that the hippocampal stem cell niche, although located deeply inside the brain, receives extrinsic cues via circulating and endothelium-derived factors [15]. Factors secreted by endothelial cells affect proliferation, differentiation, and survival of neural stem and progenitor cells and include brain derived neurotrophic factor (BDNF) [14, 16, 17] and vascular endothelial growth factor (VEGF) [15, 18, 19]. VEGF stimulates precursor cell proliferation in the SGZ and has neurogenic and angiogenic properties, indicating a direct link between the blood system and the brain [15, 19]. Following exercise, a strong physiological stimulus of hippocampal neurogenesis [2, 3], raised levels of circulating VEGF mediate the physical activity-induced increase in new neuron production [20]. Interestingly, the blockade of peripheral VEGF prevents the run-induced increase in precursor proliferation but does not affect basal neurogenesis in nonrunning animals, suggesting that peripheral factors can affect neurogenesis by different, independent mechanisms [20].

Despite their importance in maintaining a functional niche environment, the vascular niches within the two major neurogenic regions differ from each other. Unlike the hippocampus, where neurogenesis and angiogenesis appear to be coupled, no dividing endothelial cells are observed in the SVZ [15, 21]. The vascular difference between the niches could be of importance for stem cell regulation by external stimuli, such as environmental enrichment and physical exercise, that affect neurogenesis in the hippocampus but not in the SVZ [22]. In this context, signals from the CSF that repress proliferation in the SVZ cannot be excluded, as SVZ stem cells contact both blood vessels and the CSF [23–26].

The proximity of the niche to the blood vessels allows the supply of nutrients or signals to maintain homeostasis under physiological conditions, and vascular changes occur during normal aging and in the context of neurodegenerative diseases [27, 28]. The aged vascular system in the brain is characterized by decreased blood flow and the functional decline of the BBB carrier system [27], indicating an altered supply of circulating factors from the blood stream, possibly leading to either the enhanced or decreased availability of signaling molecules.

3. Brain Resident Immune Cells

The hippocampal neurogenic niche is complex and many types of cells interact to maintain a functional niche environment (Figure 1). Apart from the neural stem and neuronal lineage cells, many niche-resident cells have immunological characteristics that, in the steady state, can regulate adult neurogenesis. The largest populations are the niche-resident microglia and astrocytes, which have immune properties and are important for local surveillance [29–31].

3.1. Microglia. Microglia, although considered to be a type of immune cell, are an integral part of the brain. These resident macrophages are uniformly arranged throughout the brain and constitute approximately 10–15% of total brain cells. Microglia originate from macrophages during hematopoiesis in the yolk sac and migrate to the neural tube where they form microglia. Because bone marrow monocytes do not contribute to the pool of mature microglia in a healthy brain, it is suggested that they are sustained by local self-renewing progenitor cells [32].

Microglia located in the neurogenic niches are interesting candidates for the regulation of adult neurogenesis, both in baseline and in injury states. Under baseline conditions microglia are relatively quiescent. These resting microglia have a ramified morphology with many processes, which they use to survey the surrounding area for damage or infection [33]. Under resting conditions microglia perform important functions during development including phagocytosis of debris resulting from cell apoptosis and promote neuronal apoptosis [34] and mice deficient in microglia have severe postnatal brain development defects [35]. Microglia also play a key role in the active remodeling of the presynaptic environment by engulfing presynaptic termini [36]. This synaptic pruning is dependent on the complement system [37], whereby C1q produced by neurons activates a cascade that ultimately activates the microglial expressed C3R and these microglia preferentially engulf inactive synapses [38]. Another signaling pathway is via the neural cell secreted cytokine CX3CL1 and its corresponding microglial based receptor CX3CR1. CX3CR1 deficient knockout mice have adult neurogenesis deficits [39], impaired learning and memory, and an inability to achieve long-term potentiation (LTP) in the hippocampus [40].

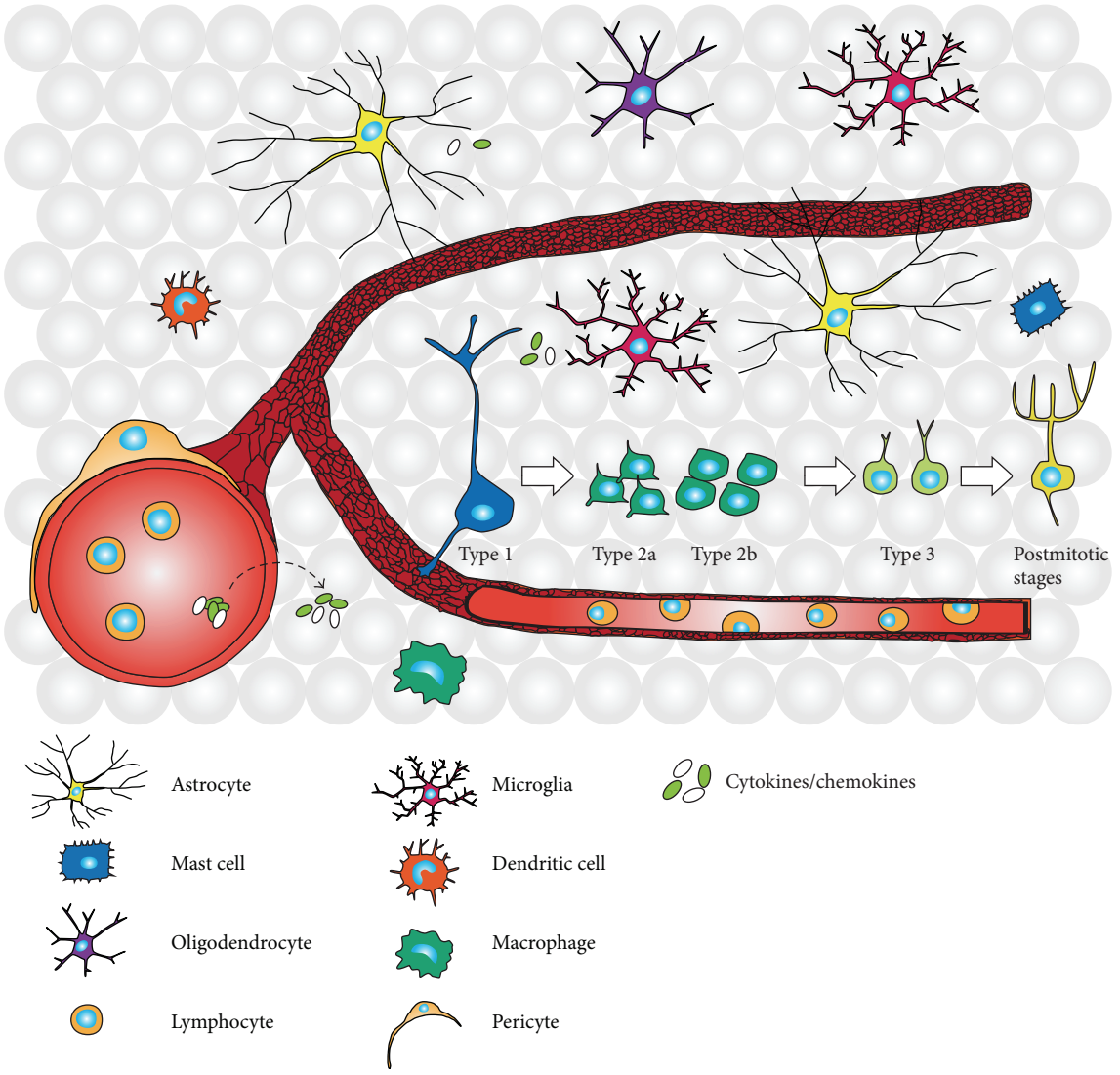

Figure 1: Neural progenitor cells and immune cells coexist in the hippocampal neurogenic niche. Type 1 neural stem cells in the subgranular zone of the hippocampal dentate gyrus mature through different developmental stages during the multistep process of new neuron formation. Type 1 radial glia-like cells give rise to transiently amplifying progenitor cells (Type 2a/Type 2b). After passing the neuronally committed Type 3 stage the cells become postmitotic after which they integrate as mature granule cells into the existing hippocampal circuitry. Within the complex niche environment peripheral and resident immune cells interact with niche cells to regulate the neurogenic process under physiological conditions. In addition, immune cells in the blood and the niche secrete immune molecules, including cytokines and chemokines, to facilitate neuroimmunological communication.

Following an insult or infection microglia respond to signals from the peripheral immune system to induce neuroinflammation. Following activation, microglia change their shape becoming amoeboid and phagocytose cell debris. They also release factors such as proteases, neurotrophic factors, cytokines, and reactive oxygen species (ROS). Microglia responses are heterogeneous and result in different activation phenotypes which are broadly classified into the classical M1 and the alternative M2 states [41]. Classical activation occurs when the microglia detect a foreign antigen and act as the first line of defense by transitioning from innate immunity into an adaptive immune response by recruiting peripheral immune cells. The most common method to experimentally induce classical microglial activation is via administration of the bacterial endotoxin lipopolysaccharide (LPS) [42]. Such work demonstrated that classically activated microglia impair adult hippocampal neurogenesis without affecting proliferation—an effect that could be blocked with the microglial inhibitor minocycline [42, 43]. Classically activated or proinflammatory M1 microglia were associated with reduced neurogenesis and this effect is believed to be due to their production of nitric oxide [44, 45] and ROS [46], whereas neuroprotective M2 microglia stimulate neurogenesis via the release of anti-inflammatory cytokines and growth factors. The neuroprotective M2 response occurs when switching from the classical inflammatory response to

a reduction of proinflammatory factors towards a production of neuroprotective factors involved in repair. This switch is induced by stimulation with anti-inflammatory cytokines such as IL-4 or IL-13 resulting in the downregulation of classical activation genes and the upregulation of repair genes [47]. Alternatively activated M2 microglia have an increased expression of a number of anti-inflammatory cytokines including interleukin-10 (IL-10), transforming growth factor beta (TGF-β), and growth factors such as insulin-like growth factor (IGF), nerve growth factor (NGF), and BDNF. It has been suggested that M2 microglia might be important for the maintenance of neurogenesis, as a number of the anti-inflammatory cytokines produced have been found to support adult neurogenesis [48].

Microglia are controlled by both intrinsic and extrinsic systems. They can be modulated by neuronal activity via neurotransmitters, as well as directly influencing neuronal activity and other regulatory molecules. They can also be regulated by astrocytes and release soluble factors in the adult brain [49]. Microglia can also upregulate major histocompatibility complex class II (MHC II), an important regulator of the cellular immune response that is responsible for the presentation of antigens to $CD4^+$ T cells [50].

Physical activity, a well-known stimulus of adult hippocampal neurogenesis, increases proliferation of microglia in both the cortex and hippocampus [51]. It has been supported by *in vitro* experiments that the exercise induced increase in hippocampal precursor proliferation is mediated via microglia and can be abolished when microglia are removed from the cultures. The potential mediator of this effect is signaling along the CX3CL1-CX3CR1 axis [52]. In addition, it was shown that microglia from exercising mice were able to activate the latent precursor cells from sedentary mice *in vitro* [52]. Conversely, microglia might also play a role in the age-related decline in neurogenesis [53]. Aging is associated with increased microglia activation, particularly towards a classic activation phenotype, with increased basal levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin-1 beta (IL-1β) [53].

3.2. Astrocytes. Astrocytes, the most abundant cell type in the brain, are located throughout the CNS. In the healthy CNS astrocytes have many important functions including providing metabolic support [54], water homeostasis [55], exchanging information between neurons and blood vessels to coordinate oxygen and glucose delivery, control of extracellular ion flux and antioxidant support by glutathione release [56], directing the development of synapses [57], modulating cerebral blood flow [58], the uptake and clearance of neurotransmitters such as GABA and glutamate [59], and helping to maintain the integrity of the BBB [60].

Astrocytes also play an important role in the brain's defense mechanism and they have phagocytic and antigen-presenting capacity [29–31]. Astrocytes can express major histocompatibility complex class I (MHC I) and MHC II antigens when stimulated by interferon gamma (IFN-γ) *in vitro*, which can lead to T cell activation [61]. Whether astrocytes express MHC II *in vivo* is controversial and may be limited to pathological conditions such as multiple sclerosis [29]. Astrocytes are also key players in the CNS as the targets and effectors of many cytokines and other inflammatory molecules. Reactive astrocytes, produced in immediate response to CNS trauma, form the glial scar and are thought to be beneficial by preventing contact between the damaged and healthy neurons. Following trauma or infection reactive astrocytes can produce a number of cytokines (e.g., CXCL10, CCL2, and IL-6) that mediate innate immune function such as recruitment of monocytes and microglia [61]. Astrocytes can also respond to a number of cytokines (TNF-α, EGF, FGF, and a number of interleukins) themselves and display a number of receptors involved in innate immunity including toll-like receptors (TLRs) [62, 63]. In contrast to the immediate activation seen following trauma, activation of astrocytes that occurs later inhibits regeneration and contributes to sustained inflammation [64]. Reactive astrocytes are key players in neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease. The strongest evidence for a role of astrocytes in CNS autoimmune disease is in neuromyelitis optica, an inflammatory disease that besides its occurrence in the context of multiple sclerosis can also be caused by specific antibodies to the water channel protein Aquaporin-4, which in the CNS is located exclusively on the surface of astrocytes [65]. It was also shown that in some multiple sclerosis patients there are autoantibodies against the potassium channel Kir4.1 that is also present on astrocytes [66].

Apart from the obvious role of a subset of astrocytes that function as the neural stem cells (NSC) under homeostatic conditions, astrocytes can also release soluble factors which influence distinct stages of adult neurogenesis including stem cell proliferation [67], neuronal differentiation [68], neuronal survival [69], and synaptic integration [70]. CNS injuries can also produce reactive astrocytes with NSC potential [71]. This is in contrast to those astrocytes activated during neurodegeneration that do not have stem cell potential [71]. Conversely, blocking reactive gliosis leads to improved integration of transplanted neural precursor cells (NPCs) in the mouse hippocampus [72].

3.3. Pericytes. Pericytes are smooth muscle-derived cells that are important in maintaining the BBB. Pericytes are often referred to as the macrophages of the brain, as due to their MHC II dependent antigen-presenting ability and their direct contact with the microvasculature they represent one of the first lines of defense [73]. In response to infection, pericytes mount an inflammatory response by increasing expression of proinflammatory cytokines, such as IL-6, TNF-α, and IL-1β [74, 75]. There are several reports that pericytes have neurogenic potential being able to differentiate into neurons *in vitro* and *in vivo* [76–78] and can be converted into neurons by direct reprogramming [79]. Recently, diffusible factors produced by pericytes were shown to increase NSC proliferation and neurogenesis *in vitro* [80].

3.4. Perivascular Macrophages. Perivascular macrophages are myeloid cells that are continuously replenished from the bone marrow progenitor cells and are located within the

perivascular space. They wrap their processes around the vasculature and help the BBB formation [81]. The major roles of perivascular macrophages include phagocytosis of cellular and pathogenic debris, brain surveillance of the interstitial space via pinocytosis, and initiation of the CNS acute phase response via the production of prostaglandins [82]. Interestingly it was shown that the percentage of $TLR4^+/CD14^+$ brain macrophages increased when mice were housed in an enriched environment potentially allowing leukocytes in the microvessels to release various neuroprotective and anti-inflammatory factors [83]. Perivascular macrophages also play a role in neurodegeneration [84], although whether they are involved in the regulation of adult neurogenesis under nonpathological conditions remains unclear.

4. Peripheral Immune Cells Influence Adult Neurogenesis

In addition to the brain resident immune cells, peripheral immune cells including T cells, B cells, natural killer cells, macrophages, mast cells, and dendritic cells can enter the CNS under normal conditions.

4.1. T Cells. Peripheral T cells can selectively enter the brain through the choroid plexus, a barrier that is composed of epithelial tight junctions and fenestrated epithelial cells, when required [85, 86]; however under physiological conditions they are present in very low numbers [87]. T cells can hone to sites of damage in the brain, become activated, and activate resident microglia, in a way that is different to the classical inflammation activation of microglia, to make them supportive of neuronal survival and proliferation. Interestingly, the $CD4^+$ T cells within the choroid plexus are distinct from those in the circulating blood as they express T cell receptors specific for CNS antigens, and they are of effector-memory type in contrast to those of the CSF which are central-memory T cells [88].

The first evidence of a role of T cells in adult neurogenesis was from Butovsky and colleagues in 2006 [89] who demonstrated that microglia activated by T helper (Th) cells produced the cytokines interleukin-4 (IL-4) and IFN-γ that promoted neurogenesis *in vitro*. Th1 and Th2 cells are generally thought to be detrimental to neurogenesis via the release of IFN-γ, although they can also be neuroprotective via the action of their main anti-inflammatory cytokine, IL-4 [90].

This evidence was closely followed by an *in vivo* corroboration of this link by Ziv and colleagues (2006) [91]. They showed that $CD4^+$ T cells promote and maintain neurogenesis by activating microglia via the release of soluble cytokines and regulating IGF-1 transport into the brain, thus regulating BDNF levels [91]. They also demonstrated that adult neurogenesis was impaired in immune-deficient severe combined immunodeficiency (SCID) and nude mice but could be restored by injection of T cells recognizing an antigen specific for the central nervous system [91]. Interestingly, $CD4^+$ T cells have been identified as the key players in this process. A study from our lab found that repopulation with $CD4^+$ but not $CD8^+$ T cells was able to restore the decrease in adult hippocampal neurogenesis observed in immune-deficient mice [92], thereby highlighting $CD4^+$ T cells as the proneurogenic T cell population under physiological conditions. An increase in activated $CD4^+$ T cells could be observed in the meninges of mice after training in the Morris water maze [93] indicating an impact of brain activity (learning) on the immune system. In these experiments, $CD4^+$ T cells displayed an activated phenotype and produced increased levels of IL-4. In addition, SCID mice that are devoid of mature T cells have dramatic impairments in hippocampal-dependent spatial learning and memory tasks including the water maze [94], the Barnes maze [95], the radial arm water maze [95], and the novel object recognition test [96]. Whether T cells also play a role in regulating the proneurogenic response to physical activity, however, remains unknown.

4.2. Regulatory T Cells. Regulatory T cells (Tregs) are a subpopulation of T cells, which normally work to suppress autoimmune responses and thus are critically involved in maintaining immune homeostasis. Interestingly, they can also produce neurotrophic factors and activate resident microglia [97]. The extent of this regulatory function is genetically determined and can differ between strains and individuals [98, 99]. $Foxp3^+$ Tregs are involved in suppressing the immune response in conditions including Alzheimer's disease, Parkinson's disease, traumatic brain injury, and stroke [100]. Tregs in the brain have mainly been studied following stroke, and their role in neurogenesis under physiological conditions is still unclear. It has been demonstrated that Tregs are present in the normal rat brain in the cortex, subcortical regions, hippocampus, and choroid plexus [97]. Recently it was shown that activated Tregs injected into the lateral ventricle of mice increased NPC proliferation, but nonactivated Tregs had no effect [101]. Activated Tregs stimulated NSC proliferation in the SVZ after middle cerebral artery occlusion but did not improve stroke outcome, as defined by infarct volume. In addition, activated Tregs enhanced the proliferation of passaged neurospheres *in vitro*, an effect that could be blocked with IL-10-specific neutralizing antibodies [101].

4.3. B Cells. B cells, the other major class of lymphocytes, can also become activated and enter the healthy human brain, albeit in very low numbers [102]. B cells however can be found in larger numbers in the brains of patients with multiple sclerosis where they play a role in the pathogenesis of the relapsing inflammatory subtype [103]. One study claimed that B cells are not required for normal hippocampal NSC proliferation [104] but the function of B cells in the healthy brain is still unknown. Given the fact that there is growing evidence of B lymphocyte functions beyond combating infection, for example, including roles in autoimmunity, this question will have to be reconsidered in the future.

4.4. Natural Killer Cells. Natural killer (NK) cells are part of the innate immune system and have heterogeneous

phenotypes and functions. Despite their primary function to kill aberrant cells, NK cells also exert immunoregulatory functions and orchestrate adaptive immune responses through the interaction with antigen-presenting cells, T cells, and B cells [105]. Although they comprise about 10% of lymphoid cells in the healthy brain [106], the role of brain resident NK cells in CNS homeostasis is not clear. Mainly studied in pathology such as in the context of multiple sclerosis, brain NK cells have neuroprotective [107] as well as neurotoxic [108] effects. During chronic brain inflammation, NK cells reside in close proximity to NSCs in the SVZ and the interaction between NK cells and NSCs results in reduced proliferation and decreased numbers of $GFAP^+BrdU^+$ cells [109]. Considering their regulatory function, the extent to which NK cells interact with NSCs during normal brain function or in response to proneurogenic stimuli such as physical exercise or environmental enrichment remains unknown.

4.5. Dendritic Cells. Dendritic cells are the antigen-presenting cells of the immune system and their role is to present antigens to T lymphocytes to induce either tolerance or adaptive immune responses. Until recently, the brain was believed to be devoid of dendritic cells under steady-state conditions. However, dendritic cells have now been shown to infiltrate into the brain via the choroid plexus, nasal epithelium, meninges, and blood in response to autoimmunity, injury, aging, and infection [110, 111]. Despite much progress, the complete picture of dendritic cell participation in the brain's immune resonance is far from being completely understood. In addition, the presence of dendritic cells in the steady-state brain of humans is unclear and warrants further investigation.

4.6. Mast Cells. Mast cells, most well known for their role in allergy and anaphylaxis, are involved in the histamine response. Mast cells are generated in the bone marrow and under normal conditions can migrate to various areas of the brain including the hippocampus. Mast cell-deficient mice have hippocampal learning deficits and deficits in hippocampal, but not SVZ, neurogenesis [112]. Mast cells are a source of serotonin and treatment of the mast cell-deficient mice with fluoxetine, a selective serotonin reuptake inhibitor, can reverse the deficit in adult neurogenesis [112]. Interestingly, mast cells can be activated by physical activity [113]. Whether these cells play a role in the increase in adult hippocampal neurogenesis in response to physical activity, however, is still unknown.

5. Immune Molecules

The various immune cells described above secrete a plethora of immune molecules, including pro- and anti-inflammatory cytokines that play an important role in brain function, both in maintaining the physiological state and in disease conditions. This regulation is tightly controlled and the outcome depends on their concentration, the particular type of cell that is activated, and other factors that are also secreted by other populations of cells in the brain.

5.1. Cytokines. Immune and neural cells share a common language for communication—cytokines. Cytokines, the chemical messengers of the immune system, although typically thought of in this context, are also important for normal brain function. In the brain, cytokines are mainly produced by activated microglia as part of the innate immune response. Increased cytokine production in the brain is generally regarded as detrimental, as it is usually associated with conditions of inflammation, infection, and neurodegeneration. The role of cytokines in normal brain function, however, is less well studied [114].

Immune cells produce a multitude of different cytokines. We will briefly outline the best studied of these, IL-6, TNF-α, IL-1β, and IFN-γ, with the major focus on their role in hippocampal neural stem cell regulation. IL-6 can act as both a pro- and anti-inflammatory cytokine and therefore its specific role in hippocampal learning and memory depends on the context. Adult hippocampal neurogenesis is dramatically reduced in the presence of IL-6 and inhibition of IL-6 can restore this deficit [42]. In addition, IL-6 is involved in regulation of synaptic plasticity, LTP, and memory [115] and there is an age dependent increase in IL-6 that is accompanied by a concomitant memory loss. Tumor necrosis factors are a family of cytokines known to cause apoptosis. TNF-α, although once considered to be purely inflammatory, can also be produced in the brain under basal conditions. Signaling by TNF-α through the TNFR1 regulates synaptic strength via modulation of AMPA receptor expression [116]. TNF-α signaling can differentially affect adult hippocampal neurogenesis, with signaling through the TNFR1 a negative and TNFR2 a positive regulator [117]. TNF-α has an antineurogenic effect on NSCs and is upregulated in a number of neurodegenerative diseases [118]. Another example of a cytokine important for normal brain function is IL-1β, which is upregulated during LTP induction and maintenance [119]. IL1R1 is expressed by NSCs of the dentate gyrus but not the SVZ [120] and IL-1β decreases hippocampal NSC proliferation, via activation of NFkB signaling [121] and elevated levels of IL-1β are observed in the brains of patients suffering neurodegenerative diseases [122]. IL-1β administration impairs spatial learning and memory in the water maze [123], spatial active avoidance test [124], radial arm maze [125], and impaired hippocampus-dependent contextual fear conditioning [126]. IFN-γ is a proinflammatory cytokine that is able to exert both positive and negative effects on adult neurogenesis depending on the neurogenic niche. IFN-γ decreases NSC proliferation in the adult SVZ both *in vitro* and *in vivo* [127] but leads to an increase in neurogenesis in the dentate gyrus of adult mice and ameliorates spatial learning and memory performance [128].

5.2. Chemokines. Chemokines are a family of small, secreted cytokines that guide the migration of cells along a concentration gradient. Stromal cell-derived factor 1-alpha (SDF-1α; CXCL12) is released from activated astrocytes and signals NSCs to migrate to the site of neuronal damage [129, 130]. It also promotes NSC proliferation [129, 130] and survival [131] via its receptors CXCR4 and CXCR7, which are highly expressed on NSCs [132, 133]. Using CXCR4 depletion mice, it

was shown that CXCR4 signaling, in addition to maintaining the NSC pool, also specified the inner third of the granule cell layer as the site of immature neuron differentiation [134]. Another chemokine that is upregulated in the brain following inflammation is monocyte chemotactic protein-1 (MCP-1; CCL2). TNF-α increases the expression of MCP-1, which induces NSC migration mediated via its receptor CCR2 that is highly expressed on NSCs [135–137].

5.3. Neurotransmitters. In addition to cytokine regulation, immune cells, most predominantly T cells, can produce and respond to common neurotransmitters such as acetylcholine, glutamate, dopamine, and serotonin [138–140]. These neurotransmitters are important for modulating learning, memory, and LTP, and they affect not only neurons but also the production and secretion of inflammatory factors from astrocytes and microglia [141]. Neurotransmitter cross talk can be bidirectional, with the CNS affecting the immune system and vice versa. Some antidepressants (including fluoxetine) act as immune modulators by shifting the T cells from a proinflammatory to an anti-inflammatory profile [139]. Microglia express functional dopamine receptors (D_1 and D_2) and their activation can decrease nitric oxide production after LPS stimulation [142, 143]. Systemically produced dopamine can transiently alleviate the suppression of autoimmune activity of Tregs allowing a neuroprotective response after injury [138]. Noradrenalin is important in maintaining brain homeostasis and noradrenalin depletion can contribute to the neuroinflammatory processes that lead to neurodegenerative diseases [144]. Noradrenalin can also change the cytokine production profile of T cells and activate hematopoietic stem cells to produce immune cells.

5.4. Major Histocompatibility Complex Class I. The MHC I functions to display fragments of non-self-proteins to cytotoxic T cells and thus is crucial for the initiation and regulation of adaptive immune responses. Until Corriveau et al. demonstrated neuronal expression in 1998 it was believed that neurons were one of the few cell types devoid of MHC I expression [145]. In the brain, it has been suggested that MHC I molecules have distinct neuronal functions that differ from their role in cellular immunity, such as the regulation of synaptic function and brain development [146, 147]. MHC I is expressed at the synapses [146] and neurons in MHC I KO mice have enhanced synaptic plasticity, increased excitability and higher frequency of miniature excitatory postsynaptic currents (mEPSPs), increased LTP, and decreased LTD [146, 148]. Furthermore, the ablation of MHC I resulted in improved behavioral recovery after stroke, indicating an enhancement of synaptic plasticity in the absence of MHC I [149]. A recent study suggests that MHC I might at least in part be involved in mediating the age-related decline in neurogenesis and cognitive function observed after exposure to an aged systemic environment, as no effects of aged blood were observed in young heterochronic parabionts with reduced MHC I cell surface expression [6, 7, 150]. The specific role of more than 50 MHC I molecules [147] and the dual role of MHC I in immune and neurogenic regulation remain unknown.

5.5. Toll-Like Receptors. Toll-like receptors (TLRs) are receptors expressed by sentinel cells at the body's first line of defense that recognize conserved molecules shared by pathogens. TLRs are also associated with the differentiation of stem cells, including hematopoietic and mesenchymal stem cells [151, 152]. Many members of the TLR family are expressed in the brain; however studies mainly focus on their role in pathological brain processes and their specific role in normal brain function is still not completely clear [153]. NSCs express TLR2 and TLR4 and in mice these receptors are involved in NSC proliferation and differentiation [154]. In rats, after TLR activation, NSCs expressing TLR2 and TLR4 produce proinflammatory cytokines, including TNF-α; however proliferation and differentiation are not affected [155].

6. Blood Modulates Brain Function

As we have shown above, the microvasculature brings circulating immune cells in close proximity to the hippocampal stem cell niche. These peripheral immune cells together with populations of brain resident immune cells secrete immune molecules and provide a common language that enables cross talk between the immune system and neurogenic niche. Aging represents one clear example of a situation in which the dramatic effect of neuroimmunological cross talk becomes apparent.

Adult hippocampal neurogenesis decreases with age, as shown by a strong decrease in proliferation, differentiation, and survival of newly born neurons, especially during the first few months of life in a rodent (or years in humans) [5, 156, 157]. Furthermore the capability to adapt to new experiences is dramatically decreased with age [158], although this statement has been questioned recently. Irrespective of this, the age-related impairment in adult neurogenesis and brain plasticity was linked to an altered systemic environment, with a parabiotic connection between the blood systems of aged and young mice reversing the aging effect in old animals [7, 8]. The systemic environment from young mice enhanced synaptic plasticity and cognition, as well as neurogenesis in the old parabionts, as determined by an increased number of proliferating BrdU-positive cells, Sox2-positive progenitor cells, and doublecortin-positive immature neurons [7]. Moreover, intravenous injections of plasma isolated from old mice into young animals showed the opposite effect, indicating that blood-borne factors mediate the decrease in adult neurogenesis during aging [7]. In a follow-up study from Villeda and colleagues, the administration of young plasma to aged mice rescued the age-related cognitive deficit with these mice displaying improvements in hippocampal-dependent learning and memory water maze tasks and contextual fear conditioning compared to aged mice receiving aged plasma [8]. Interestingly, they identified an increase in circulating immune-related factors, including the eotaxin chemokine C-C motif ligand 11 (CCL11), in the blood of aged mice and showed that this was associated with impaired cognitive function and decreased neurogenesis [7]. Of note, circulating CCL11 crosses the BBB via influx but also efflux transporters for CCL11, indicating an important role of the

BBB in controlling CCL11 levels in the brain [159]. The altered carrier capacity of the BBB in aged individuals [27] indicates a possible link between the raised systemic levels of CCL11 and the correlating cognitive impairment in old mice [7]. CCL11 is one interesting example to highlight the connection between the aging brain and the immune system; however, it is clear that systemic changes are more complex and not limited to the effect of a single factor.

Further studies confirm the relationship between systemic changes that occur during aging and adult neurogenesis. As observed in the hippocampus, Katsimpardi et al. reported the effects of young systemic factors and their capacity to enhance neurogenesis in old heterochronic parabionts in the SVZ [6]. These studies highlight the crucial role of the systemic environment and the route via the blood stream in regulating adult neurogenesis. The precise mechanisms of how the immune system, following physiological stimuli including learning and physical activity, contributes to orchestrating changes in neurogenesis still remain largely unknown.

7. From Blood to Brain: Lessons from the Crayfish

Evidence is emerging that the nervous and circulatory systems are more tightly linked than thought. Although neural stem cell research has focused on mammals, primarily rodents and humans, stem cell niches are also found in the brains of crustaceans [160]. Within this niche a pool of dividing cells differentiate into mature olfactory interneurons. Surprisingly, these cells are not self-renewing stem cells but instead more restricted progenitors that travel from a distant neurogenic niche on the ventral surface of the brain. It was recently shown that blood cells are the most likely source of the neural progenitor cells [161]. Another example where neural stem cells are externally supplied is in the primitive invertebrates, flatworms [162]. This suggests that blood derived neural stem cells are an evolutionary ancient phenomenon but one that could provide an excellent model system to study the interaction between the peripheral immune system and neural stem cells.

8. Concluding Remarks

Neuroimmunological cross talk has been predominantly studied in disease. However, there is mounting evidence that immune cells are involved not only in neuroinflammation in response to injury or disease, but also in the maintenance of brain homeostasis under nonpathological conditions. Although we are making great headway in this direction, there are still many fundamental questions that remain to be answered. For example, what is the role of infiltrating peripheral immune cells, including B cells, natural killer cells, and dendritic cells in normal brain function? What are the exact mechanism and location of T cell entry into the brain? Once inside the niche, how do T cells interact with resident immune cells including astrocytes and microglia, and what are the exact consequences of these actions under physiological conditions? Are the same mechanisms utilized for immune cell activation in steady state and disease conditions—is it just a matter of balance? Can the quiescent neural stem cells be regulated/activated by immune signals? Are immune cells involved in regulating the increase in adult hippocampal neurogenesis following stimuli such as physical exercise and learning? By addressing these questions, a more complete picture of the role that the immune system plays in normal brain function can be obtained. In particular, it seems likely that some yet unexplained aspects of adult neurogenesis may in fact be under immune regulation. We have shown above that the fields of neuroscience and immunology do indeed share a common language, and a synthesis of the two will be a fruitful area of future research.

Competing Interests

The authors declare no competing financial interests.

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References

[1] G. Kempermann, H. G. Kuhn, and F. H. Gage, "More hippocampal neurons in adult mice living in an enriched environment," *Nature*, vol. 386, no. 6624, pp. 493–495, 1997.

[2] H. van Praag, B. R. Christie, T. J. Sejnowski, and F. H. Gage, "Running enhances neurogenesis, learning, and long-term potentiation in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13427–13431, 1999.

[3] H. van Praag, G. Kempermann, and F. H. Gage, "Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus," *Nature Neuroscience*, vol. 2, no. 3, pp. 266–270, 1999.

[4] E. Gould, H. A. Cameron, D. C. Daniels, C. S. Woolley, and B. S. McEwen, "Adrenal hormones suppress cell division in the adult rat dentate gyrus," *Journal of Neuroscience*, vol. 12, no. 9, pp. 3642–3650, 1992.

[5] H. G. Kuhn, H. Dickinson-Anson, and F. H. Gage, "Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation," *The Journal of Neuroscience*, vol. 16, no. 6, pp. 2027–2033, 1996.

[6] L. Katsimpardi, N. K. Litterman, P. A. Schein et al., "Vascular and neurogenic rejuvenation of the aging mouse brain by young systemic factors," *Science*, vol. 344, no. 6184, pp. 630–634, 2014.

[7] S. A. Villeda, J. Luo, K. I. Mosher et al., "The ageing systemic milieu negatively regulates neurogenesis and cognitive function," *Nature*, vol. 477, no. 7362, pp. 90–96, 2011.

[8] S. A. Villeda, K. E. Plambeck, J. Middeldorp et al., "Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice," *Nature Medicine*, vol. 20, no. 6, pp. 659–663, 2014.

[9] A. Aspelund, S. Antila, S. T. Proulx et al., "A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules," *The Journal of Experimental Medicine*, vol. 212, no. 7, pp. 991–999, 2015.

[10] A. Louveau, I. Smirnov, T. J. Keyes et al., "Structural and functional features of central nervous system lymphatic vessels," *Nature*, vol. 523, no. 7560, pp. 337–341, 2015.

[11] N. J. Abbott, A. A. K. Patabendige, D. E. M. Dolman, S. R. Yusof, and D. J. Begley, "Structure and function of the blood-brain barrier," *Neurobiology of Disease*, vol. 37, no. 1, pp. 13–25, 2010.

[12] N. J. Abbott, L. Rönnbäck, and E. Hansson, "Astrocyte-endothelial interactions at the blood-brain barrier," *Nature Reviews Neuroscience*, vol. 7, no. 1, pp. 41–53, 2006.

[13] V. Filippov, G. Kronenberg, T. Pivneva et al., "Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes," *Molecular and Cellular Neuroscience*, vol. 23, no. 3, pp. 373–382, 2003.

[14] F. Ehret, S. Vogler, and G. Kempermann, "A co-culture model of the hippocampal neurogenic niche reveals differential effects of astrocytes, endothelial cells and pericytes on proliferation and differentiation of adult murine precursor cells," *Stem Cell Research*, vol. 15, no. 3, pp. 514–521, 2015.

[15] T. D. Palmer, A. R. Willhoite, and F. H. Gage, "Vascular niche for adult hippocampal neurogenesis," *Journal of Comparative Neurology*, vol. 425, no. 4, pp. 479–494, 2000.

[16] C. Leventhal, S. Rafii, D. Rafii, A. Shahar, and S. A. Goldman, "Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma," *Molecular and Cellular Neurosciences*, vol. 13, no. 6, pp. 450–464, 1999.

[17] Q. Shen, S. K. Goderie, L. Jin et al., "Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells," *Science*, vol. 304, no. 5675, pp. 1338–1340, 2004.

[18] S. A. Goldman and Z. Chen, "Perivascular instruction of cell genesis and fate in the adult brain," *Nature Neuroscience*, vol. 14, no. 11, pp. 1382–1389, 2011.

[19] K. Jin, Y. Zhu, Y. Sun, X. O. Mao, L. Xie, and D. A. Greenberg, "Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 18, pp. 11946–11950, 2002.

[20] K. Fabel, K. Fabel, B. Tam et al., "VEGF is necessary for exercise-induced adult hippocampal neurogenesis," *European Journal of Neuroscience*, vol. 18, no. 10, pp. 2803–2812, 2003.

[21] M. Tavazoie, L. Van der Veken, V. Silva-Vargas et al., "A specialized vascular niche for adult neural stem cells," *Cell Stem Cell*, vol. 3, no. 3, pp. 279–288, 2008.

[22] J. Brown, C. M. Cooper-Kuhn, G. Kempermann et al., "Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis," *European Journal of Neuroscience*, vol. 17, no. 10, pp. 2042–2046, 2003.

[23] F. Doetsch, I. Caille, D. A. Lim, J. M. Garcia-Verdugo, and A. Alvarez-Buylla, "Subventricular zone astrocytes are neural stem cells in the adult mammalian brain," *Cell*, vol. 97, no. 6, pp. 703–716, 1999.

[24] J. M. García-Verdugo, F. Doetsch, H. Wichterle, D. A. Lim, and A. Alvarez-Buylla, "Architecture and cell types of the adult subventricular zone: in search of the stem cells," *Journal of Neurobiology*, vol. 36, no. 2, pp. 234–248, 1998.

[25] Z. Mirzadeh, F. T. Merkle, M. Soriano-Navarro, J. M. Garcia-Verdugo, and A. Alvarez-Buylla, "Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain," *Cell Stem Cell*, vol. 3, no. 3, pp. 265–278, 2008.

[26] Q. Shen, Y. Wang, E. Kokovay et al., "Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions," *Cell Stem Cell*, vol. 3, no. 3, pp. 289–300, 2008.

[27] E. Farkas and P. G. M. Luiten, "Cerebral microvascular pathology in aging and Alzheimer's disease," *Progress in Neurobiology*, vol. 64, no. 6, pp. 575–611, 2001.

[28] J. S. Goldberg and K. K. Hirschi, "Diverse roles of the vasculature within the neural stem cell niche," *Regenerative Medicine*, vol. 4, no. 6, pp. 879–897, 2009.

[29] F. Aloisi, "Immune function of microglia," *Glia*, vol. 36, no. 2, pp. 165–179, 2001.

[30] C. Lööv, L. Hillered, T. Ebendal, and A. Erlandsson, "Engulfing astrocytes protect neurons from contact-induced apoptosis following injury," *PLoS ONE*, vol. 7, no. 3, Article ID e33090, 2012.

[31] J. D. Sokolowski and J. W. Mandell, "Phagocytic clearance in neurodegeneration," *The American Journal of Pathology*, vol. 178, no. 4, pp. 1416–1428, 2011.

[32] B. Ajami, J. L. Bennett, C. Krieger, W. Tetzlaff, and F. M. V. Rossi, "Local self-renewal can sustain CNS microglia maintenance and function throughout adult life," *Nature Neuroscience*, vol. 10, no. 12, pp. 1538–1543, 2007.

[33] K. Helmut, U.-K. Hanisch, M. Noda, and A. Verkhratsky, "Physiology of microglia," *Physiological Reviews*, vol. 91, no. 2, pp. 461–553, 2011.

[34] A. Sierra, J. M. Encinas, J. J. P. Deudero et al., "Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis," *Cell Stem Cell*, vol. 7, no. 4, pp. 483–495, 2010.

[35] B. Erblich, L. Zhu, A. M. Etgen, K. Dobrenis, and J. W. Pollard, "Absence of colony stimulation factor-1 receptor results in loss of microglia, disrupted brain development and olfactory deficits," *PLoS ONE*, vol. 6, no. 10, Article ID e26317, 2011.

[36] B. Stevens, N. J. Allen, L. E. Vazquez et al., "The classical complement cascade mediates CNS Synapse elimination," *Cell*, vol. 131, no. 6, pp. 1164–1178, 2007.

[37] Q. Shi, K. J. Colodner, S. B. Matousek et al., "Complement C3-deficient mice fail to display age-related hippocampal decline," *Journal of Neuroscience*, vol. 35, no. 38, pp. 13029–13042, 2015.

[38] D. P. Schafer, E. K. Lehrman, A. G. Kautzman et al., "Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner," *Neuron*, vol. 74, no. 4, pp. 691–705, 2012.

[39] R. C. Paolicelli, G. Bolasco, F. Pagani et al., "Synaptic pruning by microglia is necessary for normal brain development," *Science*, vol. 333, no. 6048, pp. 1456–1458, 2011.

[40] J. T. Rogers, J. M. Morganti, A. D. Bachstetter et al., "CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity," *The Journal of Neuroscience*, vol. 31, no. 45, pp. 16241–16250, 2011.

[41] S. Gordon, "Alternative activation of macrophages," *Nature Reviews Immunology*, vol. 3, no. 1, pp. 23–35, 2003.

[42] M. L. Monje, H. Toda, and T. D. Palmer, "Inflammatory blockade restores adult hippocampal neurogenesis," *Science*, vol. 302, no. 5651, pp. 1760–1765, 2003.

[43] C. T. Ekdahl, J.-H. Claasen, S. Bonde, Z. Kokaia, and O. Lindvall, "Inflammation is detrimental for neurogenesis in adult brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 23, pp. 13632–13637, 2003.

[44] C. C. Chao, S. Hu, T. W. Molitor, E. G. Shaskan, and P. K. Peterson, "Activated microglia mediate neuronal cell injury via a nitric oxide mechanism," *The Journal of Immunology*, vol. 149, no. 8, pp. 2736–2741, 1992.

[45] K. M. Boje and P. K. Arora, "Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death," *Brain Research*, vol. 587, no. 2, pp. 250–256, 1992.

[46] M. Tanaka, A. Sotomatsu, T. Yoshida, S. Hirai, and A. Nishida, "Detection of superoxide production by activated microglia using a sensitive and specific chemiluminescence assay and microglia-mediated PC12h cell death," *Journal of Neurochemistry*, vol. 63, no. 1, pp. 266–270, 1994.

[47] C. A. Colton, R. T. Mott, H. Sharpe, Q. Xu, W. E. Van Nostrand, and M. P. Vitek, "Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD," *Journal of Neuroinflammation*, vol. 3, article 27, 2006.

[48] R. A. Kohman, T. K. Bhattacharya, C. Kilby, P. Bucko, and J. S. Rhodes, "Effects of minocycline on spatial learning, hippocampal neurogenesis and microglia in aged and adult mice," *Behavioural Brain Research*, vol. 242, no. 1, pp. 17–24, 2013.

[49] M.-È. Tremblay, B. Stevens, A. Sierra, H. Wake, A. Bessis, and A. Nimmerjahn, "The role of microglia in the healthy brain," *Journal of Neuroscience*, vol. 31, no. 45, pp. 16064–16069, 2011.

[50] A. S. Harms, S. Cao, A. L. Rowse et al., "MHCII is required for α-synuclein-induced activation of microglia, CD4 T cell proliferation, and dopaminergic neurodegeneration," *The Journal of Neuroscience*, vol. 33, no. 23, pp. 9592–9600, 2013.

[51] D. Ehninger and G. Kempermann, "Regional effects of wheel running and environmental enrichment on cell genesis and microglia proliferation in the adult murine neocortex," *Cerebral Cortex*, vol. 13, no. 8, pp. 845–851, 2003.

[52] J. Vukovic, M. J. Colditz, D. G. Blackmore, M. J. Ruitenberg, and P. F. Bartlett, "Microglia modulate hippocampal neural precursor activity in response to exercise and aging," *Journal of Neuroscience*, vol. 32, no. 19, pp. 6435–6443, 2012.

[53] A. Sierra, A. C. Gottfried-Blackmore, B. S. Mcewen, and K. Bulloch, "Microglia derived from aging mice exhibit an altered inflammatory profile," *Glia*, vol. 55, no. 4, pp. 412–424, 2007.

[54] I. Allaman, M. Bélanger, and P. J. Magistretti, "Astrocyte-neuron metabolic relationships: for better and for worse," *Trends in Neurosciences*, vol. 34, no. 2, pp. 76–87, 2011.

[55] M. Simard and M. Nedergaard, "The neurobiology of glia in the context of water and ion homeostasis," *Neuroscience*, vol. 129, no. 4, pp. 877–896, 2004.

[56] J. E. Rash, "Molecular disruptions of the panglial syncytium block potassium siphoning and axonal saltatory conduction: pertinence to neuromyelitis optica and other demyelinating diseases of the central nervous system," *Neuroscience*, vol. 168, no. 4, pp. 982–1008, 2010.

[57] W.-S. Chung, L. E. Clarke, G. X. Wang et al., "Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways," *Nature*, vol. 504, no. 7480, pp. 394–400, 2013.

[58] T. Takano, G.-F. Tian, W. Peng et al., "Astrocyte-mediated control of cerebral blood flow," *Nature Neuroscience*, vol. 9, no. 2, pp. 260–267, 2006.

[59] A. Schousboe, A. Sarup, L. K. Bak, H. S. Waagepetersen, and O. M. Larsson, "Role of astrocytic transport processes in glutamatergic and GABAergic neurotransmission," *Neurochemistry International*, vol. 45, no. 4, pp. 521–527, 2004.

[60] N. J. Abbott, "Astrocyte-endothelial interactions and blood-brain barrier permeability," *Journal of Anatomy*, vol. 200, no. 6, pp. 629–638, 2002.

[61] Y. Dong and E. N. Benveniste, "Immune function of astrocytes," *Glia*, vol. 36, no. 2, pp. 180–190, 2001.

[62] T. Kielian, "Overview of toll-like receptors in the CNS," *Current Topics in Microbiology and Immunology*, vol. 336, no. 1, pp. 1–14, 2009.

[63] K. A. Kigerl, J. P. de Rivero Vaccari, W. D. Dietrich, P. G. Popovich, and R. W. Keane, "Pattern recognition receptors and central nervous system repair," *Experimental Neurology*, vol. 258, pp. 5–16, 2014.

[64] M. Pekny and M. Nilsson, "Astrocyte activation and reactive gliosis," *Glia*, vol. 50, no. 4, pp. 427–434, 2005.

[65] V. A. Lennon, T. J. Kryzer, S. J. Pittock, A. S. Verkman, and S. R. Hinson, "IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel," *Journal of Experimental Medicine*, vol. 202, no. 4, pp. 473–477, 2005.

[66] R. Srivastava, M. Aslam, S. R. Kalluri et al., "Potassium channel KIR4.1 as an immune target in multiple sclerosis," *The New England Journal of Medicine*, vol. 367, no. 2, pp. 115–123, 2012.

[67] X. Cao, L.-P. Li, X.-H. Qin et al., "Astrocytic adenosine 5′-triphosphate release regulates the proliferation of neural stem cells in the adult hippocampus," *Stem Cells*, vol. 31, no. 8, pp. 1633–1643, 2013.

[68] H. Song, C. F. Stevens, and F. H. Gage, "Astroglia induce neurogenesis from adult neural stem cells," *Nature*, vol. 417, no. 6884, pp. 39–44, 2002.

[69] J.-C. Platel, K. A. Dave, V. Gordon, B. Lacar, M. E. Rubio, and A. Bordey, "NMDA receptors activated by subventricular zone astrocytic glutamate are critical for neuroblast survival prior to entering a synaptic network," *Neuron*, vol. 65, no. 6, pp. 859–872, 2010.

[70] S. Sultan, L. Li, J. Moss et al., "Synaptic integration of adult-born hippocampal neurons is locally controlled by astrocytes," *Neuron*, vol. 88, no. 5, pp. 957–972, 2015.

[71] S. Sirko, G. Behrendt, P. A. Johansson et al., "Reactive glia in the injured brain acquire stem cell properties in response to sonic hedgehog," *Cell Stem Cell*, vol. 12, no. 4, pp. 426–439, 2013.

[72] A. Widestrand, J. Faijerson, U. Wilhelmsson et al., "Increased neurogenesis and astrogenesis from neural progenitor cells grafted in the hippocampus of GFAP-/- Vim-/- mice," *Stem Cells*, vol. 25, no. 10, pp. 2619–2627, 2007.

[73] M. M. Verbeek, J. R. Westphal, D. J. Ruiter, and R. M. W. De Waal, "T lymphocyte adhesion to human brain pericytes is mediated via very late antigen-4/vascular cell adhesion molecule-1 interactions," *Journal of Immunology*, vol. 154, no. 11, pp. 5876–5884, 1995.

[74] D. J. Alcendor, A. M. Charest, W. Q. Zhu, H. E. Vigil, and S. M. Knobel, "Infection and upregulation of proinflammatory cytokines in human brain vascular pericytes by human cytomegalovirus," *Journal of Neuroinflammation*, vol. 9, article 95, 2012.

[75] S. Nakagawa, V. Castro, and M. Toborek, "Infection of human pericytes by HIV-1 disrupts the integrity of the blood-brain barrier," *Journal of Cellular and Molecular Medicine*, vol. 16, no. 12, pp. 2950–2957, 2012.

[76] P. Dore-Duffy, A. Katychev, X. Wang, and E. Van Buren, "CNS microvascular pericytes exhibit multipotential stem cell activity," *Journal of Cerebral Blood Flow and Metabolism*, vol. 26, no. 5, pp. 613–624, 2006.

[77] G. Paul, I. Özen, N. S. Christophersen et al., "The adult human brain harbors multipotent perivascular mesenchymal stem cells," *PLoS ONE*, vol. 7, no. 4, Article ID e35577, 2012.

[78] T. Yamashima, A. B. Tonchev, I. H. Vachkov et al., "Vascular adventitia generates neuronal progenitors in the monkey hippocampus after ischemia," *Hippocampus*, vol. 14, no. 7, pp. 861–875, 2004.

[79] M. Karow, R. Sánchez, C. Schichor et al., "Reprogramming of pericyte-derived cells of the adult human brain into induced neuronal cells," *Cell Stem Cell*, vol. 11, no. 4, pp. 471–476, 2012.

[80] E. E. Crouch, C. Liu, V. Silva-Vargas, and F. Doetsch, "Regional and stage-specific effects of prospectively purified vascular cells on the adult V-SVZ neural stem cell lineage," *Journal of Neuroscience*, vol. 35, no. 11, pp. 4528–4539, 2015.

[81] M. B. Graeber, W. J. Streit, D. Büringer, D. L. Sparks, and G. W. Kreutzberg, "Ultrastructural location of major histocompatibility complex (MHC) class II positive perivascular cells in histologically normal human brain," *Journal of Neuropathology and Experimental Neurology*, vol. 51, no. 3, pp. 303–311, 1992.

[82] S. Kida, P. V. Steart, E.-T. Zhang, and R. O. Weller, "Perivascular cells act as scavengers in the cerebral perivascular spaces and remain distinct from pericytes, microglia and macrophages," *Acta Neuropathologica*, vol. 85, no. 6, pp. 646–652, 1993.

[83] J. Chabry, S. Nicolas, J. Cazareth et al., "Enriched environment decreases microglia and brain macrophages inflammatory phenotypes through adiponectin-dependent mechanisms: relevance to depressive-like behavior," *Brain, Behavior, and Immunity*, vol. 50, pp. 275–287, 2015.

[84] M. Prinz, J. Priller, S. S. Sisodia, and R. M. Ransohoff, "Heterogeneity of CNS myeloid cells and their roles in neurodegeneration," *Nature Neuroscience*, vol. 14, no. 10, pp. 1227–1235, 2011.

[85] G. Kunis, K. Baruch, N. Rosenzweig et al., "IFN-γ-dependent activation of the brain's choroid plexus for CNS immune surveillance and repair," *Brain*, vol. 136, no. 11, pp. 3427–3440, 2013.

[86] R. Shechter, A. London, and M. Schwartz, "Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates," *Nature Reviews Immunology*, vol. 13, no. 3, pp. 206–218, 2013.

[87] B. Engelhardt and R. M. Ransohoff, "The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms," *Trends in Immunology*, vol. 26, no. 9, pp. 485–495, 2005.

[88] K. Baruch and M. Schwartz, "CNS-specific T cells shape brain function via the choroid plexus," *Brain, Behavior, and Immunity*, vol. 34, pp. 11–16, 2013.

[89] O. Butovsky, Y. Ziv, A. Schwartz et al., "Microglia activated by IL-4 or IFN-γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells," *Molecular and Cellular Neuroscience*, vol. 31, no. 1, pp. 149–160, 2006.

[90] X. Xiong, G. E. Barreto, L. Xu, Y. B. Ouyang, X. Xie, and R. G. Giffard, "Increased brain injury and worsened neurological outcome in interleukin-4 knockout mice after transient focal cerebral ischemia," *Stroke*, vol. 42, no. 7, pp. 2026–2032, 2011.

[91] Y. Ziv, N. Ron, O. Butovsky et al., "Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood," *Nature Neuroscience*, vol. 9, no. 2, pp. 268–275, 2006.

[92] S. A. Wolf, B. Steiner, A. Wengner, M. Lipp, T. Kammertoens, and G. Kempermann, "Adaptive peripheral immune response increases proliferation of neural precursor cells in the adult hippocampus," *The FASEB Journal*, vol. 23, no. 9, pp. 3121–3128, 2009.

[93] N. C. Derecki, A. N. Cardani, C. H. Yang et al., "Regulation of learning and memory by meningeal immunity: a key role for IL-4," *Journal of Experimental Medicine*, vol. 207, no. 5, pp. 1067–1080, 2010.

[94] J. Kipnis, H. Cohen, M. Cardon, Y. Ziv, and M. Schwartz, "T cell deficiency leads to cognitive dysfunction: implications for therapeutic vaccination for schizophrenia and other psychiatric conditions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 21, pp. 8180–8185, 2004.

[95] A. Brynskikh, T. Warren, J. Zhu, and J. Kipnis, "Adaptive immunity affects learning behavior in mice," *Brain, Behavior, and Immunity*, vol. 22, no. 6, pp. 861–869, 2008.

[96] N. Ron-Harel, Y. Segev, G. M. Lewitus et al., "Age-dependent spatial memory loss can be partially restored by immune activation," *Rejuvenation Research*, vol. 11, no. 5, pp. 903–913, 2008.

[97] L. Xie, G. R. Choudhury, A. Winters, S.-H. Yang, and K. Jin, "Cerebral regulatory T cells restrain microglia/macrophage-mediated inflammatory responses via IL-10," *European Journal of Immunology*, vol. 45, no. 1, pp. 180–191, 2015.

[98] J. Kipnis, E. Yoles, H. Schori, E. Hauben, I. Shaked, and M. Schwartz, "Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response," *The Journal of Neuroscience*, vol. 21, no. 13, pp. 4564–4571, 2001.

[99] G. Huang, A. L. Smith, D. H. Gray et al., "A genetic and functional relationship between T cells and cellular proliferation in the adult hippocampus," *PLoS Biology*, vol. 8, no. 12, Article ID e1000561, 2010.

[100] J. Kipnis, H. Avidan, R. R. Caspi, and M. Schwartz, "Dual effect of $CD4^+CD25^+$ regulatory T cells in neurodegeneration: a dialogue with microglia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 2, pp. 14663–14669, 2004.

[101] J. Wang, L. Xie, C. Yang et al., "Activated regulatory T cell regulates neural stem cell proliferation in the subventricular zone of normal and ischemic mouse brain through interleukin 10," *Frontiers in Cellular Neuroscience*, vol. 9, article 361, 2015.

[102] I. C. Anthony, D. H. Crawford, and J. E. Bell, "B lymphocytes in the normal brain: contrasts with HIV-associated lymphoid infiltrates and lymphomas," *Brain*, vol. 126, no. 5, pp. 1058–1067, 2003.

[103] J. W. Prineas, "Multiple sclerosis: presence of lymphatic capillaries and lymphoid tissue in the brain and spinal cord," *Science*, vol. 203, no. 4385, pp. 1123–1125, 1979.

[104] S. A. Wolf, B. Steiner, A. Akpinarli et al., "CD4-positive T lymphocytes provide a neuroimmunological link in the control of adult hippocampal neurogenesis," *The Journal of Immunology*, vol. 182, no. 7, pp. 3979–3984, 2009.

[105] A. Lunemann, J. D. Lunemann, and C. Munz, "Regulatory NK-cell functions in inflammation and autoimmunity," *Molecular Medicine*, vol. 15, no. 9-10, pp. 352–358, 2009.

[106] A. Poli, J. Kmiecik, O. Domingues et al., "NK cells in central nervous system disorders," *Journal of Immunology*, vol. 190, no. 11, pp. 5355–5362, 2013.

[107] J. Hao, R. Liu, W. Piao et al., "Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology," *Journal of Experimental Medicine*, vol. 207, no. 9, pp. 1907–1921, 2010.

[108] R. Winkler-Pickett, H. A. Young, J. M. Cherry et al., "In vivo regulation of experimental autoimmune encephalomyelitis by NK cells: alteration of primary adaptive responses," *Journal of Immunology*, vol. 180, no. 7, pp. 4495–4506, 2008.

[109] Q. Liu, N. Sanai, W. Jin, A. La Cava, L. Van Kaer, and F. Shi, "Neural stem cells sustain natural killer cells that dictate recovery from brain inflammation," *Nature Neuroscience*, vol. 19, no. 2, pp. 243–252, 2016.

[110] N. Anandasabapathy, G. D. Victora, M. Meredith et al., "Flt3L controls the development of radiosensitive dendritic cells in the meninges and choroid plexus of the steady-state mouse brain," *The Journal of Experimental Medicine*, vol. 208, no. 18, pp. 1695–1705, 2011.

[111] K. Bulloch, M. M. Miller, J. Gal-Toth et al., "CD11c/EYFP transgene illuminates a discrete network of dendritic cells within the embryonic, neonatal, adult, and injured mouse brain," *Journal of Comparative Neurology*, vol. 508, no. 5, pp. 687–710, 2008.

[112] K. M. Nautiyal, C. A. Dailey, J. L. Jahn et al., "Serotonin of mast cell origin contributes to hippocampal function," *European Journal of Neuroscience*, vol. 36, no. 3, pp. 2347–2359, 2012.

[113] T. S. Hallstrand, W. A. Altemeier, M. L. Aitken, and W. R. Henderson, "Role of cells and mediators in exercise-induced bronchoconstriction," *Immunology and Allergy Clinics of North America*, vol. 33, no. 3, pp. 313–328, 2013.

[114] T. Wyss-Coray and L. Mucke, "Inflammation in neurodegenerative disease-a double-edged sword," *Neuron*, vol. 35, no. 3, pp. 419–432, 2002.

[115] H. Eyre and B. T. Baune, "Neuroplastic changes in depression: a role for the immune system," *Psychoneuroendocrinology*, vol. 37, no. 9, pp. 1397–1416, 2012.

[116] E. C. Beattie, D. Stellwagen, W. Morishita et al., "Control of synaptic strength by glial TNFα," *Science*, vol. 295, no. 5563, pp. 2282–2285, 2002.

[117] R. E. Iosif, C. T. Ekdahl, H. Ahlenius et al., "Tumor necrosis factor receptor 1 is a negative regulator of progenitor proliferation in adult hippocampal neurogenesis," *Journal of Neuroscience*, vol. 26, no. 38, pp. 9703–9712, 2006.

[118] S. L. Montgomery and W. J. Bowers, "Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system," *Journal of Neuroimmune Pharmacology*, vol. 7, no. 1, pp. 42–59, 2012.

[119] H. Schneider, F. Pitossi, D. Balschun, A. Wagner, A. Del Rey, and H. O. Besedovsky, "A neuromodulatory role of interleukin-1β in the hippocampus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 13, pp. 7778–7783, 1998.

[120] T. Ben-Hur, O. Ben-Menachem, V. Furer, O. Einstein, R. Mizrachi-Kol, and N. Grigoriadis, "Effects of proinflammatory cytokines on the growth, fate, and motility of multipotential neural precursor cells," *Molecular and Cellular Neuroscience*, vol. 24, no. 3, pp. 623–631, 2003.

[121] J. W. Koo and R. S. Duman, "IL-1β is an essential mediator of the antineurogenic and anhedonic effects of stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 2, pp. 751–756, 2008.

[122] A. Halle, V. Hornung, G. C. Petzold et al., "The NALP3 inflammasome is involved in the innate immune response to amyloid-β," *Nature Immunology*, vol. 9, no. 8, pp. 857–865, 2008.

[123] C. Song and D. Horrobin, "Omega-3 fatty acid ethyl-eicosapentaenoate, but not soybean oil, attenuates memory impairment induced by central IL-1β administration," *Journal of Lipid Research*, vol. 45, no. 6, pp. 1112–1121, 2004.

[124] W. A. Banks, S. A. Farr, M. E. La Scola, and J. E. Morley, "Intravenous human interleukin-1 α impairs memory processing in mice: dependence on blood-brain barrier transport into posterior division of the septum," *Journal of Pharmacology and Experimental Therapeutics*, vol. 299, no. 2, pp. 536–541, 2001.

[125] Y. Matsumoto, T. Yamaguchi, S. Watanabe, and T. Yamamoto, "Involvement of arachidonic acid cascade in working memory impairment induced by interleukin-1 beta," *Neuropharmacology*, vol. 46, no. 8, pp. 1195–1200, 2004.

[126] C. R. Pugh, K. T. Nguyen, J. L. Gonyea et al., "Role of interleukin-1 beta in impairment of contextual fear conditioning caused by social isolation," *Behavioural Brain Research*, vol. 106, no. 1-2, pp. 109–118, 1999.

[127] L. Li, T. L. Walker, Y. Zhang, E. W. Mackay, and P. F. Bartlett, "Endogenous interferon γ directly regulates neural precursors in the non-inflammatory brain," *Journal of Neuroscience*, vol. 30, no. 27, pp. 9038–9050, 2010.

[128] R. Baron, A. Nemirovsky, I. Harpaz, H. Cohen, T. Owens, and A. Monsonego, "IFN-γ enhances neurogenesis in wild-type mice and in a mouse model of Alzheimer's disease," *The FASEB Journal*, vol. 22, no. 8, pp. 2843–2852, 2008.

[129] J. Imitola, K. Raddassi, K. I. Park et al., "Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1α/CXC chemokine receptor 4 pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 52, pp. 18117–18122, 2004.

[130] J. Imitola, M. Comabella, A. K. Chandraker et al., "Neural stem/progenitor cells express costimulatory molecules that are differentially regulated by inflammatory and apoptotic stimuli," *American Journal of Pathology*, vol. 164, no. 5, pp. 1615–1625, 2004.

[131] K. A. Molyneaux, H. Zinszner, P. S. Kunwar et al., "The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival," *Development*, vol. 130, no. 18, pp. 4279–4286, 2003.

[132] H. T. Ni, S. Hu, W. S. Sheng et al., "High-level expression of functional chemokine receptor CXCR4 on human neural precursor cells," *Developmental Brain Research*, vol. 152, no. 2, pp. 159–169, 2004.

[133] H. Peng, Y. Huang, J. Rose et al., "Stromal cell-derived factor 1-mediated CXCR4 signaling in rat and human cortical neural progenitor cells," *Journal of Neuroscience Research*, vol. 76, no. 1, pp. 35–50, 2004.

[134] C. Schultheiß, P. Abe, F. Hoffmann et al., "CXCR4 prevents dispersion of granule neuron precursors in the adult dentate gyrus," *Hippocampus*, vol. 23, no. 12, pp. 1345–1358, 2013.

[135] A. Belmadani, P. B. Tran, D. Ren, and R. J. Miller, "Chemokines regulate the migration of neural progenitors to sites of neuroinflammation," *The Journal of Neuroscience*, vol. 26, no. 12, pp. 3182–3191, 2006.

[136] P. B. Tran, D. Ren, T. J. Veldhouse, and R. J. Miller, "Chemokine receptors are expressed widely by embryonic and adult neural progenitor cells," *Journal of Neuroscience Research*, vol. 76, no. 1, pp. 20–34, 2004.

[137] D. Widera, W. Holtkamp, F. Entschladen et al., "MCP-1 induces migration of adult neural stem cells," *European Journal of Cell Biology*, vol. 83, no. 8, pp. 381–387, 2004.

[138] J. Kipnis, M. Cardon, H. Avidan et al., "Dopamine, through the extracellular signal-regulated kinase pathway, downregulates $CD4^+CD25^+$ regulatory T-cell activity: implications for

neurodegeneration," *Journal of Neuroscience*, vol. 24, no. 27, pp. 6133–6143, 2004.

[139] M. Kubera, A.-H. Lin, G. Kenis, E. Bosmans, D. Van Bockstaele, and M. Maes, "Anti-inflammatory effects of antidepressants through suppression of the interferon-γ/interleukin-10 production ratio," *Journal of Clinical Psychopharmacology*, vol. 21, no. 2, pp. 199–206, 2001.

[140] S. Maren and G. J. Quirk, "Neuronal signalling of fear memory," *Nature Reviews Neuroscience*, vol. 5, no. 11, pp. 844–852, 2004.

[141] J. M. Pocock and H. Kettenmann, "Neurotransmitter receptors on microglia," *Trends in Neurosciences*, vol. 30, no. 10, pp. 527–535, 2007.

[142] K. Färber, U. Pannasch, and H. Kettenmann, "Dopamine and noradrenaline control distinct functions in rodent microglial cells," *Molecular and Cellular Neuroscience*, vol. 29, no. 1, pp. 128–138, 2005.

[143] S. A. Kuhn, F. K. H. van Landeghem, R. Zacharias et al., "Microglia express $GABA_B$ receptors to modulate interleukin release," *Molecular and Cellular Neuroscience*, vol. 25, no. 2, pp. 312–322, 2004.

[144] M. V. Simonini, P. E. Polak, A. Sharp, S. McGuire, E. Galea, and D. L. Feinstein, "Increasing CNS noradrenaline reduces EAE severity," *Journal of Neuroimmune Pharmacology*, vol. 5, no. 2, pp. 252–259, 2010.

[145] R. A. Corriveau, G. S. Huh, and C. J. Shatz, "Regulation of class I MHC gene expression in the developing and mature CNS by neural activity," *Neuron*, vol. 21, no. 3, pp. 505–520, 1998.

[146] C. A. Goddard, D. A. Butts, and C. J. Shatz, "Regulation of CNS synapses by neuronal MHC class I," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 16, pp. 6828–6833, 2007.

[147] C. J. Shatz, "MHC class I: an unexpected role in neuronal plasticity," *Neuron*, vol. 64, no. 1, pp. 40–45, 2009.

[148] G. S. Huh, L. M. Boulanger, H. Du, P. A. Riquelme, T. M. Brotz, and C. J. Shatz, "Functional requirement for class I MHC in CNS development and plasticity," *Science*, vol. 290, no. 5499, pp. 2155–2159, 2000.

[149] J. D. Adelson, G. E. Barreto, L. Xu et al., "Neuroprotection from stroke in the absence of MHCI or PirB," *Neuron*, vol. 73, no. 6, pp. 1100–1107, 2012.

[150] L. K. Smith, Y. He, J. Park et al., "β2-Microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis," *Nature Medicine*, vol. 21, no. 8, pp. 932–937, 2015.

[151] M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady et al., "Toll-like receptors and their ligands control mesenchymal stem cell functions," *Blood*, vol. 109, no. 4, pp. 1422–1432, 2007.

[152] Y. Nagai, K. P. Garrett, S. Ohta et al., "Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment," *Immunity*, vol. 24, no. 6, pp. 801–812, 2006.

[153] H. Lee, S. Lee, I.-H. Cho, and S. J. Lee, "Toll-like receptors: sensor molecules for detecting damage to the nervous system," *Current Protein and Peptide Science*, vol. 14, no. 1, pp. 33–42, 2013.

[154] A. Rolls, R. Shechter, A. London et al., "Toll-like receptors modulate adult hippocampal neurogenesis," *Nature Cell Biology*, vol. 9, no. 9, pp. 1081–1088, 2007.

[155] R. Covacu, L. Arvidsson, Å. Andersson et al., "TLR activation induces TNF-α production from adult neural stem/progenitor cells," *The Journal of Immunology*, vol. 182, no. 11, pp. 6889–6895, 2009.

[156] L. Bondolfi, F. Ermini, J. M. Long, D. K. Ingram, and M. Jucker, "Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice," *Neurobiology of Aging*, vol. 25, no. 3, pp. 333–340, 2004.

[157] H. van Praag, T. Shubert, C. Zhao, and F. H. Gage, "Exercise enhances learning and hippocampal neurogenesis in aged mice," *Journal of Neuroscience*, vol. 25, no. 38, pp. 8680–8685, 2005.

[158] H. W. Mahncke, A. Bronstone, and M. M. Merzenich, "Chapter 6 brain plasticity and functional losses in the aged: scientific bases for a novel intervention," *Progress in Brain Research*, vol. 157, pp. 81–109, 2006.

[159] M. A. Erickson, Y. Morofuji, J. B. Owen, and W. A. Banks, "Rapid transport of CCL11 across the blood-brain barrier: regional variation and importance of blood cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 349, no. 3, pp. 497–507, 2014.

[160] M. Schmidt, "Continuous neurogenesis in the olfactory brain of adult shore crabs, Carcinus maenas," *Brain Research*, vol. 762, no. 1-2, pp. 131–143, 1997.

[161] J. L. Benton, R. Kery, J. Li, C. Noonin, I. Söderhäll, and B. S. Beltz, "Cells from the immune system generate adult-born neurons in crayfish," *Developmental Cell*, vol. 30, no. 3, pp. 322–333, 2014.

[162] J. C. Rink, "Stem cell systems and regeneration in planaria," *Development Genes and Evolution*, vol. 223, no. 1-2, pp. 67–84, 2013.

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Review Article

Insights into the Biology and Therapeutic Applications of Neural Stem Cells

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The cerebral cortex is essential for our higher cognitive functions and emotional reasoning. Arguably, this brain structure is the distinguishing feature of our species, and yet our remarkable cognitive capacity has seemingly come at a cost to the regenerative capacity of the human brain. Indeed, the capacity for regeneration and neurogenesis of the brains of vertebrates has declined over the course of evolution, from fish to rodents to primates. Nevertheless, recent evidence supporting the existence of neural stem cells (NSCs) in the adult human brain raises new questions about the biological significance of adult neurogenesis in relation to ageing and the possibility that such endogenous sources of NSCs might provide therapeutic options for the treatment of brain injury and disease. Here, we highlight recent insights and perspectives on NSCs within both the developing and adult cerebral cortex. Our review of NSCs during development focuses upon the diversity and therapeutic potential of these cells for use in cellular transplantation and in the modeling of neurodevelopmental disorders. Finally, we describe the cellular and molecular characteristics of NSCs within the adult brain and strategies to harness the therapeutic potential of these cell populations in the treatment of brain injury and disease.

1. NSCs during Development of the Cerebral Cortex

The development of the mammalian cerebral cortex follows stepwise production of neurons, then glial cells, including astrocytes and oligodendrocytes from local NSCs. Early during embryonic development, cells of the central nervous system are derived from the neuroectoderm, which is organised as a neural tube. Over time, the neural tube invaginates to form structures including the prosencephalon, from which emerge the telencephalon and diencephalon. The cerebral cortex arises from the dorsal telencephalon (also known as the pallium), while the ventral telencephalon (also known as the subpallium) gives rise to the basal ganglia (reviewed in [1]). NSCs from the dorsal and ventral telencephalon are critical to the generation of the two main classes of cerebral cortex neurons, the excitatory projection neurons which signal using glutamate as their neurotransmitter and the inhibitory interneurons that use γ-amino butyric acid (GABA). Excitatory projection neurons are born from local NSCs residing within the dorsal telencephalon, and these neurons migrate radially to position themselves appropriately within the developing cortex and hippocampus (reviewed in [2]). Different subtypes of excitatory cortical projection neurons are generated in a temporal sequence, so as to generate defined layers I to VI. On the other hand, inhibitory interneurons are born from NSCs residing within the ventral telencephalon. These neurons undergo long-distance, tangential migration to populate the dorsal cortical structures.

A remarkable feature during cerebral corticogenesis is the synchronous development and complementary positioning of temporally derived interneurons from the ventral telencephalon and projection neurons from the dorsal telencephalon, such that functional neural circuits are established

between excitatory projection neurons and their appropriate inhibitory interneuron counterparts [2–4]. In all, the characteristic six-layered structure of the neocortex features neurons in layers V and VI which project to subcortical targets (subcerebral projection neurons and corticothalamic projection neurons, resp.), neurons in layers II and III which largely project to other cortical areas (corticocortical projection neurons, as well as callosal neurons which project to the contralateral hemisphere and whose axons comprise the corpus callosum), and neurons in layers I and IV that largely form axonal connections within the cortical hemisphere. Within each layer, cortical interneurons adopt a variety of unique dendritic morphologies so as to modulate projection neuron firing. Detailed accounts of the formation of cortical projection neuron and interneuron subtypes are the subject of several excellent reviews [2–5].

The Diversity of NSCs within the Embryonic Cerebral Cortex. NSCs are defined by their capacity to self-renew, as well as be able to generate neurons, astrocytes, and oligodendrocytes. In contrast, not all neural progenitor cells (NPCs) exhibit self-renewal capacity. The diversity of NSCs and NPCs (i.e., cells which generate neurons but are not necessarily self-renewing) is, in part, responsible for the diversity and relative population densities of neuronal subtypes within the cerebral cortex. Early in the formation of the cerebral cortex, the dorsal telencephalon comprises a uniform layer of neuroepithelial cells. The local NSCs of the germinal ventricular zone (VZ), which lines the vesicular lumen, initially undergo self-renewing, proliferative divisions. At approximately mid-gestation in rodents, a subset of NSCs transition to become lineage-restricted NPCs and accumulate as a secondary proliferative layer above the VZ, described as the subventricular zone (SVZ). A population of NSCs undergo neurogenic divisions to form an early, transient neuronal layer above the SVZ, known as the preplate (PP). As corticogenesis progresses, newborn neurons split the PP layer to form an outer marginal zone (MZ), an underlying cortical plate (CP), and a subplate (SP). The MZ comprises a small (1–3%) population of distinct progenitors [6] as well as Cajal-Retzius cells which derive from the cortical hem/antihem and septum [7] and which secrete Reelin, an essential factor for cortical layering (reviewed in [8]). Over time, newborn cortical neurons continue to migrate into the CP, resulting in its progressive enlargement. Notably, cortical neurons are added to each of the CP layers in a temporally specified manner, such that neurons which occupy deep layers IV and V are generated early during corticogenesis, while neurons of superficial layers (IV, III, and II) are generated later. Eventually, the cell-sparse MZ forms layer I, while the VZ/SVZ compartment is progressively depleted and reduced to a single-cell layer of ependymal cells, with the exception of the lateral wall of the cortical SVZ which continues to support a niche of resident glial-like NSCs which generates neurons into adulthood (discussed in Section 2).

In the last two decades, significant progress has been made to describe the cellular heterogeneity of embryonic NPCs and NSCs in the dorsal telencephalon (Figure 1). Notably, three main types of progenitor cells have been identified on the basis of their relationship with the apical surface of the dorsal telencephalon (located immediately adjacent to the ventricular lumen) relative to the superficial basal lamina of the MZ, as well as their distinct cellular/molecular features: (i) Apical Progenitors (APs); (ii) Basal Progenitors (BPs); and, more recently, (iii) Subapical Progenitors (SAPs) [47–49].

APs are NSCs that remain in contact with the luminal wall and form adherens junctions with other Apical Progenitors. APs are also identified by the apical location of their mitoses, are able to translocate their nuclei along the vertical axis in a cell cycle dependent fashion (termed interkinetic nuclear migration (INM) [50, 51]), and exhibit apicobasal polarity [49, 52]. Interestingly, APs show a temporal relationship whereby the earliest APs identified within the telencephalon are the neuroepithelial (NE) cells that undergo proliferative, symmetric divisions to expand the local pool of progenitors. As corticogenesis progresses, NE cells adopt an asymmetric mode of cell division generating apical Radial Glial (aRG) cells as well as apical Intermediate Progenitor (aIP) cells (first described as short neural precursors [53]) or, more infrequently, neurons. NE cells and aRG cells are capable of proliferative divisions, while aIPs undergo a single round of symmetric, neurogenic division to generate two identical daughter neurons [53].

The BPs, the second type of progenitor cell within the cortex, are identifiable by their detachment from adherens junction complexes within the VZ, their location within the SVZ, their expression of the transcription factor TBR2 (also known as EOMES), and their capacity to undergo basal rather than apical mitotic divisions [54, 55]. BPs are the products of cell division by NE cells and aRG cells and comprise two main cell types, namely, basal Intermediate Progenitors (bIPs) and basal Radial Glia (bRG). BPs can undergo symmetric, neurogenic divisions that ultimately deplete the pool of SVZ progenitors. However, the capacity for proliferative divisions by BPs and the size of the relative population of BP subtypes are significantly different in lissencephalic (smooth) brained rodents, compared to the gyrencephalic (convoluted) brains of primates such as humans [47]. The expansion of BPs in the human cortex that can undergo proliferative divisions has been suggested to constitute an important cellular basis for human cortical expansion and gyrification [56]. Indeed, recent findings demonstrate the preponderance of bRG cells in the human ventral forebrain, which generate large numbers of cortical interneurons [57].

More recently, a new type of cortical progenitor, SAPs, was recognised to be distinct from APs and BPs, owing to the abventricular location of their mitoses and their ventricular contact [48]. These SAPs are capable of proliferative divisions and are more abundant in the ventral versus the dorsal telencephalon. Furthermore, SAPs appear to be more numerous in the cortices of gyrencephalic brains of ferrets and sheep, compared with the lissencephalic cortex of the marmoset. Given their recent discovery by Pilz and colleagues [48], the precise contribution by SAPs to the cellular diversity of cortical neurons remains to be clarified. However, their abundance in both the dorsal and the ventral telencephalon

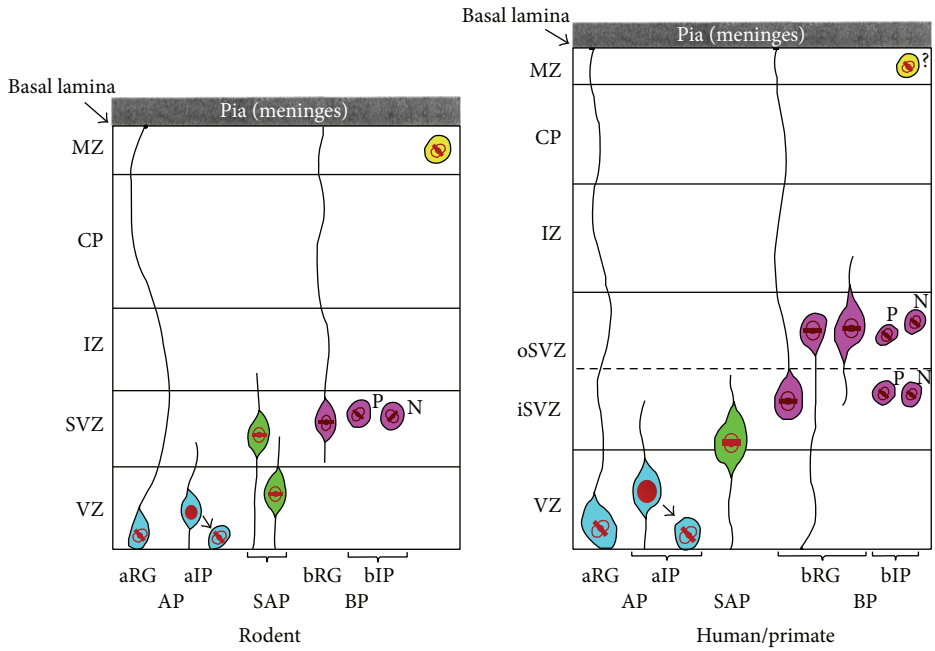

Figure 1: Summary of progenitor subtype diversity within the rodent and human/primate brain. Apical Progenitor (AP) cells (light blue) include apical Radial Glia (aRG) which attach to the basal lamina and apical Intermediate Progenitor (aIP) cells which have short processes. Both types of APs are defined by their mitotic division at the apical surface. Subapical Progenitor (SAP) cells (coloured green) are defined by their ventricular contact and abventricular mode of cell division. Basal Progenitor (BP) cells (magenta) are defined by their basal mitoses and comprise basal Radial Glia (bRG) cells attached to the basal lamina as well as basal Intermediate Progenitor (bIP) cells which undergo a proliferative division (labelled "P") or neurogenic divisions (labelled "N"), as indicated. A yellow coloured marginal zone progenitor is represented in rodent cortex. In the human/primate cortex, AP and SAP cell types have been identified, while three types of bRGs have been identified including those with a basal attachment, an apical attachment, or only emanating short processes. The bIPs cell types which undergo proliferative or neurogenic divisions have been described in the iSVZ and oSVZ. The presence of MZ progenitor cells within the human/primate cortex remains to be clarified. VZ: ventricular zone, SVZ: subventricular zone, IZ: Intermediate Zone, CP: cortical plate, MZ: marginal zone, iSVZ: inner subventricular zone, and oSVZ: outer subventricular zone as presented. Relative sizes of rodent and human/primate compartments are not drawn to scale. See text for further details.

suggests that SAPs are likely to play a significant role in cortical neuron development.

While excitatory cortical projection neurons are generated from NSCs of the dorsal telencephalon, inhibitory cortical interneurons are largely generated from the germinal zones of the ventral telencephalon. In mice, the ventral telencephalon is organised into several prominent structures termed ganglionic eminences, each with distinct NSC and NPC populations that generate specific neuronal subtypes. For example, NPCs of the lateral ganglionic eminences (LGE) generate striatal projection neurons and interneurons destined for the olfactory bulb, while NPCs residing within the medial ganglionic eminences (MGE) generate cortical interneurons that invade the dorsal telencephalon, as well as local projection neurons of the globus pallidus. Also, the anterior entopeduncular area (AEP) is recognised as a source of interneurons that populate the dorsal cortex via tangential migration [58]. Notably, it was within the considerably large SVZ of the mouse ventral telencephalon that Pilz and colleagues discovered SAPs undergo mitoses at basal positions, away from the ventricular surface of the telencephalon [48].

While it is clear that the primary source of cortical interneurons in rodents appears to be the ventral telencephalon, the developmental origin of human cortical interneurons appears to involve both the ventral and dorsal cortex, with evidence supportive of a limited contribution by dorsal cortical progenitors. Letinic and colleagues presented the first evidence that prominent numbers of interneuron progenitors could be found within the dorsal cortex, as identified by their expression of Dlx1, Dlx2, and Mash1 [59]. This finding was supported by subsequent studies demonstrating the propensity for dorsally derived cortical cells to differentiate into subtypes of interneuron [60–64]. However, Hansen and coworkers [57, 65] more recently reported that DLX2-expressing cells of the dorsal cortex are not colabelled with Ki67, a marker of cell proliferation, or incorporate the DNA-synthesis marker BrdU in studies with brain slice cultures. A similar conclusion was drawn by Ma and colleagues in their studies of human and primate (macaque) cortex, since they found that cultured slices of monkey dorsal cortex yielded an extremely low proportion of GABAergic neurons that arise from at least one cell division (marked by BrdU incorporation) [65]. These new findings thus provide compelling

evidence to support the notion that the vast majority of human cortical interneurons are of a subcortical origin and that interneuron progenitors in the cortex are postmitotic. Interestingly, in cases of human holoprosencephaly (HPE) with severe ventral forebrain hypoplasia, it was reported that only subpopulations of cortical interneurons (namely, those which express either NOS1, NPY, or SST) were absent, while calretinin-positive interneurons were still detected [66]. In such cases it would appear that certain interneuron subtypes could arise from the dorsal cortex, at least in situations in which the ventral forebrain is severely compromised. The capacity for cortical interneuron production in the human brain in the context of development and disease remains to be clarified.

Insights into the Molecular Regulation of NSCs within the Embryonic Cerebral Cortex. Over the course of embryonic cortical development, the timing of NSC proliferation and neurogenesis is guided by cell extrinsic and cell intrinsic factors. The cerebrospinal fluid (CSF) courses through the ventricular system of the neural tube to deliver numerous signalling factors that influence the proliferative potential of cortical NSCs [67]. As early as embryonic day (E) E8.5–E9.5 in the mouse, Sonic Hedgehog (Shh), Fibroblast Growth Factor (Fgf), and Bone Morphogenetic Proteins (Bmps) establish gradients across the rostrocaudal, lateromedial, and dorsoventral telencephalon [2]. Such extrinsic signals are interpreted by embryonic cortical cells to induce NSC expression of genes encoding transcription factors such as *Lim-homeodomain 2* (*Lhx2*), *Forkhead Box G1* (*FoxG1*), *Paired Box Domain 6* (*Pax6*), and *Empty Spiracles Homologue-1* and *Empty Spiracles Homologue-2* (*Emx1* and *Emx2*) in a region-specific manner. Notably, Lhx2 is detected in the entire telencephalon except for the dorsal midline, while Foxg1, Pax6, and Emx1 are expressed in cells of the dorsal telencephalon, and Emx2 is expressed throughout the telencephalon [68].

The expression patterns for these abovementioned transcription factors reflect their instructive roles for NSC proliferation and neurogenesis. For example, Lhx2 specifies cortical and hippocampal cell fates, and studies of knockout mice reveal that its absence results in the expansion of adjacent structures, including the midline structures known as the cortical hem and antihem [69, 70]. More recent investigations of *Lhx2* deficiency using lineage-specific cre-driver mice have revealed its role in NSC proliferation and neurogenesis. In studies of conditional (loxp) mice crossed with *Nestin-cre* to delete *Lhx2* throughout the developing nervous system, Lhx2 was found to regulate progenitor proliferation and neurogenesis through β-catenin signalling [71]. Deletion of *Lhx2* in telencephalic progenitors using *Emx1-cre* mice led to the formation of olfactory cortex rather than lateral cortex in a critical developmental window (E10.5) in embryonic mouse development [72]. The activity of Lhx2 appears to involve the transcriptional regulation of downstream target genes, such as *Pax6*, as revealed by Shetty and coworkers, who demonstrated that loss of *Lhx2* in mouse embryos from E11.5 onwards led to the loss of distinct neurocircuitry (namely, the barrel cortex) which accompanied changes in the regional identity of the cortex and which appeared to phenocopy *Pax6* deficiency [73].

In the case of Foxg1, its expression within the E9.5 embryo is observed as a high-rostrolateral-to-low-caudomedial gradient. Deletion of *Foxg1* in the mouse results in repatterning of the cortical field to cortical hem and hippocampus, together with the concomitant loss of cortical plate neurons [74]. In newborn cortical neurons, the precise timing of *Foxg1* expression is critical for their migration from the IZ to the CP through a mechanism which, in part, involves modulation of *Unc5D* expression [75]. In addition, the sequential production of Cajal-Retzius cells, deep layer neurons followed by upper layer neurons requires *Foxg1*, with its selective loss resulting in the commensurate disruption of this temporal sequence for the production of cortical glutamatergic neurons [76, 77]. Hence, Lhx2 and Foxg1 are necessary to specify the identity of cortical NSCs.

In contrast to Lhx2 and Foxg1, studies of mouse corticogenesis reveal that Pax6 is critical for dorsal *versus* ventral telencephalic identity [78–81]. *In situ* hybridisation studies reveal a regionalised pattern for Pax6 in the dorsal telencephalon, with high rostrolateral expression and low caudomedial expression. In contrast, Emx1 and Emx2 expression is detected in an opposing (low rostrolateral expression and high caudomedial expression) gradient [82]. Pax6 was recognised to be critical for establishing cortical identity, due to studies of the mouse mutant *small eye* that revealed that *Pax6*-deficiency led to ectopic expression of ventral telencephalic genes by dorsal telencephalic NSCs, including *Mash1*, *Gsh2*, and *Dlx1/2* [78–81]. Mutations to *Emx1/2* result in a reduction in the size of the cortex [83], but studies of compound *Pax6/Emx2* double-mutant embryos reveal that both these genes are required in concert for establishing the identity of dorsal cortical NSCs, since their compound loss results in the lack of the dorsal telencephalon, and an expansion of ventral telencephalic domains across the entire cortex [84]. Together, these studies provide examples of cell intrinsic factors that specify the identity of NSCs during cortical development.

In addition to the functions for Pax6 in cortical regionalisation, additional studies have underscored its importance in regulating the transition between APs and BPs within the embryonic cortex, as well as its role in driving neurogenesis. For example, Pax6 activates expression of the proneural basic helix-loop-helix (bHLH) transcription factor Neurog2 in APs to instruct their neuroprogenitor fate and to drive neuronal subtype specification in postmitotic neurons [79]. Pax6 also orchestrates the proliferation of APs and promotes their asymmetric division to expand the pool of BPs within the embryonic cortex [85–87]. Homozygous mutant mice for *Pax6* display a selective loss of cortical neurons destined for upper layers [88–90].

The role and interplay of intrinsic factors in cortical neuron specification and subtype identity is perhaps best exemplified in studies of Neurog2 and its related family members Neurog1 and Ascl1. Both Neurog2 and Neurog1 are specifically expressed by APs and, to a lesser extent, BPs [91]. Furthermore, studies with lineage tracer (*Neurog2*^{EGFPKI}) mice indicate that *Neurog2* is expressed in early postmitotic

neurons which have exited S/G2/M-phases of the cell cycle [91]. On the other hand, Ascl1 is detected predominantly in NSCs of the ventral telencephalon. Loss of Neurog2 leads to the reduction of early-born glutamatergic neurons destined for layers V and VI of the mouse cortex, and this phenotype is exacerbated in *Neurog1*/*Neurog2* double-mutant mice [79, 92]. In contrast, in *Ascl1* loss-of-function mutants there is depletion of NSCs of the ventral telencephalon and medial ganglionic eminence, as well as their neuronal progeny [93]. Interestingly, loss of *Neurog2*, or both *Neurog1* and *2*, leads to ectopic expression of Ascl1 by dorsal telencephalic progenitors, as well as the subsequence ectopic expression of ventral telencephalic genes, including *Dlx1*, *Dlx2*, *Dlx5*, and the GABAergic neurotransmitter genes *Gad1* and *Gad2* [92]. In support of the notion that Neurog2 suppresses Ascl1 in the cortex, these ventral telencephalic markers are not detected in *Neurog2*/*Ascl1* double-mutant embryos [79]. Thus, the specification of glutamatergic projection neurons versus GABAergic interneurons by NSCs is governed by the activities and interplay of transcription factors, such as the proneural bHLH proteins (please refer to these articles [2, 3, 58, 94] for further details).

Studies of proneural bHLH transcription factors in the embryonic cortex can also provide insights into the timing of neurogenesis versus gliogenesis by NSCs. For example, loss of both *Neurog2* and *Ascl1* leads to a significant reduction in neuronal production coupled with premature initiation of gliogenesis within the embryonic cortex [95]. The expression of Neurog2 or Ascl1 in NSCs is required to maintain their neurogenic potential and prevent activation of gliogenesis [95]. A proposed mechanism for this dual role was reported by Sun and colleagues who found that Neurog1 induced neurogenesis through the direct activation of neuronal genes, while suppressing glial differentiation through sequestration of the transcriptional coactivating factor CREB-binding protein (CBP). This sequestration prevented the association of CBP with Smad1 and Stat transcription factors and thereby the activation of the promoters of astrocyte-specific genes including *S100β* and *glial fibrillary acidic protein* (*Gfap*) [96]. More recently, studies of the zinc finger transcriptional repressor Rp58 (also known as Znf238) have identified its role in cortical development. Notably, Rp58 is detected in NSCs and postmitotic neurons of the dorsal telencephalon, and homozygous loss of *Rp58* leads to a disruption of NSCs and BPs [97], premature gliogenesis [98], and defective neurogenesis [97, 99, 100] within the embryonic cortex. Interestingly, Rp58 can bind *Neurog2*-like gene regulatory sequences, repressing candidate gene expression as well as antagonising the functions of *Neurog2*-type transcriptional activators [99]. Furthermore, Rp58 directly suppresses the expression of *Neurog2* [101] as well as *Neurog2*-target genes, such as *Rnd2* [99]. Thus, these findings suggest that Rp58 regulate the development of newborn postmitotic neuronal progeny in part through mechanisms involving neurogenins, but the precise mechanisms that underlie the function of Rp58 in APs and BPs remain to be clarified.

Insights into the regulation of neurogenesis versus gliogenesis by NSCs can also be gleaned from studies of the *nuclear factor one* (NFI) family of transcriptional regulators (comprising *Nfia*, *Nfib*, *Nfic*, and *Nfix*) [102–104]. These proteins bind target DNA sequences so as to activate or repress candidate gene transcription in a context-specific manner (reviewed in [105]). Nfia, Nfib, and Nfix are detected within the VZ of the dorsal telencephalon [106, 107], and studies of homozygous mutant mice revealed that the loss of *Nfia*, *Nfib*, or *Nfix* led to an expansion of NSCs (marked by Pax6 expression), but without an accompanying increase in BPs (marked by Tbr2) [108–110]. Curiously, *Nfi* deficiency resulted in a reduction in gliogenesis, marked by Gfap expression [109, 111, 112]. To account for these interesting phenotypes, molecular studies have begun to clarify the activities of Nfis in the coordination of NSC expansion and neuronal-glial differentiation. For example, a study by Namihira and colleagues reported that Notch signalling-induced Nfia expression in cortical NSCs was coincident with derepression of the *Gfap* regulatory region (marked by dissociation of the DNA methyltransferase Dnmt1 from this locus), which was coincident with promoter occupancy by the activator Stat3 [113]. While these observations suggest that NFIs regulate gliogenesis through indirect mechanisms, their roles in NSC expansion could be linked to their transcriptional regulatory activities on target genes that govern stem cell identity. This is supported by recent findings that demonstrate that the expression of stem cell-associated genes *Ezh2* and *Sox9* is directly suppressed by Nfib [114] and Nfix [109] *in vitro* and that *Ezh2* and *Sox9* expression is elevated in *Nfib* and *Nfix* knockout mouse embryos. Interestingly, the expression of the two Notch-induced stem cell regulators, namely, the *Hairy-Enhancer-of-Split* (*Hes*) genes *Hes1* and *Hes5*, is also elevated in *Nfia* and *Nfib* knockout mice, further suggesting a role for both NFIs in suppressing NSC self-renewal [110]. Collectively, these lines of evidence suggest that NFIs orchestrate the timely progression of NSC proliferation, neurogenesis, and gliogenesis during cortical development, likely by directly suppressing NSC genes and modulating *Gfap* expression through an indirect mechanism. Taken together, these examples provide insight into the underlying mechanisms which regulate embryonic NSCs. Given the relatively recent discovery of the extensive cellular heterogeneity of NSCs in the developing cerebral cortex, as well as the potential differences between rodent and human NSCs [115] (Figure 1), the molecular mechanisms underlying the functions for each distinct AP and BP subtype remain a significant topic of interest.

2. NSCs in the Adult Cerebral Cortex

A subset of embryonic NSCs persists into the postnatal and adult mammalian brain throughout life. Much like in the developing brain, the biology of these adult NSC populations has primarily been studied using rodent models. The biology of adult NSCs in rodents is, at face value, very similar to embryonic NSCs, with the major point of difference being that adult NSCs are long-lived and largely quiescent. In rodents, the two adult neurogenic niches are the subgranular zone (SGZ) of the hippocampal dentate gyrus and the SVZ lining the lateral ventricles. In the SGZ, NSCs give rise to Intermediate Progenitors and then immature granule

neurons, which integrate into existing hippocampal circuitry (reviewed by [116]). In the adult SVZ, NSCs (B1 cells) are located in the walls of the lateral ventricles neighbouring the hippocampus, cortex, striatum, and septum [117]. These cells give rise to Intermediate Progenitor cells, then immature neurons that migrate towards the olfactory bulb where they generate different types of interneurons (reviewed by [118]).

2.1. The Developmental Origin of Adult NSCs. The dogma concerning the developmental origin of SGZ and SVZ adult NSCs has been that NSCs within the SGZ arise from the dentate neuroepithelium during embryonic development and that NSCs within the SVZ represent a continuation of embryonic progenitors from the lateral wall that become specified during the early postnatal period. Recently however, both of these views have been challenged.

The view that SGZ NSCs solely originate from the dentate neuroepithelium was promoted by a pioneer study of rat dentate gyrus development from Altman and Bayer [119]. In this study, it was shown that the structural development of the dentate gyrus or primary neurogenesis [120] began when precursor cells migrated away from the dentate neuroepithelium to establish an abventricular site of proliferation from which the dentate gyrus would later form. Some of these cells, they argued, persisted to form the SGZ during the second postnatal week, thereby implicating the dentate neuroepithelium as the source of SGZ precursors. In 2013, Li and colleagues [121] directly challenged this idea using genetic manipulations and fate mapping experiments. Based on previous observations that Shh signalling is necessary for SGZ formation, but not for the formation of granule cell layer of the dentate gyrus [121, 122], they generated reporter mice in which cells receiving Shh signalling would be labelled. Curiously, labelled cells were mostly present in the ventricular zone of the ventral hippocampus from E14. Time course experiments using a cre-dependent reporter demonstrated that these cells in the ventral hippocampus migrated to the dentate gyrus through a septotemporal route. Reporter cells were found to label a subset of proliferating cells in the SGZ shortly after birth and also in 12-month-old mice. These results demonstrated that at least some SGZ NSCs arise from the ventral hippocampus and are specified during early development. However, as not all SGZ stem cells were labelled in the reporter mice in this study, this suggests that SGZ stem cells likely have multiple developmental origins. An interesting line of inquiry will be to establish whether there is a relationship between the developmental origin of SGZ stem cells and the emerging functional heterogeneity of this population [123].

Two recent studies have also challenged the origin of Type B1 stem cells in the rodent SVZ. Because prior studies had shown that B1 cells are derived from NSCs from multiple regions of the germinal ventricular zone surrounding the lateral ventricles during development [124, 125] and exhibit similar morphology and gene expression patterns, this had suggested a linear lineage relationship from NE cells to aRG cells to B1 cells. While this hypothesis would predict that B1 cells are specified during the early postnatal period when aRG cells become depleted, both Fuentealba and colleagues [126] and Furutachi and colleagues [31] used label-retention assays, such as thymidine analog injections, to demonstrate that the majority of B1 cells became quiescent (retained the analog label) if injected at E14, but not after this point. Further experiments using a retroviral barcoding paradigm confirmed these observations, as only aRG cells that had been transfected with the retroviral library prior to E14-E15 shared a clonal relationship with B1 cells in the postnatal brain. By delimiting the spatial and temporal origin of B1 cells, these studies have enabled a platform for future investigations to interrogate the signalling pathways involved in specification. As a window to these possibilities, in Furutachi and colleague's [31] study they identified p57 as a key molecule in generating quiescent NSCs. High expression of p57 during embryogenesis predicted a quiescent state for these cells in the adult brain, and loss of p57 led to reduced numbers of quiescent NSCs. This finding suggests that forced expression of p57 could be used to manipulate NSC number in the adult brain.

Together, these studies have shed a new light on the developmental origin of NSCs within the adult cerebral cortex. The most surprising and unifying element of these studies is that the NSCs within these niches are specified early during development, during mid-neurogenesis within the fetal brain. These findings emphasise the tight temporal regulation of adult NSC specification and suggest that the specification of these cells may not be solely due to stochastic processes during the early postnatal period. Indeed, the broad significance of these findings relates to enhancing our understanding of the developmental origin of adult NSCs in rodents and extending these investigations into primate brain development, which will be key to comprehending the basic biology of these cells in the human brain and so being able to harness their potential for use in regenerative medicine.

2.2. Regulation of Adult NSCs. Arguably just as important as studying the developmental origin of adult NSCs is to understand the niche factors and molecular signals that maintain these cellular populations throughout life. Excessive proliferation of adult NSCs (or loss of quiescence) leads to premature depletion and reduced neurogenesis in the long term [11, 127–129]. Conversely, quiescent NSCs must acutely respond to stimuli such as neural activity by proliferating and generating new neurons. The abundance of different niche and molecular factors that control this process of quiescence/proliferation/differentiation is a testament to how finely balanced this process is. Here, we review some of these niche and molecular cues.

2.2.1. The Niche Microenvironment. The spatially restricted nature of neurogenesis in the SGZ and SVZ of the adult brain suggests that there are important local cues that are released to maintain NSC populations. The structural organisation of the niche and the morphology of NSCs support this idea. For example, in the hippocampus, clusters of NSCs are located close to the tips of capillaries [130]. Likewise, B1 cells in the SVZ have a long basolateral process that terminates on blood

vessels and a thin apical tip that protrudes into the ventricular space and so is in direct contact with the CSF [131]. Moreover, most SVZ NSCs are located in the highly vascularised lateral side of the lateral ventricles [132]. Some of the factors that are released from vascular/endothelial cells and which regulate neurogenesis include the growth factors Vegf [133] and Pedf [134], the hormone erythropoietin [135], and neurotrophin NT-3 [136].

Interestingly, the cellular source of some of the most important and canonical niche signals that regulate neurogenesis, such as Notch, Wnt, and Shh pathways, is largely unknown. Some of these signals may come from the vasculature or CSF, but they could also come from other cellular components within the niche. For example, coculture of NSCs with niche astrocytes promotes neurogenesis, but this process is not evident when NSCs are cultured with astrocytes from nonneurogenic regions [137], suggesting that niche astrocytes secrete/express some of these important signalling molecules. Local microglia may also secrete ligands in response to exercise to promote neurogenesis [138]; likewise, ablation of neuronal progenitors in the hippocampus (Type 2 cells) or SVZ (Type A cells) through AraC treatment promotes NSC division, demonstrating that these progenitors are also a source of cellular feedback within the niche [117, 139]. In the hippocampus, neural activity also plays an important role in niche homeostasis. GABA released from parvalbumin-expressing interneurons maintains adult NSC in a quiescent state and inhibits self-renewal. As parvalbumin-expressing interneurons are activated by mature granule neurons of the dentate gyrus, this network may therefore suppress neurogenesis during periods of high local activity [20]. As another example, signalling through Nmdar promotes integration of newborn neurons into the existing circuitry [140]. Thus, neurotransmitter signalling within the hippocampus modulates neural activity, providing a local circuitry mechanism that influences the hippocampal NSC niche.

2.2.2. Molecular Regulation of NSC in the Adult Brain. There are many molecular regulators of NSC in the adult brain. An exhaustive discussion of all the molecules and signalling pathways that regulate NSC within the adult brain is beyond the scope of this review. Rather, here we highlight major factors that play a role in this process, which are listed in Table 1. These molecular regulators of adult NSC biology can broadly be defined as extrinsic or intrinsic factors or, alternately, grouped as signalling pathways in cases where the relationships between molecules are understood.

In adult NSCs, similar to their embryonic counterparts, some of the most well-established signalling nodes are the Notch, Bmp, and Wnt pathways. The first of these two pathways, Notch and Bmp, promote NSC quiescence [11, 128, 129], whereas the Wnt pathway promotes symmetric division of adult NSCs [14]. Another large cohort of molecules implicated in adult NSC biology via loss- or gain-of-function experiments are transcription factors. Groups of these proteins, such as members of the bHLH [35], T-box [43], Sox [41], and Nfi transcription factor family members [39], control large suites of genes and therefore act as master

Table 1: Molecular regulators of NSC in adult SGZ and SVZ. Summary of molecular regulators of adult NSC, grouped into ligands, neuropeptides, neurotransmitters, epigenetic, cell cycle regulators, and transcription factors.

Molecule/regulator	Key finding	Ref
Ligands		
Notch	Activation promotes quiescence	[9, 10]
Bmp	Activation promotes quiescence	[11–13]
Wnt	Promotes NSC symmetric division	[14]
Tgf-β	Promotes quiescence and survival	[15]
Neuropeptides		
Npy	Induces proliferation, migration, and differentiation of NSC	[16–19]
Neurotransmitters		
GABA	Maintains adult NSC quiescence	[20]
Epigenetic		
Chd7	Maintains adult NSC quiescence	[21]
Dnmt1/3a	Increased expression in differentiating NSC; upregulation favours neurogenic fate	[22, 23]
Gadd45	Required for expression of extrinsic factors from mature granule neurons that modulate neurogenesis	[24]
Hdac2	Required for NSC differentiation and appropriate expression of progenitor markers	[25]
Mbd	Loss-of-function reduces neurogenesis	[26]
Tet1	Positively regulates NSC proliferation	[27]
Cell cycle regulators		
p21	Maintains quiescence and negatively regulates SOX2 expression	[28, 29]
p27	Maintains quiescence	[30]
p57	Maintains quiescence	[31, 32]
Transcription factors		
Foxo3	Maintenance of progenitor cells and quiescence	[33, 34]
Ascl1	Controls neuron fate commitment; overexpression produces oligodendrocytes	[35–38] [39]
Nfix	Maintains NSC quiescence *in vitro*	[39]
Pax6	Maintenance of NSCs	[40]
Sox2	Maintains NSC self-renewal through Shh signalling	[41, 42]
Tbr2	Required for generation of Intermediate Progenitors in DG	[43]
Tlx	Required for NSC self-renewal through WNT and neuron fate commitment through Mash1	[36, 44, 45]
Rest	Maintenance of NSC	[46]

regulators over cellular processes such as quiescence, fate commitment, and differentiation.

Of increasing interest to the field is how epigenetic modifications modulate adult NSC behaviour. Chromatin modifications such as DNA methylation of proximal promoters affect the accessibility of chromatin and therefore transcription [141]. For example, the activity of Dnmt3a, a member of the Dnmt family that confers methylation of the 5th position of cytosine (5mC), is crucial for expression of neurogenic genes in adult NSCs [22]. Conversely Tet1, which demethylates cytosine residues by converting 5mC to 5-hydroxymethylcytosine (5hmC), is also crucial for normal adult hippocampal neurogenesis [27]. Indeed, the 5hmC mark is highly enriched in the brain and increases in the hippocampus with age [142]. Together, these examples demonstrate the important balance between methylation and demethylation during adult neurogenesis.

Looking forward, the study of noncoding RNAs will also be an important area in the adult neurogenesis field. Testament to this is a blunt experimental approach taken by Cernilogar and colleagues [143] where they deleted the RNAse III enzyme Dicer in neural tissue. This enzyme is required for the processing and generation of microRNAs and small interfering RNAs that function to silence the expression of specific protein coding transcripts. In this preliminary study, deletion of *Dicer* affected levels of the neuroblast marker doublecortin. Moreover, the RNA interference machinery comprising Dicer/Ago2 was enriched in the chromatin of differentiating versus undifferentiated neural progenitor cells. Similarly, the role of long noncoding RNAs (lncRNAs) has also recently implicated in adult neurogenesis. Of the few lncRNAs studied thus far, both negative regulators of neurogenesis, such as *Six3os* and *Dlx1as* [144], and a positive regulator, *Pnky* [145], have been identified.

Overall, the many regulators of SGZ and SVZ NSCs are testament to the inherent complexity of the cell biology of these cell populations. The future challenge will be to continue to characterise the biological regulators of adult NSCs using existing reductionist approaches and, crucially, to then place these findings in their cellular context through systems biology. In doing so, we will then be well placed to identify signalling pathways and molecules that are therapeutic targets for stem cell-based therapies for degenerative conditions. Increasingly, these efforts will also be accelerated by single-cell sequencing technologies. These technologies provide unprecedented insight into the diversity of cell types in adult neurogenic niches. For example, recent studies employing this technology have revealed subpopulations of NSCs that become activated after ischemic brain injury [146] or exposure to growth factors [147].

3. The Therapeutic Potential of NSCs

Understanding the molecular signals that regulate neurogenesis during development and within the adult neurogenic niches will help guide the development of NSC based therapies to treat human diseases and conditions. Since monitoring NSCs in human patients is restricted to only correlative postmortem studies, the use of animal models has been essential to gain insight into applying NSCs for therapeutic benefit. For example, an informed view of how fetal NSCs generate interneurons *in vivo* has guided the attempts by some to generate these cells *in vitro* to treat epilepsies through cellular transplantation. Likewise, the dysfunction of adult NSCs is increasingly thought to underlie several major disorders including depression, anxiety, and neurodegenerative disease, although direct causal evidence is lacking. Accelerating endogenous neurogenesis in these contexts may therefore improve patient outcome (Figure 2). Here we discuss some of the most promising applications of NSC based therapies and research.

3.1. Insights and Therapeutic Applications Arising from the Study of Fetal Neurogenesis. The study of fetal neurogenesis has led to the evaluation of the potential for these cells and their progeny to treat disease. An elegant study by Baraban and colleagues reported that the transplantation of cells from the E13.5 medial ganglionic eminence (MGE) of mice could reduce the incidence and duration of seizures in a genetic model of epilepsy resulting from a mutation to the potassium channel gene *Kv1.1* [148]. Notably, the authors performed transplantations in presymptomatic (postnatal) mice and observed the widespread distribution and synaptic integration of donor cells that had differentiated into interneurons, suggesting that the presence of donor cells was likely responsible for alleviating seizure-like behaviour when the mice matured to adulthood. While this provides one tantalising experimental therapy for the treatment of epilepsy in humans (deletions to the KV1.1 are associated with one form of human epilepsy), it is unclear if the procedure leads to undesirable behavioural side effects. Nevertheless, these findings reveal the capacity for transplanted fetal cortical cells to disperse broadly within the site of injection so as to modulate excitation-inhibition balance. Such properties of transplanted cells also identify them as potential vectors for the delivery of therapeutic agents.

In addition to fetal cortical sources of cells for transplantation, a study by Gaspard and coworkers pioneered the culture of mouse embryonic stem cells in the presence of a chemical inhibitor of the morphogen Shh to generate cortical glutamatergic neurons in a temporally specified manner [149]. A further study by Espuny-Camacho and colleagues applied analogous cell culture techniques with human embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs) to generate functional cortical pyramidal neurons [150]. By drawing parallels between fetal NSC activities and the progression of cortical neurogenesis, the authors of both studies collectively recognised the potential for their cell-based approaches to model fetal cortical neurogenesis, as well as evaluate the suitability of iPSC and ES cell-derived neurons of distinct subtypes to treat brain injury or disease [151]. To support the viability of this approach, a subsequent study by Michelsen and coworkers demonstrated that mouse ES cell-derived cortical neurons with the molecular properties of visual cortical neurons could restore the axonal connectivity and functional properties of the mouse visual cortex following a lesion [152]. Notably, these ES cell-derived visuocortical-like neurons could not ameliorate the effects of

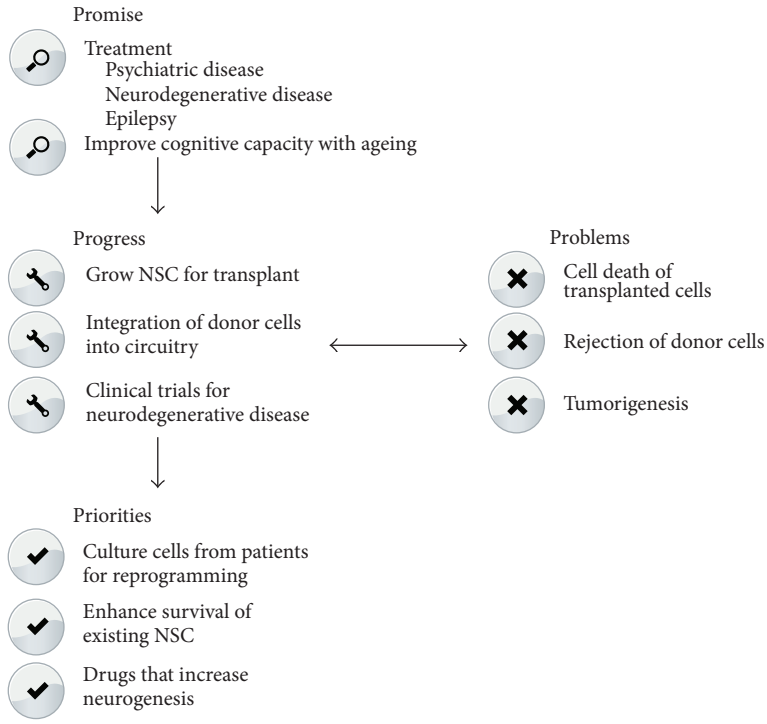

Figure 2: Summary of the promise, priorities, problems, and progress of the therapeutic application of NSCs. This schematic outlines the promise of the therapeutic application of NSCs, several of the priorities for applying NSCs for therapeutic application, some of the problems faced with using NSCs in patients, and finally what progress that has been made in the application of NSCs.

a lesion to the motor cortex, while grafting of motor cortex into the visual cortex lesion also did not lead to restoration of function. These studies demonstrate the importance for matching cell-based sources of neuronal subtypes with the graft site in order to restore region-specific brain function.

Recent technological innovations in cell culture and pluripotent stem cell biology have further converged on the capacity to study the molecular and cellular basis for developmental brain disorders using a three-dimensional culture system [153, 154]. A landmark report by Lancaster and colleagues described an extended rolling culture protocol to generate cerebral organoids from iPSCs [154]. Remarkably, these cerebral organoids recapitulated some of the features of early cortical development, including the spatial organisation of NSCs (marked by PAX6) and BPs (marked by TBR2). Crucially, parallel studies with organoids derived from a patient with microcephaly revealed premature neurodifferentiation, highlighting this as a possible mechanism that may underlie this condition. More recently, Camp and colleagues have applied single-cell gene expression approaches to study both cerebral organoids and fetal neocortical cells in order to identify the similarities and differences between the molecular profiles of cells derived from each of these sources [155]. It was interesting to note in their study that organoids comprised fewer BPs than APs, which could reflect the limitations of organoid culture or discrepancies in the time point between the organoid and fetal cortical tissue. Regardless, this technology is anticipated to accelerate our understanding of the extrinsic and intrinsic factors that influence human cortical development and disease.

While an understanding of fetal neurogenesis can guide our development of cell-based methods to model cortical neurogenesis and developmental brain disorders, a better understanding of the fetal NSC compartment will enable us to identify sources of repair cells which could be mobilized in times of injury or stress. Looking to vertebrates such as zebrafish, a recent excellent study by Barbosa and colleagues described the maintenance of MZ-like stem cells in the periphery of the forebrain from birth to adulthood, and these stem cells can be activated to restore lost brain tissue upon injury [156]. Given the identification of self-renewing MZ progenitors in the embryonic mouse [6] it remains to be determined if common molecular mechanisms could be drawn between MZ NSCs in mice and adult zebrafish NSCs to enable us to engineer mammalian MZ NSCs capable of extensive self-renewal and repair. Such approaches could be extended to account for the molecular mechanisms that underlie the self-renewal capacity of all progenitor cell types in the fetal cortex which have been described in this review.

Knowledge of the molecular mechanisms that drive fetal NSC neurogenesis could also lead to the development of novel cellular substrates for cell transplantation therapy. Guided by insights into the neurogenic programming potential for proneural bHLH factors, Masserdotti and colleagues have recently reported that forced expression of Ascl1 or Neurog2 in postnatal astrocytes and mouse embryonic

fibroblasts led to their reprogramming into neurons [157]. However, after prolonged culture astrocytes displayed a loss of neural reprogramming capacity by Neurog2, because of increased competition with the repressor REST complex for the neuronal target gene *NeuroD4* [157]. Such studies are extremely valuable to assess potential sources of patient-derived cells and appropriate culture conditions for neurogenic reprogramming for cell transplantation therapies, as well as the potential to stimulate neurogenesis and repair by endogenous NSCs in the postnatal brain.

3.2. Insights and Therapeutic Applications of Studying Adult NSCs. Harnessing existing endogenous populations of adult NSCs could likewise have numerous therapeutic applications in disease and for improving brain function in healthy individuals (Figure 2). For example, the stimulation of neurogenesis in the adult rodent brain is associated with several beneficial effects. Exercise enhances neurogenesis, with positive effects on learning [158, 159]; moreover, the generation of new neurons in the hippocampus is associated with improved spatial memory performance [158, 160–166] and contextual fear learning [167, 168]. Conversely, dysfunction of adult hippocampal NSCs is associated with depression and anxiety and has led to the neurogenic theory of depression [169, 170] that postulates two key features: depression accompanies decreased levels of neurogenesis and, secondly, that restoration of neurogenesis will ameliorate the symptoms. There is widespread evidence to support the first of these claims of this theory. For example, subjecting rodents to repeat restraint stress [171], unpredictable mild stress [172], social defeat stress [173], and social isolation stress [174] results in depression-like behaviours and impaired neurogenesis.

In support of the second claim of the neurogenic theory of depression, electroconvulsive therapy (ECT), which is a well-established tool for treating depression, increases hippocampal neurogenesis in adult rodents [175, 176]. Furthermore, chronic treatment with antidepressant drugs such as fluoxetine, reboxetine, and tranylcypromine also increases hippocampal neurogenesis in adult rodents [177]. In nonhuman primates, chronic fluoxetine and ECT increase hippocampal neurogenesis [178, 179], though whether this correlates with increased hippocampal-dependent learning and memory is not clear. Moreover, rodents with blocked neurogenesis do not recover from depression-like behaviours when antidepressants are chronically administered to them [179–183]. Thus, many treatments for depression enhance adult neurogenesis, and at least in rodents, neurogenesis is required for some aspects of antidepressant function. Confusingly, some recent studies have suggested there is a neurogenesis-independent mechanism of action of antidepressant drugs, showing there is no effect or only modest effects of reducing neurogenesis on the efficacy of antidepressants [180, 183–189]. Thus, while these aforementioned studies highlight an association between neurogenesis and depression, to what extent changes in neurogenesis and depression are causally linked and whether selectively enhancing adult neurogenesis in humans is sufficient or necessary to treat depression are still unclear [190].

Despite these unresolved issues, designing drugs that selectively increase hippocampal neurogenesis stands out as a logical therapeutic strategy in the treatment of depression, particularly since there have been few new antidepressant drugs with novel modes of action in the last decade (Figure 2). Importantly, two decades of research has broadened our understanding of the molecules and signalling pathways that serve to amplify adult hippocampal neurogenesis, providing new therapeutic targets. For example, inhibiting effectors of the Notch and Bmp signalling pathways that mediate NSC quiescence [11, 12, 128, 129] could result in a short-term increase in neurogenesis that may be of therapeutic benefit, though this needs to be considered in parallel with the potential depletion of the quiescent NSC pool that may arise from such a treatment. Other targets include boosting the expression of growth factors that decline with age [191, 192]. Likewise, the discovery that quiescent NSCs uniquely metabolise lipids compared to proliferating NSCs in both the hippocampus [193] and SVZ [194] opens a range of new drug targets. Most promisingly to date, an unbiased screen for compounds that increase neurogenesis in rodents uncovered P7C3 as a potential target [195]. This compound, which promotes the survival of newborn neurons [196], is currently in clinical trials to ameliorate neurodegenerative diseases and it also has known antidepressant effects. With an increased understanding of the molecular pathways regulating adult neurogenesis, drugs that are already on the market could be used off-label if they are known to affect these pathways. An example of this is metformin, a well-tolerated oral medication for diabetes that has recently been shown to have proneurogenic effects in mice [197].

Another potential therapeutic intervention of NSCs is to apply them to ameliorate the age-related cognitive decline of the brain. Ageing is associated with a decline in neurogenesis ([198–201]; plus see review [161]). Ageing seems to affect various aspects of neurogenesis. For example, many studies report a significant age-related decline in cell proliferation [199, 200, 202–205]. The greatest decline in cell proliferation tends to occur by middle-age, and only modest additional declines are reported between middle-age and senescence [201, 206–211]. Not only is cell proliferation affected, but also the capacity of neurons to migrate is compromised with ageing [212, 213].

Are these age-related changes in neurogenesis associated with compromised cognitive capacity? Studies report conflicting results. Aged rats that perform better in the Morris water maze test of spatial learning and memory have more proliferating cells and newborn neurons than age-matched controls [208, 214]. Other studies show no correlation or a negative correlation between proliferating cells and performance [215, 216]. Thus, facilitating neurogenesis during ageing could have beneficial impacts on cognitive function, but further studies are needed.

Several mechanisms have been put forward to explain the age-related decline in neurogenesis. Ageing has been associated with changes in the hippocampal NSC niche vasculature [217]. Moreover, blood-borne factors such as circulating chemokines can inhibit or promote neurogenesis in an age-dependent manner [218]. Growth factors that have

important roles in neurogenesis such as Vegf, Fgf2, Bdnf, and Wnt signalling decrease with age [219–221]. Indeed, it is largely these extrinsic factors and alterations to neurogenic niche environment that contribute to the decline in neurogenesis observed with age rather than changes intrinsic to the neural precursors themselves [222]. Recently, chronic administration in aged rats of a peptide known to have neuroprotective properties was shown to restore neurogenesis, synaptic plasticity, and memory [223], suggesting that induction of neurogenesis has beneficial effects on cognition during ageing. Moreover, increasing neuronal activity in the aged brain through seizures can induce quiescent NSCs to reenter the cell cycle and restore proliferation to a level comparable to the one observed in young animals [224]. Thus, targeting key molecules involved in neurogenesis or reactivating NSC offers therapeutic promise in reversing or ameliorating ageing-related changes in brain function (Figure 2).

4. Concluding Remarks

In this paper, we have highlighted the cellular and molecular diversity of NSCs in the fetal and adult cerebral cortex. This research is critical as a basis for our understanding of the dynamic properties of embryonic and adult NSCs and how we might be able to manipulate them at the cellular and molecular level. This work has been facilitated by rapid advances in molecular and cellular techniques, as well as sequencing modalities and lineage tracing paradigms. Indeed, this suite of basic research has served as a springboard to drive the therapeutic applications of NSCs towards the treatment of brain injury and disease. While many of these therapeutic approaches are in the early, preclinical stage, it is likely that the knowledge gleaned from the ongoing study of embryonic and adult NSCs will enable the continual refinement of cellular replacement techniques and the identification of therapeutic targets that will lead to real treatments for brain injury and disease in the clinic. Such achievements will realise the promise of NSC research, which has for a long time held the imagination and fuelled the hope for researchers, clinicians, patients, and the broader community.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lachlan Harris and Oressia Zalucki contributed equally to this work.

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References

[1] M. Rallu, J. G. Corbin, and G. Fishell, "Parsing the prosencephalon," *Nature Reviews Neuroscience*, vol. 3, no. 12, pp. 943–951, 2002.

[2] L. C. Greig, M. B. Woodworth, M. J. Galazo, H. Padmanabhan, and J. D. Macklis, "Molecular logic of neocortical projection neuron specification, development and diversity," *Nature Reviews Neuroscience*, vol. 14, no. 11, pp. 755–769, 2013.

[3] C. P. Wonders and S. A. Anderson, "The origin and specification of cortical interneurons," *Nature Reviews Neuroscience*, vol. 7, no. 9, pp. 687–696, 2006.

[4] M. B. Woodworth, L. Custo Greig, A. R. Kriegstein, and J. D. Macklis, "Snapshot: cortical development," *Cell*, vol. 151, no. 4, p. 918.e1, 2012.

[5] H. Markram, M. Toledo-Rodriguez, Y. Wang, A. Gupta, G. Silberberg, and C. Wu, "Interneurons of the neocortical inhibitory system," *Nature Reviews Neuroscience*, vol. 5, no. 10, pp. 793–807, 2004.

[6] M. R. Costa, N. Kessaris, W. D. Richardson, M. Götz, and C. Hedin-Pereira, "The marginal zone/layer I as a novel niche for neurogenesis and gliogenesis in developing cerebral cortex," *Journal of Neuroscience*, vol. 27, no. 42, pp. 11376–11388, 2007.

[7] F. Bielle, A. Griveau, N. Narboux-Nême et al., "Multiple origins of Cajal-Retzius cells at the borders of the developing pallium," *Nature Neuroscience*, vol. 8, no. 8, pp. 1002–1012, 2005.

[8] J. A. Cooper, "A mechanism for inside-out lamination in the neocortex," *Trends in Neurosciences*, vol. 31, no. 3, pp. 113–119, 2008.

[9] P. Chapouton, P. Skupien, B. Hesl et al., "Notch activity levels control the balance between quiescence and recruitment of adult neural stem cells," *The Journal of Neuroscience*, vol. 30, no. 23, pp. 7961–7974, 2010.

[10] I. Imayoshi and R. Kageyama, "The role of notch signaling in adult neurogenesis," *Molecular Neurobiology*, vol. 44, no. 1, pp. 7–12, 2011.

[11] M. A. Bonaguidi, C.-Y. Peng, T. McGuire et al., "Noggin expands neural stem cells in the adult hippocampus," *Journal of Neuroscience*, vol. 28, no. 37, pp. 9194–9204, 2008.

[12] H. Mira, Z. Andreu, H. Suh et al., "Signaling through BMPR-IA regulates quiescence and long-term activity of neural stem cells in the adult hippocampus," *Cell Stem Cell*, vol. 7, no. 1, pp. 78–89, 2010.

[13] A. M. Bond, G. L. Ming, and H. Song, "Adult mammalian neural stem cells and neurogenesis: five decades later," *Cell Stem Cell*, vol. 17, no. 4, pp. 385–395, 2015.

[14] D. Piccin and C. M. Morshead, "Wnt signaling regulates symmetry of division of neural stem cells in the adult brain and in response to injury," *Stem Cells*, vol. 29, no. 3, pp. 528–538, 2011.

[15] M. Kandasamy, B. Lehner, S. Kraus et al., "TGF-beta signalling in the adult neurogenic niche promotes stem cell quiescence as well as generation of new neurons," *Journal of Cellular and Molecular Medicine*, vol. 18, no. 7, pp. 1444–1459, 2014.

[16] F. Agasse, L. Bernardino, H. Kristiansen et al., "Neuropeptide Y promotes neurogenesis in murine subventricular zone," *Stem Cells*, vol. 26, no. 6, pp. 1636–1645, 2008.

[17] M. Decressac, L. Prestoz, J. Veran, A. Cantereau, M. Jaber, and A. Gaillard, "Neuropeptide Y stimulates proliferation, migration and differentiation of neural precursors from the subventricular zone in adult mice," *Neurobiology of Disease*, vol. 34, no. 3, pp. 441–449, 2009.

[18] O. W. Howell, K. Doyle, J. H. Goodman et al., "Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus," *Journal of Neurochemistry*, vol. 93, no. 3, pp. 560–570, 2005.

[19] N. Thiriet, F. Agasse, C. Nicoleau et al., "NPY promotes chemokinesis and neurogenesis in the rat subventricular zone," *Journal of Neurochemistry*, vol. 116, no. 6, pp. 1018–1027, 2011.

[20] J. Song, C. Zhong, M. A. Bonaguidi et al., "Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision," *Nature*, vol. 489, no. 7414, pp. 150–154, 2012.

[21] K. M. Jones, N. Saric, J. P. Russell, C. L. Andoniadou, P. J. Scambler, and M. A. Basson, "CHD7 maintains neural stem cell quiescence and prevents premature stem cell depletion in the adult hippocampus," *STEM CELLS*, vol. 33, no. 1, pp. 196–210, 2015.

[22] H. Wu, V. Coskun, J. Tao et al., "Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes," *Science*, vol. 329, no. 5990, pp. 444–447, 2010.

[23] R. P. Singh, K. Shiue, D. Schomberg, and F. C. Zhou, "Cellular epigenetic modifications of neural stem cell differentiation," *Cell Transplantation*, vol. 18, no. 10-11, pp. 1197–1211, 2009.

[24] D. K. Ma, M.-H. Jang, J. U. Guo et al., "Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis," *Science*, vol. 323, no. 5917, pp. 1074–1077, 2009.

[25] M. Jawerka, D. Colak, L. Dimou et al., "The specific role of histone deacetylase 2 in adult neurogenesis," *Neuron Glia Biology*, vol. 6, no. 2, pp. 93–107, 2010.

[26] X. Zhao, T. Ueba, B. R. Christie et al., "Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 11, pp. 6777–6782, 2003.

[27] R.-R. Zhang, Q.-Y. Cui, K. Murai et al., "Tet1 regulates adult hippocampal neurogenesis and cognition," *Cell Stem Cell*, vol. 13, no. 2, pp. 237–245, 2013.

[28] T. E. Kippin, D. J. Martens, and D. van der Kooy, "p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity," *Genes & Development*, vol. 19, no. 6, pp. 756–767, 2005.

[29] M. Á. Marqués-Torrejón, E. Porlan, A. Banito et al., "Cyclin-dependent kinase inhibitor p21 controls adult neural stem cell expansion by regulating Sox2 gene expression," *Cell Stem Cell*, vol. 12, no. 1, pp. 88–100, 2013.

[30] Z. Andreu, M. A. Khan, P. González-Gõmez et al., "The cyclin-dependent kinase inhibitor $p27^{kip1}$ regulates radial stem cell quiescence and neurogenesis in the adult hippocampus," *Stem Cells*, vol. 33, no. 1, pp. 219–229, 2015.

[31] S. Furutachi, H. Miya, T. Watanabe et al., "Slowly dividing neural progenitors are an embryonic origin of adult neural stem cells," *Nature Neuroscience*, vol. 18, no. 5, pp. 657–665, 2015.

[32] S. Furutachi, A. Matsumoto, K. I. Nakayama, and Y. Gotoh, "p57 controls adult neural stem cell quiescence and modulates the pace of lifelong neurogenesis," *The EMBO Journal*, vol. 32, no. 7, pp. 970–981, 2013.

[33] J.-H. Paik, Z. Ding, R. Narurkar et al., "FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis," *Cell Stem Cell*, vol. 5, no. 5, pp. 540–553, 2009.

[34] V. M. Renault, V. A. Rafalski, A. A. Morgan et al., "FoxO3 regulates neural stem cell homeostasis," *Cell Stem Cell*, vol. 5, no. 5, pp. 527–539, 2009.

[35] S. Jessberger, N. Toni, G. D. Clemenson Jr., J. Ray, and F. H. Gage, "Directed differentiation of hippocampal stem/progenitor cells in the adult brain," *Nature Neuroscience*, vol. 11, no. 8, pp. 888–893, 2008.

[36] M. Elmi, Y. Matsumoto, Z.-J. Zeng et al., "TLX activates MASH1 for induction of neuronal lineage commitment of adult hippocampal neuroprogenitors," *Molecular and Cellular Neuroscience*, vol. 45, no. 2, pp. 121–131, 2010.

[37] E. J. Kim, C. T. Leung, R. R. Reed, and J. E. Johnson, "In vivo analysis of Ascl1 defined progenitors reveals distinct developmental dynamics during adult neurogenesis and gliogenesis," *Journal of Neuroscience*, vol. 27, no. 47, pp. 12764–12774, 2007.

[38] E. J. Kim, J. L. Ables, L. K. Dickel, A. J. Eisch, and J. E. Johnson, "Ascl1 (Mash1) defines cells with long-term neurogenic potential in subgranular and subventricular zones in adult mouse brain," *PLoS ONE*, vol. 6, no. 3, Article ID e18472, 2011.

[39] B. Martynoga, J. L. Mateo, B. Zhou et al., "Epigenomic enhancer annotation reveals a key role for NFIX in neural stem cell quiescence," *Genes & Development*, vol. 27, no. 16, pp. 1769–1786, 2013.

[40] M. Maekawa, N. Takashima, Y. Arai et al., "Pax6 is required for production and maintenance of progenitor cells in postnatal hippocampal neurogenesis," *Genes to Cells*, vol. 10, no. 10, pp. 1001–1014, 2005.

[41] R. Favaro, M. Valotta, A. L. M. Ferri et al., "Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh," *Nature Neuroscience*, vol. 12, no. 10, pp. 1248–1256, 2009.

[42] A. L. M. Ferri, M. Cavallaro, D. Braida et al., "Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain," *Development*, vol. 131, no. 15, pp. 3805–3819, 2004.

[43] R. D. Hodge, B. R. Nelson, R. J. Kahoud et al., "Tbr2 is essential for hippocampal lineage progression from neural stem cells to intermediate progenitors and neurons," *Journal of Neuroscience*, vol. 32, no. 18, pp. 6275–6287, 2012.

[44] W. Niu, Y. Zou, C. Shen, and C.-L. Zhang, "Activation of postnatal neural stem cells requires nuclear receptor TLX," *Journal of Neuroscience*, vol. 31, no. 39, pp. 13816–13828, 2011.

[45] Q. Qu, G. Sun, W. Li et al., "Orphan nuclear receptor TLX activates Wnt/beta-catenin signalling to stimulate neural stem cell proliferation and self-renewal," *Nature Cell Biology*, vol. 12, no. 1, pp. 31–409, 2010.

[46] Z. Gao, K. Ure, P. Ding et al., "The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells," *The Journal of Neuroscience*, vol. 31, no. 26, pp. 9772–9786, 2011.

[47] M. Florio and W. B. Huttner, "Neural progenitors, neurogenesis and the evolution of the neocortex," *Development*, vol. 141, no. 11, pp. 2182–2194, 2014.

[48] G.-A. Pilz, A. Shitamukai, I. Reillo et al., "Amplification of progenitors in the mammalian telencephalon includes a new radial glial cell type," *Nature Communications*, vol. 4, article 2125, 2013.

[49] M. Götz and W. B. Huttner, "The cell biology of neurogenesis," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 10, pp. 777–788, 2005.

[50] M. E. Sauer and B. E. Walker, "Radioautographic study of interkinetic nuclear migration in the neural tube," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 101, no. 3, pp. 557–560, 1959.

[51] E. Taverna and W. B. Huttner, "Neural progenitor nuclei IN motion," *Neuron*, vol. 67, no. 6, pp. 906–914, 2010.

[52] W. B. Huttner and M. Brand, "Asymmetric division and polarity of neuroepithelial cells," *Current Opinion in Neurobiology*, vol. 7, no. 1, pp. 29–39, 1997.

[53] J. S. Gal, Y. M. Morozov, A. E. Ayoub, M. Chatterjee, P. Rakic, and T. F. Haydar, "Molecular and morphological heterogeneity of neural precursors in the mouse neocortical proliferative zones," *Journal of Neuroscience*, vol. 26, no. 3, pp. 1045–1056, 2006.

[54] C. Englund, A. Fink, C. Lau et al., "Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex," *Journal of Neuroscience*, vol. 25, no. 1, pp. 247–251, 2005.

[55] S. Cappello, A. Attardo, X. Wu et al., "The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface," *Nature Neuroscience*, vol. 9, no. 9, pp. 1099–1107, 2006.

[56] J. H. Lui, D. V. Hansen, and A. R. Kriegstein, "Development and evolution of the human neocortex," *Cell*, vol. 146, no. 1, pp. 18–36, 2011.

[57] D. V. Hansen, J. H. Lui, P. Flandin et al., "Non-epithelial stem cells and cortical interneuron production in the human ganglionic eminences," *Nature Neuroscience*, vol. 16, no. 11, pp. 1576–1587, 2013.

[58] O. Marin and J. L. R. Rubenstein, "A long, remarkable journey: tangential migration in the telencephalon," *Nature Reviews Neuroscience*, vol. 2, no. 11, pp. 780–790, 2001.

[59] K. Letinic, R. Zoncu, and P. Rakic, "Origin of GABAergic neurons in the human neocortex," *Nature*, vol. 417, no. 6889, pp. 645–649, 2002.

[60] S. Rakic and N. Zecevic, "Emerging complexity of layer I in human cerebral cortex," *Cerebral Cortex*, vol. 13, no. 10, pp. 1072–1083, 2003.

[61] I. Jakovcevski, N. Mayer, and N. Zecevic, "Multiple origins of human neocortical interneurons are supported by distinct expression of transcription factors," *Cerebral Cortex*, vol. 21, no. 8, pp. 1771–1782, 2011.

[62] N. Zecevic, F. Hu, and I. Jakovcevski, "Interneurons in the developing human neocortex," *Developmental Neurobiology*, vol. 71, no. 1, pp. 18–33, 2011.

[63] N. V. Radonjić, A. E. Ayoub, F. Memi et al., "Diversity of cortical interneurons in primates: the role of the dorsal proliferative niche," *Cell Reports*, vol. 9, no. 6, pp. 2139–2151, 2014.

[64] N. Al-Jaberi, S. Lindsay, S. Sarma, N. Bayatti, and G. J. Clowry, "The early fetal development of human neocortical GABAergic interneurons," *Cerebral Cortex*, vol. 25, no. 3, pp. 631–645, 2015.

[65] T. Ma, C. Wang, L. Wang et al., "Subcortical origins of human and monkey neocortical interneurons," *Nature Neuroscience*, vol. 16, no. 11, pp. 1588–1597, 2013.

[66] S. Fertuzinhos, E. Krsnik, Y. I. Kawasawa et al., "Selective depletion of molecularly defined cortical interneurons in human holoprosencephaly with severe striatal hypoplasia," *Cerebral Cortex*, vol. 19, no. 9, pp. 2196–2207, 2009.

[67] M. K. Lehtinen, M. W. Zappaterra, X. Chen et al., "The cerebrospinal fluid provides a proliferative niche for neural progenitor cells," *Neuron*, vol. 69, no. 5, pp. 893–905, 2011.

[68] M. Gulisano, V. Broccoli, C. Pardini, and E. Boncinelli, "Emx1 and emx2 show different patterns of expression during proliferation and differentiation of the developing cerebral cortex in the mouse," *European Journal of Neuroscience*, vol. 8, no. 5, pp. 1037–1050, 1996.

[69] S. Bulchand, E. A. Grove, F. D. Porter, and S. Tole, "LIM-homeodomain gene Lhx2 regulates the formation of the cortical hem," *Mechanisms of Development*, vol. 100, no. 2, pp. 165–175, 2001.

[70] V. S. Mangale, K. E. Hirokawa, P. R. V. Satyaki et al., "Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate," *Science*, vol. 319, no. 5861, pp. 304–309, 2008.

[71] L. C. Hsu, S. Nam, Y. Cui et al., "Lhx2 regulates the timing of β-catenin-dependent cortical neurogenesis," *Proceedings of the National Academy of Sciences*, vol. 112, no. 39, pp. 12199–12204, 2015.

[72] S.-J. Chou, C. G. Perez-Garcia, T. T. Kroll, and D. D. M. O'Leary, "Lhx2 specifies regional fate in Emx1 lineage of telencephalic progenitors generating cerebral cortex," *Nature Neuroscience*, vol. 12, no. 11, pp. 1381–1389, 2009.

[73] A. S. Shetty, G. Godbole, U. Maheshwari et al., "Lhx2 regulates a cortex-specific mechanism for barrel formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 50, pp. E4913–E4921, 2013.

[74] C. Hanashima, S. C. Li, L. Shen, E. Lai, and G. Fishell, "Foxg1 suppresses early cortical cell fate," *Science*, vol. 303, no. 5654, pp. 56–59, 2004.

[75] G. Miyoshi and G. Fishell, "Dynamic FoxG1 expression coordinates the integration of multipolar pyramidal neuron precursors into the cortical plate," *Neuron*, vol. 74, no. 6, pp. 1045–1058, 2012.

[76] K. Toma, T. Kumamoto, and C. Hanashima, "The timing of upper-layer neurogenesis is conferred by sequential derepression and negative feedback from deep-layer neurons," *Journal of Neuroscience*, vol. 34, no. 39, pp. 13259–13276, 2014.

[77] T. Kumamoto, K.-I. Toma, Gunadi et al., "Foxg1 coordinates the switch from nonradially to radially migrating glutamatergic subtypes in the neocortex through spatiotemporal repression," *Cell Reports*, vol. 3, no. 3, pp. 931–945, 2013.

[78] T. T. Kroll and D. D. M. O'Leary, "Ventralized dorsal telencephalic progenitors in Pax6 mutant mice generate GABA interneurons of a lateral ganglionic eminence fate," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 20, pp. 7374–7379, 2005.

[79] C. Schuurmans, O. Armant, M. Nieto et al., "Sequential phases of cortical specification involve neurogenin-dependent and -independent pathways," *The EMBO Journal*, vol. 23, no. 14, pp. 2892–2902, 2004.

[80] A. Stoykova, D. Treichel, M. Hallonet, and P. Gruss, "Pax6 modulates the dorsoventral patterning of the mammalian telencephalon," *The Journal of Neuroscience*, vol. 20, no. 21, pp. 8042–8050, 2000.

[81] H. Toresson, S. S. Potter, and K. Campbell, "Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2," *Development*, vol. 127, no. 20, pp. 4361–4371, 2000.

[82] K. M. Bishop, G. Goudreau, and D. D. M. O'Leary, "Regulation of area identity in the mammalian neocortex by Emx2 and Pax6," *Science*, vol. 288, no. 5464, pp. 344–349, 2000.

[83] K. M. Bishop, S. Garel, Y. Nakagawa, J. L. R. Rubenstein, and D. D. M. O'Leary, "Emx1 and Emx2 cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical efferents, and thalamocortical pathfinding," *Journal of Comparative Neurology*, vol. 457, no. 4, pp. 345–360, 2003.

[84] L. Muzio, B. Di Benedetto, A. Stoykova, E. Boncinelli, P. Gruss, and A. Mallamaci, "Conversion of cerebral cortex into basal ganglia in $Emx2^{-/-}Pax6^{Sey/Sey}$ double-mutant mice," *Nature Neuroscience*, vol. 5, no. 8, pp. 737–745, 2002.

[85] G. Estivill-Torrus, H. Pearson, V. van Heyningen, D. J. Price, and P. Rashbass, "Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors," *Development*, vol. 129, no. 2, pp. 455–466, 2002.

[86] M. Gotz, A. Stoykova, and P. Gruss, "Pax6 controls radial glia differentiation in the cerebral cortex," *Neuron*, vol. 21, no. 5, pp. 1031–1044, 1998.

[87] J. C. Quinn, M. Molinek, B. S. Martynoga et al., "Pax6 controls cerebral cortical cell number by regulating exit from the cell cycle and specifies cortical cell identity by a cell autonomous mechanism," *Developmental Biology*, vol. 302, no. 1, pp. 50–65, 2007.

[88] M. Nieto, E. S. Monuki, H. Tang et al., "Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II-IV of the cerebral cortex," *Journal of Comparative Neurology*, vol. 479, no. 2, pp. 168–180, 2004.

[89] V. Tarabykin, A. Stoykova, N. Usman, and P. Gruss, "Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression," *Development*, vol. 128, no. 11, pp. 1983–1993, 2001.

[90] C. Zimmer, M.-C. Tiveron, R. Bodmer, and H. Cremer, "Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons," *Cerebral Cortex*, vol. 14, no. 12, pp. 1408–1420, 2004.

[91] O. Britz, P. Mattar, L. Nguyen et al., "A role for proneural genes in the maturation of cortical progenitor cells," *Cerebral Cortex*, vol. 16, supplement 1, pp. i138–i151, 2006.

[92] C. Fode, Q. Ma, S. Casarosa, S.-L. Ang, D. J. Anderson, and F. Guillemot, "A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons," *Genes and Development*, vol. 14, no. 1, pp. 67–80, 2000.

[93] S. Casarosa, C. Fode, and F. Guillemot, "Mash1 regulates neurogenesis in the ventral telencephalon," *Development*, vol. 126, no. 3, pp. 525–534, 1999.

[94] B. J. Molyneaux, P. Arlotta, J. R. L. Menezes, and J. D. Macklis, "Neuronal subtype specification in the cerebral cortex," *Nature Reviews Neuroscience*, vol. 8, no. 6, pp. 427–437, 2007.

[95] M. Nieto, C. Schuurmans, O. Britz, and F. Guillemot, "Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors," *Neuron*, vol. 29, no. 2, pp. 401–413, 2001.

[96] Y. Sun, M. Nadal-Vicens, S. Misono et al., "Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms," *Cell*, vol. 104, no. 3, pp. 365–376, 2001.

[97] H. Okado, C. Ohtaka-Maruyama, Y. Sugitani et al., "The transcriptional repressor RP58 is crucial for cell-division patterning and neuronal survival in the developing cortex," *Developmental Biology*, vol. 331, no. 2, pp. 140–151, 2009.

[98] S. Hirai, A. Miwa, C. Ohtaka-Maruyama et al., "RP58 controls neuron and astrocyte differentiation by downregulating the expression of Id1-4 genes in the developing cortex," *The EMBO Journal*, vol. 31, no. 5, pp. 1190–1202, 2012.

[99] J. I. Heng, Z. Qu, C. Ohtaka-Maruyama et al., "The zinc finger transcription factor RP58 negatively regulates Rnd2 for the control of neuronal migration during cortical development," *Cerebral Cortex*, vol. 25, no. 3, pp. 806–816, 2015.

[100] C. Xiang, V. Baubet, S. Pal et al., "RP58/ZNF238 directly modulates proneurogenic gene levels and is required for neuronal differentiation and brain expansion," *Cell Death and Differentiation*, vol. 19, no. 4, pp. 692–702, 2012.

[101] C. Ohtaka-Maruyama, S. Hirai, A. Miwa et al., "RP58 regulates the multipolar-bipolar transition of newborn neurons in the developing cerebral cortex," *Cell Reports*, vol. 3, no. 2, pp. 458–471, 2013.

[102] U. Kruse, F. Qian, and A. E. Sippel, "Identification of a fourth nuclear factor I gene in chicken by cDNA cloning: NFI-X," *Nucleic Acids Research*, vol. 19, no. 23, p. 6641, 1991.

[103] R. A. W. Rupp, U. Kruse, G. Multhaup, U. Göbel, K. Beyreuther, and A. E. Sippel, "Chicken NFI/TGGCA proteins are encoded by at least three independent genes: NFI-A, NFI-B and NFI-C with homologues in mammalian genomes," *Nucleic Acids Research*, vol. 18, no. 9, pp. 2607–2616, 1990.

[104] L. Harris, L. A. Genovesi, R. M. Gronostajski, B. J. Wainwright, and M. Piper, "Nuclear factor one transcription factors: divergent functions in developmental versus adult stem cell populations," *Developmental Dynamics*, vol. 244, no. 3, pp. 227–238, 2015.

[105] R. M. Gronostajski, "Roles of the NFI/CTF gene family in transcription and development," *Gene*, vol. 249, no. 1-2, pp. 31–45, 2000.

[106] C. E. Campbell, M. Piper, C. Plachez et al., "The transcription factor Nfix is essential for normal brain development," *BMC Developmental Biology*, vol. 8, article 52, 2008.

[107] C. Plachez, C. Lindwall, N. Sunn et al., "Nuclear factor I gene expression in the developing forebrain," *Journal of Comparative Neurology*, vol. 508, no. 3, pp. 385–401, 2008.

[108] J. Betancourt, S. Katzman, and B. Chen, "Nuclear factor one B regulates neural stem cell differentiation and axonal projection of corticofugal neurons," *Journal of Comparative Neurology*, vol. 522, no. 1, pp. 6–35, 2014.

[109] Y. H. E. Heng, R. C. McLeay, T. J. Harvey et al., "NFIX regulates neural progenitor cell differentiation during hippocampal morphogenesis," *Cerebral Cortex*, vol. 24, no. 1, pp. 261–279, 2014.

[110] M. Piper, G. Barry, J. Hawkins et al., "NFIA controls telencephalic progenitor cell differentiation through repression of the Notch effector Hes1," *Journal of Neuroscience*, vol. 30, no. 27, pp. 9127–9139, 2010.

[111] M. Piper, L. Harris, G. Barry et al., "Nuclear factor one X regulates the development of multiple cellular populations in the postnatal cerebellum," *Journal of Comparative Neurology*, vol. 519, no. 17, pp. 3532–3548, 2011.

[112] T. Shu, K. G. Butz, C. Plachez, R. M. Gronostajski, and L. J. Richards, "Abnormal development of forebrain midline glia and commissural projections in Nfia knock-out mice," *The Journal of Neuroscience*, vol. 23, no. 1, pp. 203–212, 2003.

[113] M. Namihira, J. Kohyama, K. Semi et al., "Committed neuronal precursors confer astrocytic potential on residual neural precursor cells," *Developmental Cell*, vol. 16, no. 2, pp. 245–255, 2009.

[114] M. Piper, G. Barry, T. J. Harvey et al., "NFIB-mediated repression of the epigenetic factor Ezh2 regulates cortical development," *Journal of Neuroscience*, vol. 34, no. 8, pp. 2921–2930, 2014.

[115] M. Florio, M. Albert, E. Taverna et al., "Human-specific gene ARHGAP11B promotes basal progenitor amplification and neocortex expansion," *Science*, vol. 347, no. 6229, pp. 1465–1470, 2015.

[116] G.-L. Ming and H. Song, "Adult neurogenesis in the mammalian brain: significant answers and significant questions," *Neuron*, vol. 70, no. 4, pp. 687–702, 2011.

[117] F. Doetsch, I. Caille, D. A. Lim, J. M. Garcia-Verdugo, and A. Alvarez-Buylla, "Subventricular zone astrocytes are neural stem cells in the adult mammalian brain," *Cell*, vol. 97, no. 6, pp. 703–716, 1999.

[118] G. Lepousez, M. T. Valley, and P.-M. Lledo, "The impact of adult neurogenesis on olfactory bulb circuits and computations," *Annual Review of Physiology*, vol. 75, pp. 339–363, 2013.

[119] J. Altman and S. A. Bayer, "Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods," *Journal of Comparative Neurology*, vol. 301, no. 3, pp. 365–381, 1990.

[120] Z. Nicola, K. Fabel, and G. Kempermann, "Development of the adult neurogenic niche in the hippocampus of mice," *Frontiers in Neuroanatomy*, vol. 9, article 53, 2015.

[121] G. Li, L. Fang, G. Fernández, and S. Pleasure, "The ventral hippocampus is the embryonic origin for adult neural stem cells in the dentate gyrus," *Neuron*, vol. 78, no. 4, pp. 658–672, 2013.

[122] Y.-G. Han, N. Spassky, M. Romaguera-Ros et al., "Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells," *Nature Neuroscience*, vol. 11, no. 3, pp. 277–284, 2008.

[123] D. J. Jhaveri, I. O'Keeffe, G. J. Robinson et al., "Purification of neural precursor cells reveals the presence of distinct, stimulus-specific subpopulations of quiescent precursors in the adult mouse hippocampus," *Journal of Neuroscience*, vol. 35, no. 21, pp. 8132–8144, 2015.

[124] F. T. Merkle, A. D. Tramontin, J. M. García-Verdugo, and A. Alvarez-Buylla, "Radial glia give rise to adult neural stem cells in the subventricular zone," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 50, pp. 17528–17532, 2004.

[125] K. M. Young, M. Fogarty, N. Kessaris, and W. D. Richardson, "Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb," *Journal of Neuroscience*, vol. 27, no. 31, pp. 8286–8296, 2007.

[126] L. C. Fuentealba, S. B. Rompani, J. I. Parraguez et al., "Embryonic origin of postnatal neural stem cells," *Cell*, vol. 161, no. 7, pp. 1644–1655, 2015.

[127] J. R. Valcourt, J. M. S. Lemons, E. M. Haley, M. Kojima, O. O. Demuren, and H. A. Coller, "Staying alive: metabolic adaptations to quiescence," *Cell Cycle*, vol. 11, no. 9, pp. 1680–1696, 2012.

[128] I. Imayoshi, M. Sakamoto, M. Yamaguchi, K. Mori, and R. Kageyama, "Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains," *Journal of Neuroscience*, vol. 30, no. 9, pp. 3489–3498, 2010.

[129] O. Ehm, C. Göritz, M. Covic et al., "RBPJκ-dependent signaling is essential for long-term maintenance of neural stem cells in the adult hippocampus," *Journal of Neuroscience*, vol. 30, no. 41, pp. 13794–13807, 2010.

[130] T. D. Palmer, A. R. Willhoite, and F. H. Gage, "Vascular niche for adult hippocampal neurogenesis," *Journal of Comparative Neurology*, vol. 425, no. 4, pp. 479–494, 2000.

[131] Z. Mirzadeh, F. T. Merkle, M. Soriano-Navarro, J. M. Garcia-Verdugo, and A. Alvarez-Buylla, "Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain," *Cell Stem Cell*, vol. 3, no. 3, pp. 265–278, 2008.

[132] I. Kazanis, J. D. Lathia, T. J. Vadakkan et al., "Quiescence and activation of stem and precursor cell populations in the subependymal zone of the mammalian brain are associated with distinct cellular and extracellular matrix signals," *The Journal of Neuroscience*, vol. 30, no. 29, pp. 9771–9781, 2010.

[133] K. Jin, Y. Zhu, Y. Sun, X. O. Mao, L. Xie, and D. A. Greenberg, "Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 18, pp. 11946–11950, 2002.

[134] C. Ramírez-Castillejo, F. Sánchez-Sánchez, C. Andreu-Agulló et al., "Pigment epithelium-derived factor is a niche signal for neural stem cell renewal," *Nature Neuroscience*, vol. 9, no. 3, pp. 331–339, 2006.

[135] T. Shingo, S. Todd Sorokan, T. Shimazaki, and S. Weiss, "Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells," *The Journal of Neuroscience*, vol. 21, no. 24, pp. 9733–9743, 2001.

[136] A. C. Delgado, S. R. Ferrón, D. Vicente et al., "Endothelial NT-3 delivered by vasculature and CSF promotes quiescence of subependymal neural stem cells through nitric oxide induction," *Neuron*, vol. 83, no. 3, pp. 572–585, 2014.

[137] H. Song, C. F. Stevens, and F. H. Gage, "Astroglia induce neurogenesis from adult neural stem cells," *Nature*, vol. 417, no. 6884, pp. 39–44, 2002.

[138] J. Vukovic, M. J. Colditz, D. G. Blackmore, M. J. Ruitenberg, and P. F. Bartlett, "Microglia modulate hippocampal neural precursor activity in response to exercise and aging," *Journal of Neuroscience*, vol. 32, no. 19, pp. 6435–6443, 2012.

[139] B. Seri, J. M. García-Verdugo, B. S. McEwen, and A. Alvarez-Buylla, "Astrocytes give rise to new neurons in the adult mammalian hippocampus," *The Journal of Neuroscience*, vol. 21, no. 18, pp. 7153–7160, 2001.

[140] A. Tashiro, V. M. Sandler, N. Toni, C. Zhao, and F. H. Gage, "NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus," *Nature*, vol. 442, no. 7105, pp. 929–933, 2006.

[141] B. Yao and P. Jin, "Unlocking epigenetic codes in neurogenesis," *Genes and Development*, vol. 28, no. 12, pp. 1253–1271, 2014.

[142] M. Münzel, D. Globisch, T. Brückl et al., "Quantification of the sixth DNA base hydroxymethylcytosine in the brain," *Angewandte Chemie—International Edition*, vol. 49, no. 31, pp. 5375–5377, 2010.

[143] F. M. Cernilogar, R. Di Giaimo, F. Rehfeld, S. Cappello, and D. C. Lie, "RNA interference machinery-mediated gene regulation in mouse adult neural stem cells," *BMC Neuroscience*, vol. 16, article 60, 2015.

[144] A. D. Ramos, A. Diaz, A. Nellore et al., "Integration of genome-wide approaches identifies lncRNAs of adult neural stem cells and their progeny in vivo," *Cell Stem Cell*, vol. 12, no. 5, pp. 616–628, 2013.

[145] A. D. Ramos, R. E. Andersen, S. J. Liu et al., "The long noncoding RNA *Pnky* regulates neuronal differentiation of

embryonic and postnatal neural stem cells," *Cell Stem Cell*, vol. 16, no. 4, pp. 439–447, 2015.

[146] E. Llorens-Bobadilla, S. Zhao, A. Baser, G. Saiz-Castro, K. Zwadlo, and A. Martin-Villalba, "Single-cell transcriptomics reveals a population of dormant neural stem cells that become activated upon brain injury," *Cell Stem Cell*, vol. 17, no. 3, pp. 329–340, 2015.

[147] Y. Luo, V. Coskun, A. Liang et al., "Single-cell transcriptome analyses reveal signals to activate dormant neural stem cells," *Cell*, vol. 161, no. 5, pp. 1175–1186, 2015.

[148] S. C. Baraban, D. G. Southwell, R. C. Estrada et al., "Reduction of seizures by transplantation of cortical GABAergic interneuron precursors into Kv1.1 mutant mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 36, pp. 15472–15477, 2009.

[149] N. Gaspard, T. Bouschet, R. Hourez et al., "An intrinsic mechanism of corticogenesis from embryonic stem cells," *Nature*, vol. 455, no. 7211, pp. 351–357, 2008.

[150] I. Espuny-Camacho, K. A. Michelsen, D. Gall et al., "Pyramidal neurons derived from human pluripotent stem cells integrate efficiently into mouse brain circuits in vivo," *Neuron*, vol. 77, no. 3, pp. 440–456, 2013.

[151] N. Gaspard and P. Vanderhaeghen, "Mechanisms of neural specification from embryonic stem cells," *Current Opinion in Neurobiology*, vol. 20, no. 1, pp. 37–43, 2010.

[152] K. A. Michelsen, S. Acosta-Verdugo, M. Benoit-Marand et al., "Area-specific reestablishment of damaged circuits in the adult cerebral cortex by cortical neurons derived from mouse embryonic stem cells," *Neuron*, vol. 85, no. 5, pp. 982–997, 2015.

[153] M. A. Lancaster and J. A. Knoblich, "Generation of cerebral organoids from human pluripotent stem cells," *Nature Protocols*, vol. 9, no. 10, pp. 2329–2340, 2014.

[154] M. A. Lancaster, M. Renner, C.-A. Martin et al., "Cerebral organoids model human brain development and microcephaly," *Nature*, vol. 501, no. 7467, pp. 373–379, 2013.

[155] J. G. Camp, F. Badsha, M. Florio et al., "Human cerebral organoids recapitulate gene expression programs of fetal neocortex development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 51, pp. 15672–15677, 2015.

[156] J. S. Barbosa, R. Sanchez-Gonzalez, R. Di Giaimo et al., "Live imaging of adult neural stem cell behavior in the intact and injured zebrafish brain," *Science*, vol. 348, no. 6236, pp. 789–793, 2015.

[157] G. Masserdotti, S. Gillotin, B. Sutor et al., "Transcriptional mechanisms of proneural factors and rest in regulating neuronal reprogramming of astrocytes," *Cell Stem Cell*, vol. 17, no. 1, pp. 74–88, 2015.

[158] H. van Praag, B. R. Christie, T. J. Sejnowski, and F. H. Gage, "Running enhances neurogenesis, learning, and long-term potentiation in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13427–13431, 1999.

[159] H. van Praag, T. Shubert, C. Zhao, and F. H. Gage, "Exercise enhances learning and hippocampal neurogenesis in aged mice," *Journal of Neuroscience*, vol. 25, no. 38, pp. 8680–8685, 2005.

[160] W. Deng, M. D. Saxe, I. S. Gallina, and F. H. Gage, "Adult-born hippocampal dentate granule cells undergoing maturation modulate learning and memory in the brain," *Journal of Neuroscience*, vol. 29, no. 43, pp. 13532–13542, 2009.

[161] E. Drapeau and D. N. Abrous, "Stem cell review series: role of neurogenesis in age-related memory disorders," *Aging Cell*, vol. 7, no. 4, pp. 569–589, 2008.

[162] D. Dupret, J.-M. Revest, M. Koehl et al., "Spatial relational memory requires hippocampal adult neurogenesis," *PLoS ONE*, vol. 3, no. 4, Article ID e1959, 2008.

[163] E. Gould, A. Beylin, P. Tanapat, A. Reeves, and T. J. Shors, "Learning enhances adult neurogenesis in the hippocampal formation," *Nature Neuroscience*, vol. 2, no. 3, pp. 260–265, 1999.

[164] I. Imayoshi, M. Sakamoto, T. Ohtsuka et al., "Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain," *Nature Neuroscience*, vol. 11, pp. 1153–1161, 2008.

[165] M. Nilsson, E. Perfilieva, U. Johansson, O. Orwar, and P. S. Eriksson, "Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory," *Journal of Neurobiology*, vol. 39, no. 4, pp. 569–578, 1999.

[166] V. Ramirez-Amaya, D. F. Marrone, F. H. Gage, P. F. Worley, and C. A. Barnes, "Integration of new neurons into functional neural networks," *Journal of Neuroscience*, vol. 26, no. 47, pp. 12237–12241, 2006.

[167] K. Pham, B. S. McEwen, J. E. Ledoux, and K. Nader, "Fear learning transiently impairs hippocampal cell proliferation," *Neuroscience*, vol. 130, no. 1, pp. 17–24, 2005.

[168] M. D. Saxe, F. Battaglia, J.-W. Wang et al., "Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 46, pp. 17501–17506, 2006.

[169] B. L. Jacobs, H. van Praag, and F. H. Gage, "Adult brain neurogenesis and psychiatry: a novel theory of depression," *Molecular Psychiatry*, vol. 5, no. 3, pp. 262–269, 2000.

[170] A. Sahay and R. Hen, "Adult hippocampal neurogenesis in depression," *Nature Neuroscience*, vol. 10, no. 9, pp. 1110–1115, 2007.

[171] K. Pham, J. Nacher, P. R. Hof, and B. S. McEwen, "Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus," *European Journal of Neuroscience*, vol. 17, no. 4, pp. 879–886, 2003.

[172] K.-J. Lee, S.-J. Kim, S.-W. Kim et al., "Chronic mild stress decreases survival, but not proliferation, of new-born cells in adult rat hippocampus," *Experimental and Molecular Medicine*, vol. 38, no. 1, pp. 44–54, 2006.

[173] P. Van Bokhoven, C. A. Oomen, W. J. G. Hoogendijk, A. B. Smit, P. J. Lucassen, and S. Spijker, "Reduction in hippocampal neurogenesis after social defeat is long-lasting and responsive to late antidepressant treatment," *European Journal of Neuroscience*, vol. 33, no. 10, pp. 1833–1840, 2011.

[174] A. Dranovsky, A. M. Picchini, T. Moadel et al., "Experience dictates stem cell fate in the adult hippocampus," *Neuron*, vol. 70, no. 5, pp. 908–923, 2011.

[175] T. M. Madsen, A. Treschow, J. Bengzon, T. G. Bolwig, O. Lindvall, and A. Tingström, "Increased neurogenesis in a model of electroconvulsive therapy," *Biological Psychiatry*, vol. 47, no. 12, pp. 1043–1049, 2000.

[176] B. W. Scott, J. M. Wojtowicz, and W. M. Burnham, "Neurogenesis in the dentate gyrus of the rat following electroconvulsive shock seizures," *Experimental Neurology*, vol. 165, no. 2, pp. 231–236, 2000.

[177] J. E. Malberg, A. J. Eisch, E. J. Nestler, and R. S. Duman, "Chronic antidepressant treatment increases neurogenesis in

adult rat hippocampus," *Journal of Neuroscience*, vol. 20, no. 24, pp. 9104–9110, 2000.

[178] T. D. Perera, J. D. Coplan, S. H. Lisanby et al., "Antidepressant-induced neurogenesis in the hippocampus of adult nonhuman primates," *The Journal of Neuroscience*, vol. 27, no. 18, pp. 4894–4901, 2007.

[179] T. D. Perera, A. J. Dwork, K. A. Keegan et al., "Necessity of hippocampal neurogenesis for the therapeutic action of antidepressants in adult nonhuman primates," *PLoS ONE*, vol. 6, no. 4, Article ID e17600, 2011.

[180] D. J. David, B. A. Samuels, Q. Rainer et al., "Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression," *Neuron*, vol. 62, no. 4, pp. 479–493, 2009.

[181] J. L. Onksen, E. J. Brown, and J. A. Blendy, "Selective deletion of a cell cycle checkpoint kinase (ATR) reduces neurogenesis and alters responses in rodent models of behavioral affect," *Neuropsychopharmacology*, vol. 36, no. 5, pp. 960–969, 2011.

[182] L. Santarelli, M. Saxe, C. Gross et al., "Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants," *Science*, vol. 301, no. 5634, pp. 805–809, 2003.

[183] A. Surget, M. Saxe, S. Leman et al., "Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal," *Biological Psychiatry*, vol. 64, no. 4, pp. 293–301, 2008.

[184] J. M. Bessa, D. Ferreira, I. Melo et al., "The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling," *Molecular Psychiatry*, vol. 14, no. 8, pp. 764–773, 2009.

[185] D. J. David, K. C. Klemenhagen, K. A. Holick et al., "Efficacy of the MCHR1 antagonist *N*-[3-(1-{[4-(3,4-difluorophenoxy)phenyl]methyl}(4-piperidyl))-4-methylphenyl]-2-methylpropanamide (SNAP 94847) in mouse models of anxiety and depression following acute and chronic administration is independent of hippocampal neurogenesis," *Journal of Pharmacology and Experimental Therapeutics*, vol. 321, no. 1, pp. 237–248, 2007.

[186] K. A. Holick, D. C. Lee, R. Hen, and S. C. Dulawa, "Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A receptor," *Neuropsychopharmacology*, vol. 33, no. 2, pp. 406–417, 2008.

[187] P. Jedynak, T. Kos, C. Sandi, L. Kaczmarek, and R. K. Filipkowski, "Mice with ablated adult brain neurogenesis are not impaired in antidepressant response to chronic fluoxetine," *Journal of Psychiatric Research*, vol. 56, no. 1, pp. 106–111, 2014.

[188] D. Meshi, M. R. Drew, M. Saxe et al., "Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment," *Nature Neuroscience*, vol. 9, no. 6, pp. 729–731, 2006.

[189] M. Nollet, P. Gaillard, A. Tanti, V. Girault, C. Belzung, and S. Leman, "Neurogenesis-independent antidepressant-like effects on behavior and stress axis response of a dual orexin receptor antagonist in a rodent model of depression," *Neuropsychopharmacology*, vol. 37, no. 10, pp. 2210–2221, 2012.

[190] B. R. Miller and R. Hen, "The current state of the neurogenic theory of depression and anxiety," *Current Opinion in Neurobiology*, vol. 30, pp. 51–58, 2015.

[191] E. Nieves-Martinez, W. E. Sonntag, A. Wilson et al., "Early-onset GH deficiency results in spatial memory impairment in mid-life and is prevented by GH supplementation," *Journal of Endocrinology*, vol. 204, no. 1, pp. 31–36, 2010.

[192] D. G. Blackmore, J. Vukovic, M. J. Waters, and P. F. Bartlett, "GH mediates exercise-dependent activation of SVZ neural precursor cells in aged mice," *PLoS ONE*, vol. 7, no. 11, Article ID e49912, 2012.

[193] M. Knobloch, S. M. G. Braun, L. Zurkirchen et al., "Metabolic control of adult neural stem cell activity by Fasn-dependent lipogenesis," *Nature*, vol. 493, no. 7431, pp. 226–230, 2013.

[194] L. K. Hamilton, M. Dufresne, S. E. Joppé et al., "Aberrant lipid metabolism in the forebrain niche suppresses adult neural stem cell proliferation in an animal model of Alzheimer's disease," *Cell Stem Cell*, vol. 17, no. 4, pp. 397–411, 2015.

[195] A. A. Pieper, S. Xie, E. Capota et al., "Discovery of a proneurogenic, neuroprotective chemical," *Cell*, vol. 142, no. 1, pp. 39–51, 2010.

[196] A. K. Walker, P. D. Rivera, Q. Wang et al., "The P7C3 class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis," *Molecular Psychiatry*, vol. 20, pp. 500–508, 2015.

[197] J. Wang, D. Gallagher, L. M. DeVito et al., "Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation," *Cell Stem Cell*, vol. 11, no. 1, pp. 23–35, 2012.

[198] N. M.-B. Ben Abdallah, L. Slomianka, A. L. Vyssotski, and H.-P. Lipp, "Early age-related changes in adult hippocampal neurogenesis in C57 mice," *Neurobiology of Aging*, vol. 31, no. 1, pp. 151–161, 2010.

[199] G. Kempermann, H. G. Kuhn, and F. H. Gage, "Experience-induced neurogenesis in the senescent dentate gyrus," *Journal of Neuroscience*, vol. 18, no. 9, pp. 3206–3212, 1998.

[200] H. G. Kuhn, H. Dickinson-Anson, and F. H. Gage, "Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation," *The Journal of Neuroscience*, vol. 16, no. 6, pp. 2027–2033, 1996.

[201] T. Seki and Y. Arai, "Age-related production of new granule cells in the adult dentate gyrus," *NeuroReport*, vol. 6, no. 18, pp. 2479–2482, 1995.

[202] H. A. Cameron and R. D. G. McKay, "Restoring production of hippocampal neurons in old age," *Nature Neuroscience*, vol. 2, no. 10, pp. 894–897, 1999.

[203] V. Lemaire, M. Koehl, M. Le Moal, and D. N. Abrous, "Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 20, pp. 11032–11037, 2000.

[204] V. Lemaire, S. Lamarque, M. Le Moal, P.-V. Piazza, and D. N. Abrous, "Postnatal stimulation of the pups counteracts prenatal stress-induced deficits in hippocampal neurogenesis," *Biological Psychiatry*, vol. 59, no. 9, pp. 786–792, 2006.

[205] D. G. Blackmore, M. G. Golmohammadi, B. Large, M. J. Waters, and R. L. Rietze, "Exercise increases neural stem cell number in a growth hormone-dependent manner, augmenting the regenerative response in aged mice," *Stem Cells*, vol. 27, no. 8, pp. 2044–2052, 2009.

[206] J. L. Bizon and M. Gallagher, "Production of new cells in the rat dentate gyrus over the lifespan: relation to cognitive decline," *European Journal of Neuroscience*, vol. 18, no. 1, pp. 215–219, 2003.

[207] L. Bondolfi, F. Ermini, J. M. Long, D. K. Ingram, and M. Jucker, "Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice," *Neurobiology of Aging*, vol. 25, no. 3, pp. 333–340, 2004.

[208] I. Driscoll, S. R. Howard, J. C. Stone et al., "The aging hippocampus: a multi-level analysis in the rat," *Neuroscience*, vol. 139, no. 4, pp. 1173–1185, 2006.

[209] G. Kronenberg, A. Bick-Sander, E. Bunk, C. Wolf, D. Ehninger, and G. Kempermann, "Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus," *Neurobiology of Aging*, vol. 27, no. 10, pp. 1505–1513, 2006.

[210] R. J. Lichtenwalner, M. E. Forbes, S. A. Bennett, C. D. Lynch, W. E. Sonntag, and D. R. Riddle, "Intracerebroventricular infusion of insulin-like growth factor-I ameliorates the age-related decline in hippocampal neurogenesis," *Neuroscience*, vol. 107, no. 4, pp. 603–613, 2001.

[211] M. S. Rao, B. Hattiangady, and A. K. Shetty, "The window and mechanisms of major age-related decline in the production of new neurons within the dentate gyrus of the hippocampus," *Aging Cell*, vol. 5, no. 6, pp. 545–558, 2006.

[212] V. M. Heine, S. Maslam, M. Joëls, and P. J. Lucassen, "Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation," *Neurobiology of Aging*, vol. 25, no. 3, pp. 361–375, 2004.

[213] M. S. Rao, B. Hattiangady, A. Abdel-Rahman, D. P. Stanley, and A. K. Shetty, "Newly born cells in the ageing dentate gyrus display normal migration, survival and neuronal fate choice but endure retarded early maturation," *European Journal of Neuroscience*, vol. 21, no. 2, pp. 464–476, 2005.

[214] E. Drapeau, W. Mayo, C. Aurousseau, M. Le Moal, P.-V. Piazza, and D. N. Abrous, "Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 2, pp. 14385–14390, 2003.

[215] J. L. Bizon, H. J. Lee, and M. Gallagher, "Neurogenesis in a rat model of age-related cognitive decline," *Aging Cell*, vol. 3, no. 4, pp. 227–234, 2004.

[216] D. A. Merrill, R. Karim, M. Darraq, A. A. Chiba, and M. H. Tuszynski, "Hippocampal cell genesis does not correlate with spatial learning ability in aged rats," *Journal of Comparative Neurology*, vol. 459, no. 2, pp. 201–207, 2003.

[217] B. Hattiangady and A. K. Shetty, "Aging does not alter the number or phenotype of putative stem/progenitor cells in the neurogenic region of the hippocampus," *Neurobiology of Aging*, vol. 29, no. 1, pp. 129–147, 2008.

[218] S. A. Villeda, J. Luo, K. I. Mosher et al., "The ageing systemic milieu negatively regulates neurogenesis and cognitive function," *Nature*, vol. 477, no. 7362, pp. 90–96, 2011.

[219] G. M. Bernal and D. A. Peterson, "Phenotypic and gene expression modification with normal brain aging in GFAP-positive astrocytes and neural stem cells," *Aging Cell*, vol. 10, no. 3, pp. 466–482, 2011.

[220] B. Hattiangady, M. S. Rao, G. A. Shetty, and A. K. Shetty, "Brain-derived neurotrophic factor, phosphorylated cyclic AMP response element binding protein and neuropeptide Y decline as early as middle age in the dentate gyrus and CA1 and CA3 subfields of the hippocampus," *Experimental Neurology*, vol. 195, no. 2, pp. 353–371, 2005.

[221] A. K. Shetty, B. Hattiangady, and G. A. Shetty, "Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes," *Glia*, vol. 51, no. 3, pp. 173–186, 2005.

[222] D. R. Riddle and R. J. Lichtenwalner, "Neurogenesis in the adult and aging brain," in *Brain Aging: Models, Methods, and Mechanisms*, D. R. Riddle, Ed., chapter 6, CRC Press, Boca Raton, Fla, USA, 2007.

[223] S. Bolognin, M. Buffelli, J. Puoliväli, and K. Iqbal, "Rescue of cognitive-aging by administration of a neurogenic and/or neurotrophic compound," *Neurobiology of Aging*, vol. 35, no. 9, pp. 2134–2146, 2014.

[224] S. Lugert, O. Basak, P. Knuckles et al., "Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging," *Cell Stem Cell*, vol. 6, no. 5, pp. 445–456, 2010.

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Research Article

Innervation of Cochlear Hair Cells by Human Induced Pluripotent Stem Cell-Derived Neurons *In Vitro*

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Induced pluripotent stem cells (iPSCs) may serve as an autologous source of replacement neurons in the injured cochlea, if they can be successfully differentiated and reconnected with residual elements in the damaged auditory system. Here, we explored the potential of hiPSC-derived neurons to innervate early postnatal hair cells, using established *in vitro* assays. We compared two hiPSC lines against a well-characterized hESC line. After ten days' coculture *in vitro*, hiPSC-derived neural processes contacted inner and outer hair cells in whole cochlear explant cultures. Neural processes from hiPSC-derived neurons also made contact with hair cells in denervated sensory epithelia explants and expressed synapsin at these points of contact. Interestingly, hiPSC-derived neurons cocultured with hair cells at an early stage of differentiation formed synapses with a higher number of hair cells, compared to hiPSC-derived neurons cocultured at a later stage of differentiation. Notable differences in the innervation potentials of the hiPSC-derived neurons were also observed and variations existed between the hiPSC lines in their innervation efficiencies. Collectively, these data illustrate the promise of hiPSCs for auditory neuron replacement and highlight the need to develop methods to mitigate variabilities observed amongst hiPSC lines, in order to achieve reliable clinical improvements for patients.

1. Introduction

The ANs are responsible for faithfully transmitting acoustic information from the inner ear to the brain. The cell bodies of these neurons reside in a bony channel called Rosenthal's canal, which is located in the middle (modiolus) of the cochlea. Each of these cell bodies extends a peripheral process towards the organ of Corti to innervate the sensory hair cells, while the central processes project into the auditory nerve and ultimately synapse with neurons in the cochlear nucleus. In severe sensorineural hearing loss, the numbers of these neurons are significantly reduced or lost [1, 2], resulting in a breakdown of sound transmission to the brain. A variety of measures have been explored over the last two decades to restore or replace the damaged ANs following hearing loss, one being the use of stem cells. In order for stem cells to be used as a therapy for AN replacement, it is important that the donor cells are derived from a suitable source and are capable of innervating the appropriate cells/tissues in the peripheral and central auditory system [3].

Several studies have explored the capacity for various human stem cell types to grow towards sensory hair cells [3, 4] and cochlear nucleus tissues [5, 6] *in vitro*. These studies demonstrated that the cocultured stem cell-derived neural progenitors are capable of innervating developing hair cells [3, 5] and differentiating into both neuronal and glial lineages [4]. Importantly, several studies have observed the expression of synaptic markers including synapsin 1 and GluR2/3 in the stem cell-derived neural processes at regions adjoining or adjacent to the hair cells (detected using immunochemistry;

[3, 4, 7–9]). Additionally, Matsumoto and colleagues [8] revealed that after coculturing mouse embryonic stem cells (ESCs) with denervated cochlear explants for one week *in vitro*, the ESC-derived neural processes appeared to be highly vesiculated and in direct contact with the inner hair cell (IHC) membranes (substantiated by transmission electron microscopy; [8]). Whilst these data indicate the potential for stem cell-derived neurons to innervate developing hair cells, publications describing the innervation of IHCs and/or outer hair cells (OHCs) by hiPSC-derived neurons are not yet available.

Several promising *in vivo* studies also support the use of stem cells for AN replacement [4, 10]. For instance, following transplantation of hESC-derived neurons into the cochlea of deafened animals, hESC-derived neurons extended their neural processes towards and contacted the base of the hair cells [4, 10]. Moreover, these stem cell-derived neural fibres were reported to express GluR2 and NKAα3 at the basal pole of the IHCs, a marker of afferent nerve terminals [10]. Chen and colleagues also reported that transplanted hESC-derived auditory neural progenitors could synapse with their central (cochlear nucleus) targets in a deafened (auditory neuropathy) experimental model [10]. Importantly, the authors reported a 46% restoration in auditory function following stem cell transplantation. Whilst these exciting findings have very promising clinical implications, the long-term consequences of allotransplantation of hESCs into the cochlea, including the instigation of an immunogenic response and/or teratoma formation, remain unresolved. The use of autologous stem cell types for AN replacement [11], including hiPSCs, may minimize the risks associated with immunorejection and thus warrant further investigation.

To our knowledge, there has only been one published study that has explored the potential for iPSC-derived neurons to make contacts with sensory hair cells *in vitro* [9]. In this study, the authors' differentiated mouse induced pluripotent stem cells (miPSC) toward a neural lineage using the stromal cell-derived inducing activity method and then cocultured these neurons with developing cochlear explants. After seven days of coculture, miPSC-derived neural processes extended toward hair cells in 50% ($n = 6$) of the cochlear explants examined. Of the three cocultures that reported neurite extension, iPSC-derived neural processes grew in close proximity to the hair cells. Whilst these data are encouraging, it is yet to be determined if miPSC-derived neurons are capable of making direct contact and/or forming synaptic connections with the sensory hair cells *in vitro* or *in vivo*. Furthermore, for this therapy to be clinically applicable, it is vital that the donor cells are of human origin. Therefore, the focus of the current study was to determine whether hiPSC-derived sensory neurons could innervate the sensory hair cells *in vitro*, whether there were differences in their ability to do so (comparing two hiPSC lines to a control hESC line), and the efficacy with which any innervation occurred. The present study employed a well-characterized assay for producing high numbers of AN-like sensory neurons from human pluripotent stem cell lines, as recently described [12].

2. Materials and Methods

2.1. Cell Lines. The iPS1 and iPS2 ([13]; WiCell) and the hESC line, H9 ([14]; WA-09; WiCell), were used in this study. Passage numbers ranged from 71 to 94 (iPS1), 33 to 48 (iPS2), and 85 to 140 (H9). The tissue culture procedures were performed using aseptic techniques in class II biological safety cabinets, as recently described [12]. Stem cells were maintained at 37°C, 5% CO_2, and differentiated at 37°C, 10% CO_2 in humidified incubators.

2.2. Neural Differentiation. The stem cells were maintained and differentiated using procedures previously described [12]. Briefly, the undifferentiated cells were maintained in Knockout Serum Replacement media (1 : 1 DMEM/F12 with Glutamax, 20% Knockout Serum Replacement, 10 mM Nonessential Amino Acids, and 55 mM β-Mercaptoethanol; all purchased from Life Technologies) supplemented with 10 μg/mL of basic fibroblast growth factor (bFGF; Peprotech) on a layer of mitotically inactivated human feeders (CCD-1079Sk human foreskin fibroblast cell line; ATCC). The cells were routinely passaged each week.

The stem cells were differentiated towards a neural lineage by treating cells with Noggin media, which contained neurobasal media (NBM; neurobasal A with 1% N2, 2% B27, 2 mM L-Glutamine, and 0.5% Penicillin/Streptomycin; all purchased from Life Technologies), 500 ng/mL of Recombinant Noggin (R&D Systems), and 4 ng/mL of bFGF for two weeks. To promote neurosphere formation, the cell colonies were mechanically dissected into small sections and transferred into 96-well low attachment plate (Sigma-Aldrich) containing NBM supplemented with epidermal growth factor (EGF; Peprotech) and bFGF (20 ng/mL each). After four days (18 DIV), the neurospheres were plated onto gelatinized organ culture dishes that contained a layer of inactivated human feeders (CCD-1079Sk; ATCC) and NBM supplemented with EGF and bFGF (20 ng/mL each), followed by treatment with the Rho-kinase inhibitor Y27632 (25 μM, Sigma-Aldrich) at 19 and 20 DIV [12]. The neurospheres were cocultured with the early postnatal cochlear explants at 21 and 28 DIV.

2.3. Animals and Experimental Groups. Time-mated pregnant Hooded-Wistar rats were obtained from the Biological Research Centre at the University of Adelaide, Australia. Two coculture assays were employed. The first assay involved coculturing stem cell-derived neurons with cochlear explants (hair cells and peripheral AN fibres) obtained from postnatal day 3/4 (P3/4) rat pups. Explant only controls were set up identically for each experiment. The second coculture assay involved culturing the stem cell-derived neurons with denervated cochlear explants (hair cells only [3, 15]) dissected from P2/3 rat pups. Each experiment was repeated in triplicate using animals from three different litters.

2.4. Cochlear Explant Dissections and Coculture Experiments. The cochlear explants were dissected from early postnatal rats using methods previously described [16]. For synaptogenesis assays, the hair cells were dissected from the peripheral

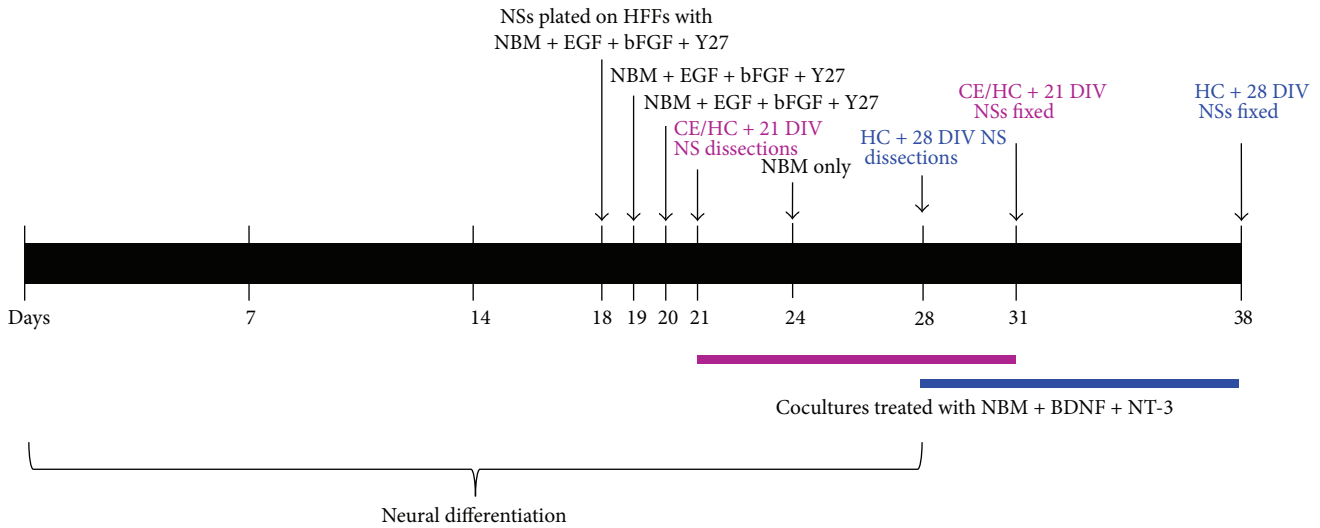

Figure 1: Timeline for coculture experiments. The stem cell lines were differentiated towards a neuronal lineage for either 21 or 28 days *in vitro* (DIV). For the cochlear explant cocultures, 21 DIV neurospheres were cocultured with cochlear explants for up to 10 days (red). For the synapse forming assay experiments, 21 (red) and 28 (blue) DIV neurospheres were cocultured with hair cells for 10 days in each case. The cocultures were treated with NBM, BDNF, and NT-3. DIV: days *in vitro*; NBM: neurobasal media; EGF: epidermal growth factor; bFGF: basic fibroblast growth factor; HFF: human foreskin fibroblasts; CE: cochlear explant; HC: hair cell; NS: neurosphere; and Y27: small molecule Y27632.

processes of ANs, thereby denervating the cochlear explant, using techniques previously described [3, 17]. The cochlear explants and denervated cochlear explants (hair cells only) were grown on 0.4 μm organotypic membranes (Millipore) in coculture media containing NBM supplemented with brain-derived neurotrophic factor and neurotrophin-3 (each added to give a final concentration of 10 ng/mL; Chemicon).

Whole cochlear explants were cocultured with either 21-day-old hiPSC or hESC neurospheres (Figure 1). Denervated cochlear explants were cocultured with either 21 DIV or 28 DIV hiPSC or hESC neurospheres. The cocultures were incubated at 37°C, 10% CO_2 for 1 DIV (explant only control), or 10 DIV (stem cell + explant/denervated explant cocultures). The coculture media were replenished every 2 DIV (100 μL/membrane).

2.5. Immunochemistry. The cocultures and explants were fixed by immersing in 4% paraformaldehyde for approximately 10 minutes, followed by careful rinsing (three times for five minutes) with phosphate buffered saline (PBS). The explant only controls were fixed after 1 DIV, whereas the experimental cocultures were fixed after 10 DIV. All explants were immunostained with a relevant combination of the following primary antibodies: rabbit anti-Myosin VIIa (1 : 100; Sapphire Bioscience; 25-6790), mouse anti-human Neurofilament (IgG1) (hNFM; 1 : 1500; Sapphire Bioscience; MAB5186), chicken anti-Neurofilament H (IgG) (1 : 1000; Millipore; AB5735), goat anti-Prestin (1 : 400; ThermoFisher Scientific; sc-22692), mouse anti-Peripherin (IgG1) (1 : 1000; Millipore; MAB1527), rabbit anti-synapsin 1 (1 : 200; Life Technologies; A6442), and goat anti-parvalbumin (1 : 3000; Swant; PVG-214).

For the cochlear explant cocultures, the primary antibodies were diluted in primary blocking solution (0.1% Triton-X (Sigma-Aldrich), containing 2% of the relevant serum, either goat (Abacus ALS) or donkey (Millipore) diluted in PBS) and then added to the membranes at 200 μL per well before being stored overnight at 4°C in a humidified container. The following day, the cells were rinsed thrice for five minutes in primary blocking solution. The Alexa Fluor-conjugated secondary antibodies (all purchased from Life Technologies) diluted in secondary blocking solution (0.1% Tween (Sigma-Aldrich), 2% goat or donkey serum in PBS, as described above) were added to the membranes at a volume of 200 μL per well. The slides were then transferred into a foil covered, humidified container and left for 2 hours at room temperature in the dark with gentle rotation. After 2 hours, the cells were washed thrice for five minutes in PBS, mounted with ProLong-Gold antifade reagent containing the nuclear stain 4′, 6-diamidino-2-phenylindole (DAPI; Invitrogen), and sealed with varnish the next day.

For the denervated cochlear explants, the cocultures were blocked in primary blocking solution (0.1% Triton-X, 10% goat serum in PBS) for 1 hour with gentle rotation. A higher percentage of serum was used to minimize background staining for the synapse assay cocultures. The primary antibodies were diluted in primary blocking solution at 200 μL per well, added to the membranes, and stored overnight at 4°C in a humidified container. The next day the cells were rinsed in primary blocking solution (8 × 10 minutes). Alexa Fluor-conjugated secondary antibodies were diluted in the secondary blocking solution (0.1% Tween, 10% goat, or donkey serum in PBS) and added to the membranes at 200 μL per well. The secondary antibodies were spun before and after

dilution in secondary blocking solution. The slides were transferred into a foil covered, humidified container and left for 1.5 hours at room temperature in the dark with gentle rotation. After 1.5 hours, the membranes were washed thrice for five minutes in PBS, mounted with ProLong-Gold antifade reagent containing DAPI, and sealed with varnish the next day.

2.6. Microscopy. Stained explants were visualized using confocal microscopy and images were taken using an LSM 510 META confocal scanning laser system with a Zeiss AxioImagerZ1 microscope. Optical slice thickness was set to 8.49 μm. Zen digital imaging software (Carl Zeiss) was used to process and analyze the images.

2.7. Quantification. For the cochlear explant cocultures, four regions of the explant were selected at random and quantified under 40x magnification. The number of stem cell-derived neural processes making contact with the hair cells was counted relative to the total number of hair cells present in the selected region. Additionally, we noted the total number of explants in which stem cell-derived hair cell innervation was observed and compared this to the total number of explants in the study. We used this later number in order to be able to directly compare our results with the recent relevant publication using mouse iPSC-derived neurons [9].

For the synaptogenesis assays, the total number of hair cells in the denervated cochlear explant was counted under 63x magnification. The number of hair cells making contact with synapsin 1 positive stem cell-derived neural processes was counted [17], relative to the total number of hair cells.

Statistical analysis of the coculture data was performed using GraphPad Prism (Version 6). Where data was normally distributed (Anderson-Darling test), a Student's t-test was used. For nonparametric data, a Kruskal-Wallis one-way analysis of variance (ANOVA) was used to determine statistical significance between the groups compared. Values of $p < 0.05$ were considered statistically significant, with ranges in significance from $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. Data are presented as the mean ± standard error of mean (SEM).

3. Results

3.1. Human iPSC-Derived Neural Progenitors Contacted Sensory Hair Cells in Whole Mammalian Cochlear Explant Cocultures. In order to investigate whether hiPSC-derived neurons could contact their peripheral targets (the sensory hair cells), 21 DIV neural progenitors derived from two hiPSC lines (iPS1 and iPS2) and one hESC (H9; control) line were cocultured with P3/4 whole cochlear explants (Figure 2(a)). After 10 DIV, the hiPSC- and hESC-derived neurons preferentially extended their processes toward and into the explant. Compared to the organized innervation pattern observed in the directly isolated cochlear explant (Figure 2(b)), the stem cell-derived neural processes grew in a disorganized manner (Figures 2(c)–2(e)). Additionally, the neural processes were observed to extend towards and along the rows of hair cells in the cochlear explant. The neural processes grew in fasciculating bundles along the basolateral surface of the hair cells in several of the stem cell cocultures, similar to the pattern of normal OHC innervation in the mammalian cochlea (Figures 2(d) and 2(e)). Under higher magnification, both the hiPSC- and hESC-derived neurons were observed to make direct contact with the sensory hair cells in the explant. However, the hiPSC-derived neural processes made contact with fewer hair cells compared to the hESCs (iPS1 16.9 + 4.8%, $n = 16$, $^{*}p < 0.05$; iPS2 16.4 + 3.6%, $n = 17$, $^{*}p < 0.05$; H9 39.5 + 12.1%, $n = 12$; Figure 2(f)). Overall, there was no significant difference in the average number of hair cells that the iPS1 and iPS2 cell lines were contacting throughout the cocultures examined ($p = 0.38$; Figure 2(f)). However, when the data was analyzed in terms of the number of explants in which hair cells were contacted by stem cell-derived neurons, the iPS2 cell line demonstrated a greater consistency of innervation in comparison to the iPS1 line, (iPS1 $n = 16$, 62.5% of explants innervated; iPS2 $n = 17$, 94.2% of explants innervated; $^{*}p < 0.05$).

3.2. Human iPSC-Derived Neural Progenitors Grew toward Both the Inner and Outer Hair Cells in Whole Cochlear Explant Cocultures. To investigate whether the stem cell-derived neural processes were contacting the IHCs, OHCs, or both, a Prestin antibody was used to distinguish the OHCs in the explant cocultures (Figure 3(a)). All hair cells are Myosin VIIa positive, but only the outer hair cells express the motor protein Prestin. As expected, after 1 DIV there were a significantly higher number of OHCs in the explants compared to the IHCs (Figure 3(e); $^{***}p < 0.001$). At 10 DIV, there was a significant decrease in the overall numbers of surviving IHCs and OHCs, demonstrating a decline in the relative proportion of IHC and OHC normally observed within the cochlea. In cocultures, no significant differences were observed in the number of IHCs that the stem cell-derived neural processes contacted, when compared to the numbers of OHCs that these cells contacted (Figures 3(b)–3(d) and 3(e); iPS1 IHCs = 12.3 + 5.8%, OHCs = 9.4 + 5.9%, $n = 6$, $p = 0.79$; iPS2 IHCs = 18.5 + 12.2%, OHCs = 23.2 + 12.2%, $n = 7$, $p = 0.73$; and H9 IHCs = 27.1 + 12.4%, OHCs = 32.9 + 5.8%, $n = 6$, $p = 0.68$). Notably, the iPS1-derived neurons showed significantly less OHCs innervation in comparison to the H9-derived neurons ($^{*}p < 0.05$; Figure 3(e)).

3.3. Human iPSC-Derived Neural Progenitors Made Synaptic Connections with Hair Cells in Denervated Cochlear Explant Cocultures. As the innervation of iPS2-derived neurons was more efficient when considered in terms of both the overall percentage of explants innervated (94.2%, $^{*}p < 0.05$) and fewer numbers of OHCs innervated by the iPS1-derived neurons after 10 DIV ($^{*}p < 0.05$; Figure 3(e)), the synaptic capacity of the iPS2- and hESC-derived neurons was compared next. We first examined the denervated cochlear explants at 1 and 10 DIV (Figures 4(a)–4(d), resp.). Distal AN processes were absent in the denervated cochlear explants after 1 DIV, but an accumulation of residual NFM within hair cell somata (Figure 4(a)) and synapsin 1 positive puncta

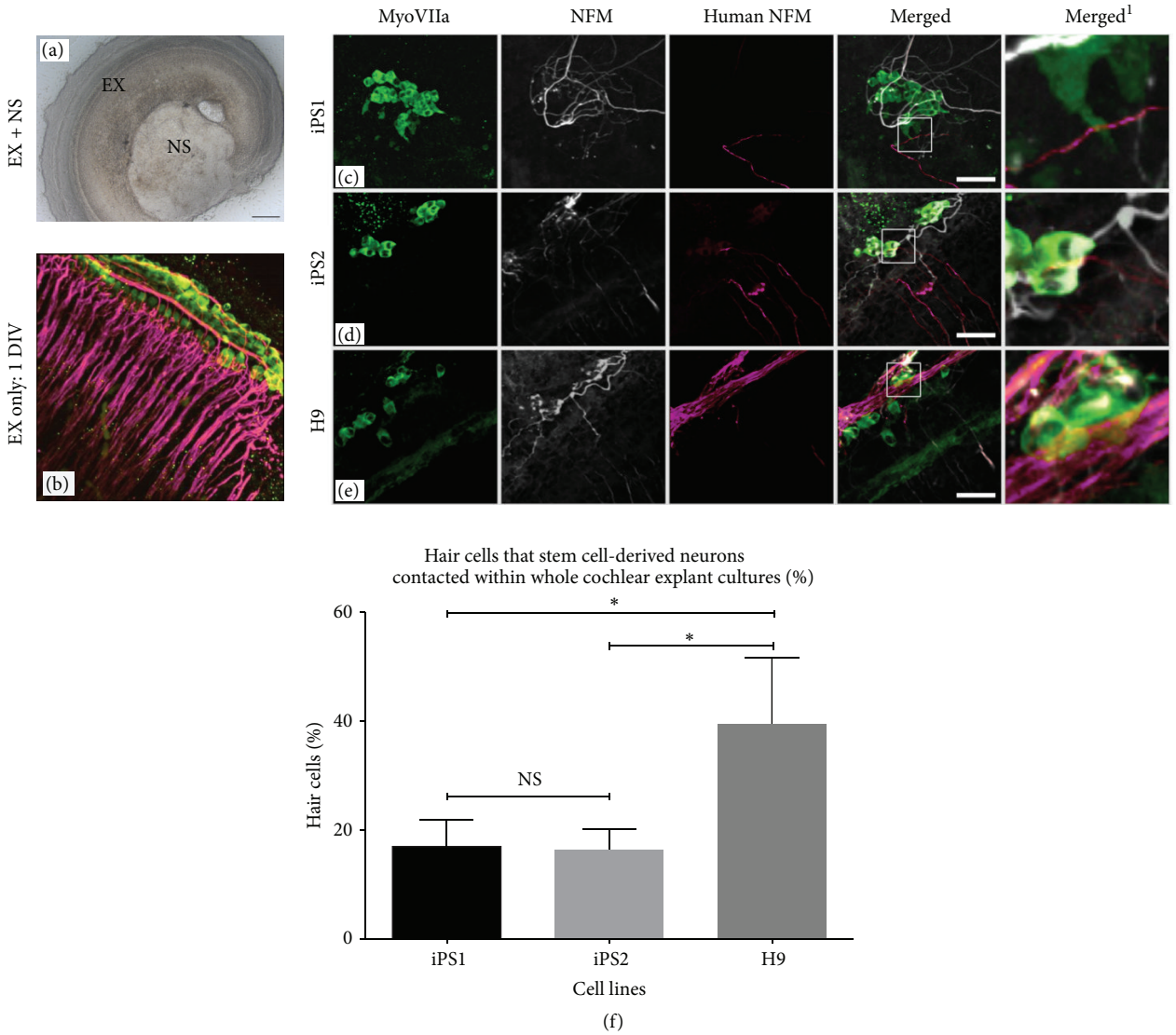

Figure 2: Coculture of cochlear explants and hESC- and hiPSC-derived neural progenitors. (a) The light microscope image depicts the cochlear explant cocultured with a stem cell-derived neurosphere (NS). (b) The explant only control obtained from P3 mice shows the normal innervation pattern of ANs after 1 DIV. (c–e) In cocultures, growth of hESC- and hiPSC-derived neural processes towards and along the rows of the hair cells was observed. Myosin VIIa (green) labels the hair cells, Neurofilament (NFM; grey) labels the endogenous neural processes, and human NFM (hNFM; red) labels the stem cell-derived neural processes. The merged[1] images represent higher magnification images of the boxed inserts and depict the contacts made between the stem cell-derived neural processes and hair cells: for example, three points of contact are shown in (c) merged[1]. Scale bar = 50 μm, relevant for all the images. (f) The hiPSC-derived neural processes made contact with fewer hair cells compared to the hESCs (iPS1 $n = 16$, $p < 0.05$; iPS2 $n = 17$, $p < 0.05$; H9 $n = 12$). There was no significant difference in the number of hair cells making contact with the iPS1 and iPS2 cell lines ($p = 0.38$). A Kruskal-Wallis one-way ANOVA was utilized to determine statistical significance between the groups compared. Data are presented as the mean ± SEM. Values of $^*p < 0.05$ were considered statistically significant. NS: not significant.

(Figure 4(b)) was observed at this time point. Conversely, after 10 DIV, there were reduced NFM accumulations within the hair cell somata (Figure 4(c)) and synaptic puncta were not detected (Figure 4(d)). Following the coculture of stem cell-derived neurospheres with denervated cochlear explants (Figure 4(e)), neural processes projecting towards isolated sensory hair cells were observed (Figures 4(f)–4(i)). The neural processes of 21 DIV neurospheres were found to express high levels of synapsin 1, and synapsin 1 positive puncta were observed to colocalize with the parvalbumin-positive sensory hair cells in the denervated explant (Figures 4(f) and 4(g)). Moreover, the synaptic connections between the hair cells and hiPSC-derived neurons were observed to occur in an *en passant* manner: that is, single stem cell-derived neurites made multiple synaptic connections with multiple hair cells. Similar observations were reported for

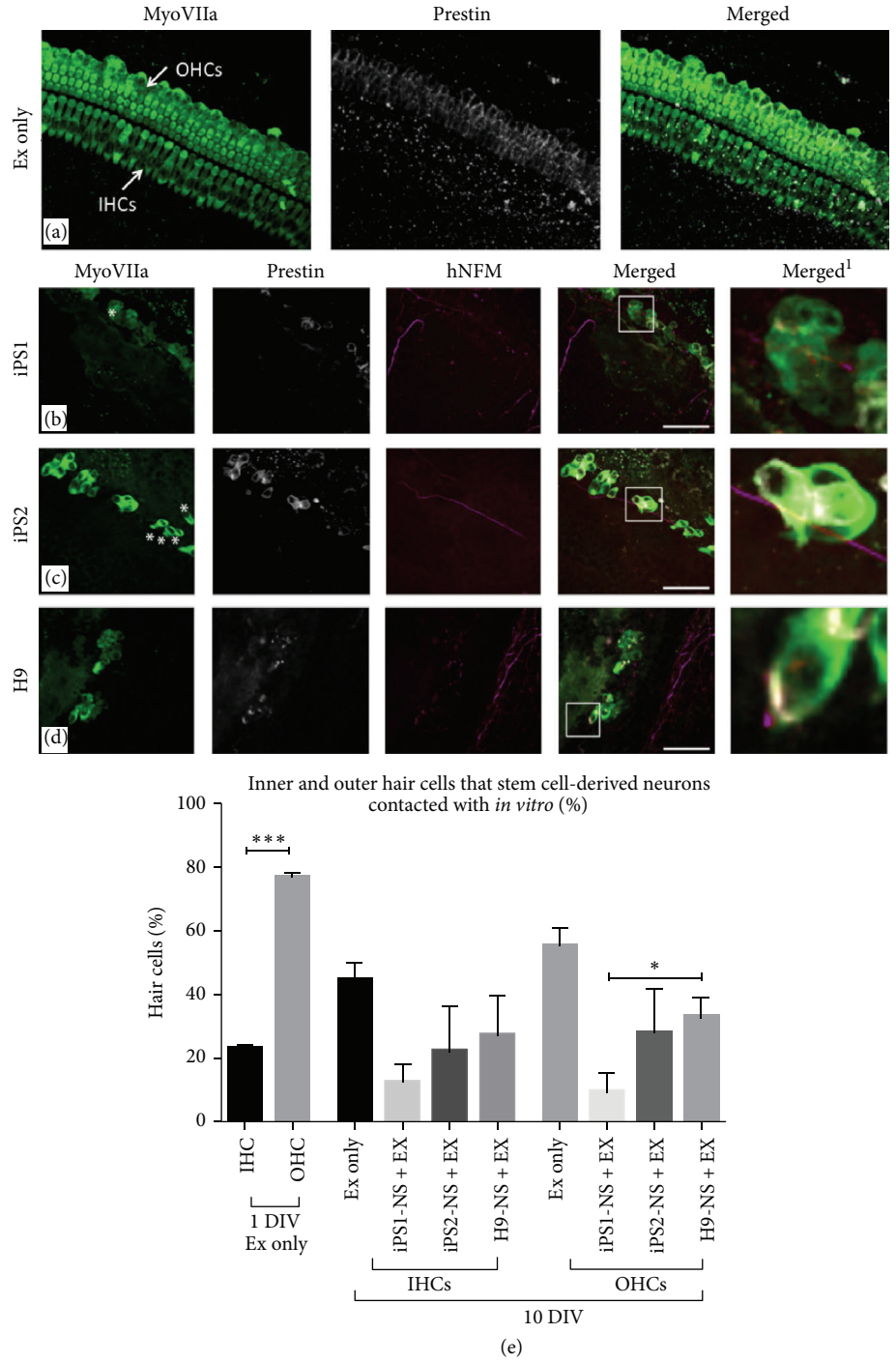

Figure 3: Innervation of inner hair cells versus outer hair cells by stem cell-derived neurons. (a) The specific labeling of Prestin (grey) to the OHCs is depicted in the explant only control. (b–d) The hiPSC- and hESC-derived neural processes made contact with both the IHCs and the OHCs. The merged[1] images represent higher magnification images of the boxed inserts and depict the contacts made between the stem cell-derived neural processes and hair cells. Scale bar = 50 μm, relevant for all the images. (e) Total numbers of hair cells (inner and outer) were quantified in each group using antibodies against MyoVIIa (all hair cells) and Prestin (outer hair cells only). Data was compared to controls grown for one day *in vitro*. There was a significantly higher number of OHCs compared to IHCs in the explant only controls after 1 DIV ($p < 0.001$). At 10 DIV, there was a decline in the number of IHCs and OHCs present in the explants. There were no significant differences in the number of OHCs that the stem cell-derived neural processes contacted, when compared to the numbers of IHCs these cells contacted (iPS1 $n = 6$, $p = 0.79$; iPS2: $n = 7$, $p = 0.73$; and H9 $n = 6$, $p = 0.68$). The iPS1-derived neurons made contact with significantly fewer OHCs compared to the hESC-derived neurons ($p < 0.05$). A Kruskal-Wallis one-way ANOVA was utilized to determine statistical significance between the groups compared. Values of $^{*}p < 0.05$ were considered statistically significant and data presented as the mean ± SEM. OHCs: outer hair cells; IHCs: inner hair cells; DIV: days *in vitro*. The asterisks ∗ highlight the IHCs present in the cultures.

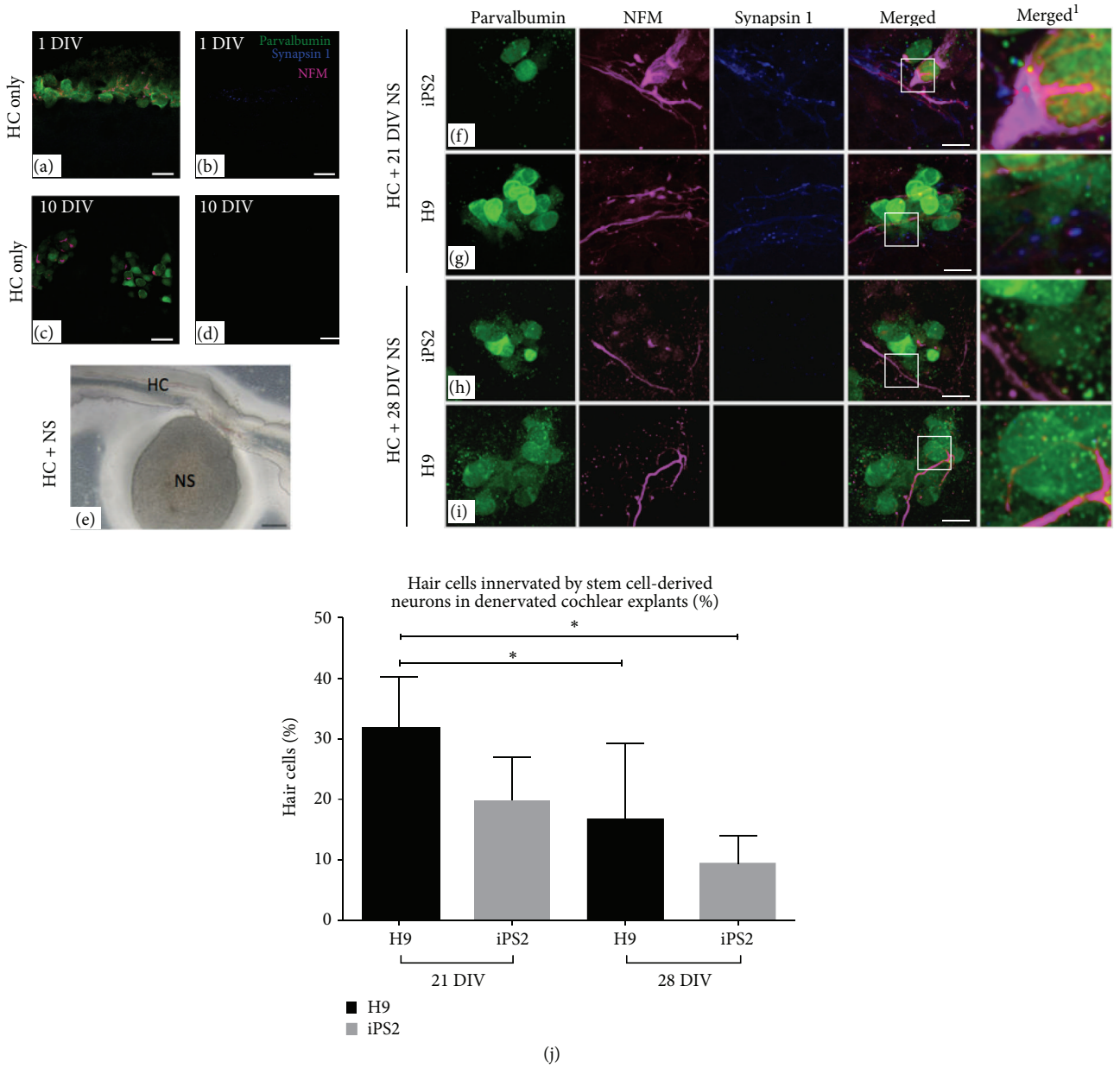

Figure 4: The synaptic potential of 21- and 28-day-old hiPSC- and hESC-derived neurons. (a-b) In the denervated cochlear explant controls at 1 DIV, some accumulation of residual NFM within hair cell somata and a few synaptic puncta were observed. (c-d) After 10 DIV, there appeared to be fewer hair cells with NFM accumulation and synapsin 1 was undetectable. (e) Light microscope image of denervated cochlear explant cocultured with stem cell-derived neurosphere. (f and g) The hiPSC and hESC neurospheres cocultured at 21 DIV extended their neural processes towards hair cells in the denervated explants. Punctate-like synapsin 1 expression was observed along the neural processes of both the hiPSC- and hESC-derived neurites making contact with hair cells. (h and i) The hiPSC and hESC neurospheres cocultured at 28 DIV projected fewer neural processes towards hair cells in the denervated explants. Synapsin 1 was rarely observed along the neural processes of both the hiPSC- and hESC-derived neurites making contact with hair cells. The merged[1] images represent higher magnification images of the boxed inserts and depict the punctate-like synaptic contacts made between the stem cell-derived neural processes and hair cells. Scale bar = 20 μm (applicable for all images). (j) The 21-day-old hiPSC- and hESC-derived neurons made contact with a significantly higher number of hair cells, compared to the 28-day-old neurons (H9: $p < 0.05$). Furthermore, the 21-day-old hESC neurons made contact with a significantly higher number of hair cells ($p < 0.05$). A Student's t-test was used to determine statistical significance. Values of $^{*}p < 0.05$ were considered statistically significant. Data are presented as the mean ± SEM.

hESC-derived neurons (Figure 4(g); [3]). The microisolate assay utilized in the current publication results in disorganized hair cell growth and some hair cell senescence after two weeks *in vitro* [3, 17]. This is due primarily to fibroblasts and supporting cells in the culture, which divide after explant isolation and disrupt the normal hair cell arrangement during their proliferation. As a result of the microdissection of the organ of Corti from the explant, the hair cells often do not retain their arrangement in rows due to lack of ultrastructure normally provided in whole tissue.

3.4. Innervation of Hair Cells in Denervated Cochlear Explants Was Significantly Greater Using "Early Stage" Compared to "Late Stage" Neurospheres. We next quantified the differences observed in the synapse forming capacity of the iPS2- and H9-derived neurons when cocultured at either an early stage of differentiation (21 DIV) or a later stage of differentiation (28 DIV) in denervated cochlear explants. The 21 DIV cocultures contained greater numbers of stem cell-derived processes which contained synapsin 1 at points of contact with the sensory hair cells, when compared to the cocultures examined at 28 DIV (Figures 4(f)–4(i)). More specifically, the neurons derived from 21 DIV H9 neurospheres made significantly more synaptic connections with hair cells, compared to those derived from 28 DIV neurospheres (H9 21 DIV (31.1 + 9.1%) and 28 DIV (16.7 + 12.5%): $n = 8$; $^{*}p < 0.05$; Figure 4(j)). In addition, the H9-derived neurons from 21 DIV neurospheres also innervated a significantly greater number of hair cells, compared to the iPS2-derived neurons from 28 DIV neurospheres (iPS2 28 DIV (9.3 + 4.6%): $n = 8$; $p < 0.05$; Figure 4(j)).

4. Discussion

The intricate wiring of ANs and the refined tonotopic organization of the inner ear facilitate the transmission of accurate sound information from the external environment to the brain. Prompting stem cells to differentiate and replicate the refined innervation pattern and functionalities of primary ANs is a significant challenge. Several previous studies have reported the potential for hESC to establish new synapses with hair cells in the auditory periphery [3, 4, 10]; however, this remains to be demonstrated for hiPSCs. Here, we report that hiPSC-derived neurons can make direct contact with and form synapses on developing sensory hair cells *in vitro*. However, the hiPSC-derived neurons had a lower innervation capacity compared to hESC-derived neurons. These observations are consistent with our recent investigations which examined the variable differentiation potentials of the cell lines described herein [12].

4.1. Establishment of Contacts between the hiPSC-Derived Neurites and Hair Cells in Cochlear Explant Cocultures. In cochlear explant cultures, hiPSC-derived neurons were observed to extend their neural processes towards the sensory epithelium and make direct contact with both IHCs and OHCs *in vitro*, an important first step in the reestablishment of synaptic input (Figure 3). However, compared to the orderly innervation pattern of adult ANs, the hiPSC-derived neurites appeared to grow towards the sensory epithelium in a disorganized manner, resembling the pattern of innervation of developing ANs (Figures 2(c)–2(e); [18–20]). This is supported by observations during the very early stages of mouse development (E18-P0), where neurites from the auditory neurons exhibit an immature and highly branched morphology whereby Type I and Type II ANs innervate both IHCs and OHCs *in vivo* [18–20]. As development progresses, these neurons undergo significant synaptic pruning and neural refinement, such that Type I neurons ultimately contact only IHCs and Type II neurons only contact OHCs [18].

4.2. hiPSC-Derived Neurons Form Immature Synaptic Terminals on Sensory Hair Cells in Denervated Cochlear Explants. It has previously been observed that the afferent dendrites of early postnatal ANs isolated from the sensory epithelium express synaptic markers (synapsin 1 and synaptophysin) as they regenerate their connections with hair cells *in vitro* [8, 21, 22]. Various stem cell-derived neurites have also been found to express synapsin 1 at regions adjacent to hair cells in coculture models [3, 4, 7, 8]. Therefore, the expression of synapsin 1 is considered to be a preliminary indicator of the capacity for stem cell-derived neurons/ANs to regenerate or form potentially functional synapses with hair cells [23]. In the present study, extensive punctate-like synapsin 1 expression was observed along hiPSC-derived neural processes making contact with hair cells in denervated explant cultures (Figure 4(f)). Furthermore, stem cell-derived synaptic puncta were observed to colocalize at the basolateral surface of hair cells, indicating the capacity for hiPSC-derived neurons to form synapses with sensory hair cells *in vitro* (Figure 4(f)).

We recently reported that hiPSC-derived neurons had higher levels of sensory neural marker expression at the later time points of differentiation (28–35 DIV; [12]). We therefore suspected that stem cell-derived neurons from these later stages of differentiation would have different potentials to innervate hair cells compared to the same neurons derived from earlier stages of differentiation. We found that hiPSC- and hESC-derived neurons cocultured at an earlier stage of differentiation (21 DIV) formed synapses with a higher number of hair cells, when compared to the more differentiated stem cell-derived neurons (28 DIV). These findings emphasize the importance of the stage of differentiation on the functional integration capacity of stem cell-derived neurons and support the rationale that neural progenitors may have better functional outcomes *in vivo*, compared to more differentiated neurons [24]. This hypothesis is further supported by recent *in vivo* studies reporting that partial hearing function recovery can be achieved following the transplantation of early stage hESC-derived otic neural progenitors into deafened gerbil cochleae [10].

4.3. Variability Amongst Stem Cell Lines. Whilst hiPSC-derived neurons were capable of directly contacting the sensory hair cells *in vitro*, they were also observed to make contact with fewer hair cells compared to the hESC-derived

neurons. Moreover, there was no significant difference in the number of hair cells that the iPS1- and iPS2-derived neurons made direct contact with *in vitro*, suggesting comparable innervation efficiencies. Interestingly, however, if the data were quantified based upon the number of cultures in which innervation was observed, inconsistencies between the iPSC lines examined were apparent. Specifically, the iPS1-derived neurons had a lower number of cultures with hair cell innervation, compared to the iPS2- and hESC-derived neurons ($p < 0.05$; Figure 2). We (and others) have previously reported that hiPSCs have a more variable differentiation potential compared to hESCs [12, 25, 26]. Consistent with these findings, the data presented here indicate that differences exist among human pluripotent stem cell-derived neurons, particularly hiPSC-derived neurons in their innervation potentials *in vitro*. However, it needs to be noted here that differences may be present among cell lines in their optimal differentiation protocol (e.g., the exact timing of a particular treatment). As such, future studies assessing multiple cell lines using optimized differentiation protocols will be required to assess cell line capacity. On the other hand, it is possible that the variabilities observed could be due to the presence of genetic and epigenetic abnormalities recently detected in hiPSC lines derived from viral integration reprogramming methods [23]. Nevertheless, the findings of the current study highlight a potential concern associated with the use of human pluripotent stem cell lines for stem cell transplants.

In terms of cell transplantation therapies, the presence of variability amongst cell lines could have several consequences. For instance, there could be differences in the numbers of ANs generated from each patient and variabilities in their integration capacities, which could consequently lead to inconsistencies in the functional outcomes patients achieve with the therapy. In further support of this idea, Chen and colleagues have reported varying degrees of auditory function recovery following the transplantation of hESC-derived neurons into gerbil cochleae [10], which may be due to underlying variability in cell differentiation. These findings reiterate the necessity to mitigate potential inconsistencies in functional outcomes between patients, prior to the clinical translation of stem cell therapies. The use of hiPSC lines generated using nonviral integration methods could potentially abate some of these variabilities and this is worthy of investigation prior to the transplantation of hiPSC-derived neurons into the cochlea. The described *in vitro* cocultures provide a proof-of-concept model from which to test the capacity of different stem cell lines at various stages of differentiation prior to their *in vivo* delivery.

In addition to cell transplantation therapies, the capacity to successfully differentiate hiPSCs towards functional ANs could facilitate the development of patient-specific cell lines to model AN degeneration "in a dish." More specifically, the derivation of hiPSC-derived ANs from patients with genetic forms of SNHL, including Usher's syndrome, Branchiootorenal syndrome, or Waardenburg syndrome (to name a few), will enable us to obtain a deeper understanding of the mechanisms underlying these conditions. Furthermore, it will permit the use of the latest gene editing technologies such as clustered regularly interspaced short palindromic repeat-(CRISPR-) associated endonucleases [24, 27, 28] to correct genetic abnormalities in these disease-specific cell lines and also provide a platform for large-scale drug screening to potentially suppress AN degeneration [29]. The benefits of modeling diseases using hiPSCs are clearly numerous; therefore, this study provides useful insight into the potential of hiPSCs to recapitulate the functionality of ANs.

4.4. Summary. In conclusion, the present study demonstrated that hiPSC-derived neurons could make direct contact with and form presynaptic connections on developing hair cells *in vitro*. Furthermore, it was observed that neural progenitors derived from pluripotent stem cells cocultured at an earlier stage of differentiation have a higher innervation potential compared to the neural progenitors cocultured at a later stage. These promising findings have directly informed our hiPSC transplantation studies in the deaf cochlea and serve as a foundation from which to further investigate the use of induced pluripotent stem cells for auditory neural replacement.

Abbreviations

AN: Auditory neuron
hiPSC: Human induced pluripotent stem cells
hESCs: Human embryonic stem cells
DIV: Days *in vitro*.

Ethical Approval

All experiments for iPS and human ES cell lines were performed in accordance with approvals obtained from the University of Melbourne Human Ethics Committee (#0605017 and #0830010). The Animal Research and Ethics Committee of the Royal Victorian Eye and Ear Hospital approved the care and use of the animals in this study (approval number, 12/253AU and 13/280AU).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] N. A. Hardie and R. K. Shepherd, "Sensorineural hearing loss during development: morphological and physiological response of the cochlea and auditory brainstem," *Hearing Research*, vol. 128, no. 1-2, pp. 147–165, 1999.

[2] P. A. Leake and G. T. Hradek, "Cochlear pathology of long term neomycin induced deafness in cats," *Hearing Research*, vol. 33, no. 1, pp. 11–33, 1988.

[3] B. A. Nayagam, A. S. Edge, K. Needham et al., "An in vitro model of developmental synaptogenesis using cocultures of human neural progenitors and cochlear explants," *Stem Cells and Development*, vol. 22, no. 6, pp. 901–912, 2013.

[4] F. Shi, C. E. Corrales, M. C. Liberman, and A. S. B. Edge, "BMP4 induction of sensory neurons from human embryonic stem cells and reinnervation of sensory epithelium," *European Journal of Neuroscience*, vol. 26, no. 11, pp. 3016–3023, 2007.

[5] A. Glavaski-Joksimovic, C. Thonabulsombat, M. Wendt, M. Eriksson, H. Ma, and P. Olivius, "Morphological differentiation of tau-green fluorescent protein embryonic stem cells into neurons after co-culture with auditory brain stem slices," *Neuroscience*, vol. 162, no. 2, pp. 472–481, 2009.

[6] T. Hyakumura, M. Dottori, K. Needham, and B. A. Nayagam, "Innervation of peripheral and central auditory tissues by human embryonic stem cell-derived neurons in vitro," in *Proceedings of the International Society for Stem Cell Research 10th Annual Meeting*, Yokohama, Japan, 2012.

[7] M. Matsumoto, T. Nakagawa, T. Higashi et al., "Innervation of stem cell-derived neurons into auditory epithelia of mice," *NeuroReport*, vol. 16, no. 8, pp. 787–790, 2005.

[8] M. Matsumoto, T. Nakagawa, K. Kojima, T. Sakamoto, F. Fujiyama, and J. Ito, "Potential of embryonic stem cell-derived neurons for synapse formation with auditory hair cells," *Journal of Neuroscience Research*, vol. 86, no. 14, pp. 3075–3085, 2008.

[9] K. Nishimura, T. Nakagawa, K. Ono et al., "Transplantation of mouse induced pluripotent stem cells into the cochlea," *Neuroreport*, vol. 20, no. 14, pp. 1250–1254, 2009.

[10] W. Chen, N. Jongkamonwiwat, L. Abbas et al., "Restoration of auditory evoked responses by human ES-cell-derived otic progenitors," *Nature*, vol. 490, no. 7419, pp. 278–282, 2012.

[11] M. A. Huisman and M. N. Rivolta, "Neural crest stem cells and their potential application in a therapy for deafness," *Frontiers in Bioscience*, vol. 4, no. 1, pp. 121–132, 2012.

[12] N. Gunewardene, N. Van Bergen, D. Crombie, K. Needham, M. Dottori, and B. A. Nayagam, "Directing human induced pluripotent stem cells into a neurosensory lineage for auditory neuron replacement," *BioResearch Open Access*, vol. 3, no. 4, pp. 162–175, 2014.

[13] J. Yu, M. A. Vodyanik, K. Smuga-Otto et al., "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, no. 5858, pp. 1917–1920, 2007.

[14] J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro et al., "Embryonic stem cell lines derived from human blastocysts," *Science*, vol. 282, no. 5391, pp. 1145–1147, 1998.

[15] J. Flores-Otero, Z. X. Hui, and R. L. Davis, "Reciprocal regulation of presynaptic and postsynaptic proteins in bipolar spiral ganglion neurons by neurotrophins," *The Journal of Neuroscience*, vol. 27, no. 51, pp. 14023–14034, 2007.

[16] B. A. Nayagam and R. L. Minter, "A comparison of *in vitro* treatments for directing stem cells toward a sensory neural fate," *American Journal of Otolaryngology: Head and Neck Medicine and Surgery*, vol. 33, no. 1, pp. 37–46, 2012.

[17] J. Flores-Otero, H. Z. Xue, and R. L. Davis, "Reciprocal regulation of presynaptic and postsynaptic proteins in bipolar spiral ganglion neurons by neurotrophins," *The Journal of Neuroscience*, vol. 27, no. 51, pp. 14023–14034, 2007.

[18] L.-C. Huang, P. R. Thorne, G. D. Housley, and J. M. Montgomery, "Spatiotemporal definition of neurite outgrowth, refinement and retraction in the developing mouse cochlea," *Development*, vol. 134, no. 16, pp. 2925–2933, 2007.

[19] E. J. Koundakjian, J. L. Appler, and L. V. Goodrich, "Auditory neurons make stereotyped wiring decisions before maturation of their targets," *Journal of Neuroscience*, vol. 27, no. 51, pp. 14078–14088, 2007.

[20] J. M. Appler and L. V. Goodrich, "Connecting the ear to the brain: molecular mechanisms of auditory circuit assembly," *Progress in Neurobiology*, vol. 93, no. 4, pp. 488–508, 2011.

[21] M. Tong, A. Brugeaud, and A. S. B. Edge, "Regenerated synapses between postnatal hair cells and auditory neurons," *Journal of the Association for Research in Otolaryngology*, vol. 14, no. 3, pp. 321–329, 2013.

[22] R. Martinez-Monedero, C. E. Corrales, M. P. Cuajungco, S. Heller, and A. S. B. Edge, "Reinnervation of hair cells by auditory neurons after selective removal of spiral ganglion neurons," *Journal of Neurobiology*, vol. 66, no. 4, pp. 319–331, 2006.

[23] X. Fu and Y. Xu, "Challenges to the clinical application of pluripotent stem cells: towards genomic and functional stability," *Genome Medicine*, vol. 4, no. 6, article 55, 2012.

[24] P. Mali, L. Yang, K. M. Esvelt et al., "RNA-guided human genome engineering via Cas9," *Science*, vol. 339, no. 6121, pp. 823–826, 2013.

[25] B.-Y. Hu, J. P. Weick, J. Yu et al., "Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 9, pp. 4335–4340, 2010.

[26] M. Koyanagi-Aoi, M. Ohnuki, K. Takahashi et al., "Differentiation-defective phenotypes revealed by large-scale analyses of human pluripotent stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20569–20574, 2013.

[27] L. Cong, F. A. Ran, D. Cox et al., "Multiplex genome engineering using CRISPR/Cas systems," *Science*, vol. 339, no. 6121, pp. 819–823, 2013.

[28] P. D. Hsu, E. S. Lander, and F. Zhang, "Development and applications of CRISPR-Cas9 for genome engineering," *Cell*, vol. 157, no. 6, pp. 1262–1278, 2014.

[29] K. Takahashi and S. Yamanaka, "Induced pluripotent stem cells in medicine and biology," *Development*, vol. 140, no. 12, pp. 2457–2461, 2013.

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Review Article

Low Density Lipoprotein Receptor Related Proteins as Regulators of Neural Stem and Progenitor Cell Function

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The central nervous system (CNS) is a highly organised structure. Many signalling systems work in concert to ensure that neural stem cells are appropriately directed to generate progenitor cells, which in turn mature into functional cell types including projection neurons, interneurons, astrocytes, and oligodendrocytes. Herein we explore the role of the low density lipoprotein (LDL) receptor family, in particular family members LRP1 and LRP2, in regulating the behaviour of neural stem and progenitor cells during development and adulthood. The ability of LRP1 and LRP2 to bind a diverse and extensive range of ligands, regulate ligand endocytosis, recruit nonreceptor tyrosine kinases for direct signal transduction and signal in conjunction with other receptors, enables them to modulate many crucial neural cell functions.

1. Low Density Lipoprotein Receptor Related Proteins 1 and 2

The LDL receptor family is a large family of multiligand receptors. Core family members include the LDL receptor; very low density lipoprotein (VLDL) receptor [1]; LDL receptor related protein (LRP)1, also known as CD91 and the α-2-macroglobulin receptor [2–4]; LRP2, also known as GP330 and Megalin [5]; LRP5 [6]; LRP6 [7]; and LRP8, also known as the apolipoprotein receptor-2 [8]. Each family member is a single-pass transmembrane receptor, containing two or more extracellular cysteine-rich complement type repeats, which act as ligand binding domains [9].

At 600 kDa, LRP1 and LRP2 are the largest and most promiscuous members of the LDL receptor family. Transcription of the *Lrp1* gene can be activated by a number of transcription factors including sterol regulatory element binding protein 2 [10], hypoxia-induced factor 1α [11], and nitric oxide-dependent transcription factors [12], but is negatively regulated by naturally occurring antisense transcripts that are inversely coded within exons 5 and 6 of the *Lrp1* gene [13]. The *Lrp1* gene codes for a precursor protein that binds to the receptor associated protein (RAP), a chaperone that occupies the ligand binding domains of the precursor [14] to prevent the binding of other ligands [15], and ensure its correct folding in the endoplasmic reticulum [16, 17] (Figure 1). RAP remains bound to the LRP1 precursor and transports it to the Golgi apparatus. This transport involves the proximal NPXY motif in the intracellular domain of the protein [18]. In the trans-Golgi network, the low pH of the secretory pathway causes protonation of the histidine residues in domain 3 of RAP [19], triggering its dissociation from the LRP1 precursor [14, 20]. The protease Furin then cleaves the LRP1 precursor at the RX(K/R)R consensus sequence, to generate a large α-chain (515 kDa) and a smaller β-chain (85 kDa) [21]. The two fragments remain noncovalently linked on their way to the cell membrane, where they are embedded as one functional unit, comprising mature LRP1 (Figure 1). LRP2 is similarly chaperoned by RAP [22] and also contains an RX(K/R)R consensus sequence, but there is no evidence that LRP2 undergoes intracellular proteolytic processing prior to its insertion into the plasma membrane [5].

1.1. Soluble LRP1 and LRP2. Once LRP1 is inserted into the plasma membrane, the soluble extracellular domain (sLRP1) can be cleaved from the cell surface by enzymes such as

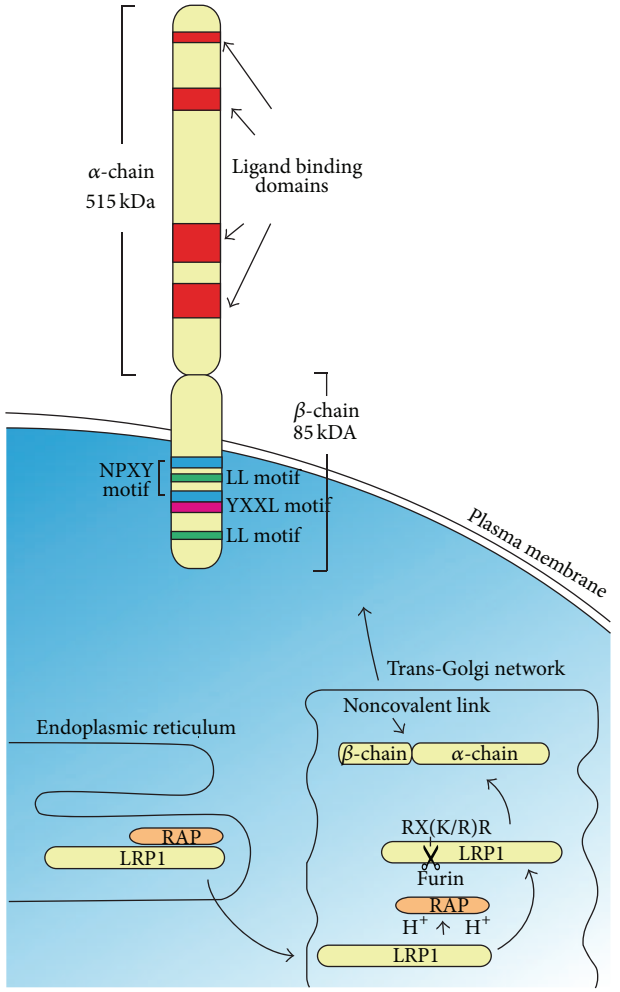

Figure 1: LRP1 maturation and structure. This schematic depicts the LRP1 precursor protein, which is synthesized in the endoplasmic reticulum and is bound to the chaperone protein, receptor associated protein (RAP). The LRP1 precursor is transported to the trans-Golgi network where the low pH causes RAP to dissociate. The protease Furin cleaves the LRP1 precursor at the RX(K/R)R consensus sequence to generate a large α-chain (515 kDa) and a smaller β-chain (85 kDa) which are noncovalently linked and shuttled to the cell membrane, where they are embedded as one functional unit. The α-chain contains four ligand-binding domains (red) that interact with a large number of ligands. The β-chain contains a small extracellular region, a transmembrane region which anchors the LRP1 protein within the plasma membrane, as well as two dileucine (LL, green) motifs and two asparagine-proline-x-tyrosine (NPXY, blue) motifs, where the distal motif is contiguous with a tyrosine-x-x-leucine (YXXL, pink) motif which interact with intracellular adaptor proteins and the endocytotic machinery.

the beta-site APP cleaving enzyme 1 (BACE1) [23] and metalloproteinase [24] (Figure 2). sLRP1 contains the α-chain and a 55 kDa fragment of the β-chain [25] and can be detected in plasma and cerebral spinal fluid [26, 27]. Similarly, soluble fragments of LRP2 have been shown to be released from cultured choroid plexus epithelial cells and can be detected in cerebral spinal fluid [28]. LRP1 and LRP2 can also undergo intramembrane proteolysis mediated by γ-secretase, in either the plasma or endosomal membrane [29], to liberate an intracellular fragment which reportedly enters the nucleus [30, 31] (Figure 2(a)). The physiological function of soluble LRP fragments in normal neural cell development is poorly understood, but they have the potential to bind LRP ligands and prevent them from binding to full-length LRPs or, in the case of the intracellular domain, modulate gene transcription.

1.2. LRP1 and LRP2 as Mediators of Endocytosis. While the proteolytic processing of these receptors is becoming increasingly well understood, LRP1 and LRP2 remain best known for their role in mediating endocytosis (Figure 2(b)). Following ligand binding to mature LRP1 in the plasma membrane, it was originally believed that the two NPXY motifs of the cytoplasmic domain interacted with the endocytotic machinery to mediate rapid clathrin-dependant endocytosis of the receptor-ligand complex, as has been previously shown for other members of this receptor family [32]. However for LRP1, the YXXL motif and the distal dileucine motif independently mediate endocytosis, and the NPXY motifs are not required [33]. The rate of endocytosis is regulated by cAMP-dependent protein kinase A, which constitutively phosphorylates LRP1, predominantly at serine 76 of the cytoplasmic tail [34].

Like LRP1, LRP2 has two intracellular NPXY domains [5]; however unlike LRP1, the distal NPXY motif of LRP2 has been shown to interact with the phosphotyrosine-binding domain of Disabled-2 [35], a clathrin-associated sorting protein, to mediate endocytosis [29, 36, 37]. Interestingly, endocytosis does not occur during mitosis, due to the phosphorylation of Disabled-2, which removes it from the cell surface, so that it no longer colocalizes with clathrin and cannot mediate this process [38]. LRP2-directed endocytosis may still occur via clathrin-independent pathways, instead relying on the small GTPase Arf6 and caveolin 1 [39, 40]. Furthermore, LRP1- and LRP2-mediated endocytosis can be influenced by the expression of miR199a and miR199b family members, which regulate the expression of a number of genes critical for clathrin-dependent and clathrin-independent endocytosis [41]. Following endocytosis, the extracellular beta-propeller regions of LRP1 and LRP2 facilitate ligand dissociation [42], so that the ligands and receptors can be differentially sorted in early endosomes.

The mechanisms regulating the recycling of LRP1 back to the plasma membrane are not fully characterised and may vary between cell types. However, it is known that this process requires binding of the adaptor protein sorting nexin 17 to the first NPXY domain of LRP1 in early endosomes [43, 44], so that LRP1 is recycled back to the cell surface in approximately 30 minutes [45]. In early endosomes, the first NPXY domain of LRP2 instead binds the phosphotyrosine-binding domain of autosomal recessive hypercholesterolemia (ARH) [46], a clathrin-associated sorting protein that couples LRP2 to the dynein motor complex [47] and transports it from the sorting endosomes to the endocytic recycling compartment [29]. The constitutive phosphorylation of LRP2 by GSK3β is also involved in directing LRP2 to the endocytic recycling

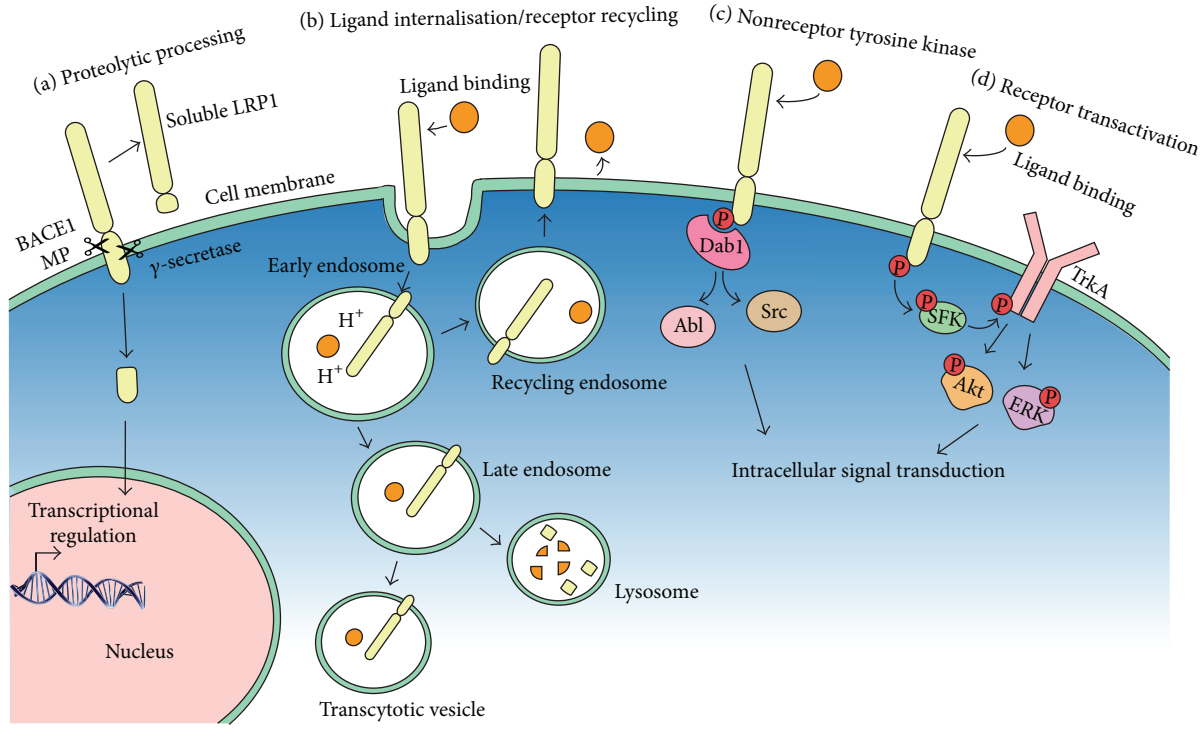

FIGURE 2: Signalling mechanisms employed by LRP1. (a) The extracellular domain of LRP1 can be shed following cleavage by beta-site APP cleaving enzyme 1 (BACE1) and metalloproteinases (MP) producing a soluble form of LRP1 (sLRP1). The intracellular domain can be cleaved by γ-secretase and is thought to translocate to the nucleus to influence gene transcription. (b) Ligand binding to LRP1 can result in receptor and ligand internalisation. Once internalised, the ligand/receptor complex can be processed in a multitude of ways, including degradation by lysosomes or resecretion via transcytotic and recycling vesicles. Note that while they are depicted together, ligand and receptor/s are trafficked independently. (c) Specific regions on the intracellular region of LRP1 interact with adaptor proteins such as Disabled-1 (Dab1), which interacts with the NPXY motifs and can recruit nonreceptor tyrosine kinases such as Src and Abl allowing signal transduction. (d) Activation of LRP1 by specific ligands can transactivate other receptors such as tropomyosin receptor kinase A (TrkA), which can then activate downstream signalling pathways to regulate cell function.

compartment, from which it is slowly recycled to the plasma membrane [48].

But what happens to the internalised ligand? LRP1 and LRP2 have been shown to bind upwards of 40 different ligands, many of which are structurally and functionally unrelated, and the list is always evolving [49]. They both have four LDL receptor homology regions which are the extracellular ligand-binding domains [50, 51] and bind common ligands including tissue-type plasminogen activator [52–55], apolipoprotein E, lactoferrin [17, 52], and metallothioneins I and II [56]; however not all ligands have been shown to bind both receptors. α2-Macroglobulin is a high affinity ligand for LRP1 [57, 58], and like prion protein has only been demonstrated to bind to LRP1 [59], while transthyretin [60] and the complex of vitamin D with the vitamin D binding protein have only been shown to bind LRP2 [61]. Once endocytosed, ligands may be degraded in lysosomes, resecreted from recycling endosomes, or trafficked in transcytotic vesicles from the apical to the basolateral membrane (or vice versa) before being secreted [62] (Figure 2(b)).

1.3. LRP1 and LRP2 Intracellular Signal Transduction. The true complexity of LRP1 and LRP2 signalling lies in the fact that these receptors not only trigger endocytosis but also influence signal transduction. Upon ligand binding, the NPXY motifs can function as a docking sites for intracellular adaptor proteins. LRP1 can bind cytosolic ligands in a phosphorylation-dependent manner, via two dileucine motifs and one YXXL motif in the intracellular domain. For example, the adaptor proteins Disabled-1 and FE65 can bind to the NPXY motifs of LRP1, to recruit and activate nonreceptor tyrosine kinases such as Src and Abl [63] (Figure 2(c)), allowing the receptor to transduce an intracellular signal or form signalling hubs through the binding of coreceptors [49] (Figure 2(d)). A number of coreceptors of LRP1 have been identified, including platelet-derived growth factor receptor (PDGFR) β [64, 65], tropomyosin-related kinase receptor A [66], amyloid precursor protein [67], and insulin-like growth factor 1 receptor [68]. These associations increase the number of intracellular pathways by which distinct LRP ligands may elicit their effects.

2. LRPs as Regulators of Nervous System Development

Despite the large number of common ligands and the structural similarities that exist between LRP1 and LRP2, the two genes are not functionally redundant during development.

Both *Lrp1* and *Lrp2* single knockout mice have severe developmental phenotypes. *Lrp1* knockout blastocysts fail to implant and therefore do not develop into embryos [69]. *Lrp2* knockout mice are mostly embryonic lethal, presenting with defects including a cleft palate, failure to form an olfactory bulb, and fusion of the forebrain hemispheres, resulting in a single ventricle (holoprosencephaly) [70]. The small number of *Lrp2* knockout mice that survive until birth experience severe vitamin D3 deficiency, as the reabsorption of vitamin D and the vitamin D binding protein from the kidney proximal tubule is LRP2-dependant, but die of respiratory failure [61, 70]. Human mutations in *Lrp2* are known to cause facio-oculo-acoustico-renal syndrome/Donnai-Barrow syndrome, an autosomal recessive disorder associated with disrupted brain formation, including agenesis of the corpus callosum [71].

The very early developmental defect observed in the *Lrp1* knockout mouse, and the gross neural phenotype of the *Lrp2* knockout mouse, do not allow us to investigate the importance of these receptors for the functioning of individual neural cell types. However, a variety of expression studies performed alongside knockdown and conditional knockout approaches demonstrate that both receptors mediate ligand endocytosis and intracellular signalling in a number of immature neural cell types. LRP1 is more widely expressed in the CNS than LRP2, being detected in mature neurons, particularly those of the entorhinal cortex, hippocampus [72] and cerebellum [73], and all CNS glia [74]. In contrast, LRP2 expression is restricted to the apical surface of the neural tube and subsequently to the forebrain, optic stalk, and otic vesicle during development [75, 76]. In the CNS of adult mice, LRP2 is predominantly expressed by cells of the choroid plexus [77] and ependymal cells [78] but has also been detected in oligodendrocytes of the spinal cord [79]. The expression patterns of LRP1 and LRP2 are largely spatially and temporally distinct, reflecting their different roles in CNS regulation.

3. LRP1 and LRP2 as Regulators of Neural Stem Cell Function

3.1. Neural Stem Cells in the Developing and Adult CNS. The early neural tube is a pseudostratified epithelium composed of neuroepithelial precursor cells. These early neural stem cells divide symmetrically, expanding their population, before switching to include asymmetric divisions that generate neuroblasts. This switch coincides with a change in gene expression, as the neuroepithelial precursor cells transition into radial glial stem cells, which comprise two molecularly distinct subgroups in the developing human brain, corresponding to those in the outer subventricular zone and those in the ventricular zone [80]. Following neuroblast generation, radial glia switch to glial generation starting with the production of oligodendrocyte progenitor cells (OPCs) and concluding with the production of astrocytic precursors [81]. Towards the end of development a subset of radial glial stem cells adopt a more astrocytic gene expression profile and give rise to the adult neural stem cells [82].

In adulthood neural stem cells reside in two key niches, the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus, where they proliferate to generate intermediate progenitor cells and ultimately neuroblasts [83]. Neural stem cells in the subventricular zone also produce a small number of OPCs under normal physiological conditions [84]. The behaviour of neural stem cells (and their intermediate progenitors) is highly controlled by mitogenic and morphogenic signalling. While key ligands and receptors for these pathways are well described, the role of LRP1 and LRP2 in these pathways has only recently been elucidated.

3.2. LRPs as Regulators of Cell Fate Specification. LRP1 and LRP2 have both been shown to facilitate the internalisation of the potent morphogen, sonic hedgehog [85–87], a finding that has provided insight into the significant neurodevelopmental defects observed in patients and mice lacking normal functioning *Lrp2* [70, 71, 75]. LRP2 is expressed by neuroepithelial cells, on the apical side of the neural plate, as early as E7.5 in the mouse. After neural tube closure at E9.5, LRP2 expression becomes increasingly restricted to the midline, ultimately being localized to the clathrin-coated pit regions of the apical cell membrane, clustered at the base of the primary cilium (a cellular organelle essential to sonic hedgehog signalling) [88] and in the subapical endosomes of the radial glia [89]. At E8 sonic hedgehog is produced by cells of the axial mesoderm (the notochord and prechordal plate) and by E8.5 its expression expands to include the radial glia at the ventral midline of the rostral diencephalon. This expansion does not occur in *Lrp2* knockout embryos, as LRP2 is required for the radial glia to bind and sequester sonic hedgehog as it diffuses, regulating morphogen presentation to the neural stem cells [89].

Once sonic hedgehog is bound to LRP2 it can also bind its receptor patched-1, and the complex undergoes clathrin-mediated endocytosis [89]. All components can then be found within early endosomes and recycling endosomes but do not appear to be targeted to the lysosome for degradation. The internalisation of patched-1 by LRP2 results in activation of the effector smoothened, leading to changes in gene transcription mediated by the Gli transcription factors. Therefore, in the absence of LRP2, radial glia show reduced expression of the sonic hedgehog target genes *gli1* and *six3* [89]. The loss of sonic hedgehog and Gli3-mediated transcriptional repression has secondary consequences for neural development, including aberrant bone morphogenic protein 4 expression in the dorsal forebrain [75, 89, 90] and disrupted fibroblast growth factor 8 and noggin expression [89]. These data indicate that LRP2 regulates the patched-1-dependent internalisation and trafficking of sonic hedgehog [89], which is necessary for neural stem cell specification and ventral forebrain patterning.

Later in development, the expression of LRP2 by spinal cord radial glial is also necessary for glial cell specification. *Lrp2* knockout mice completely lack oligodendrocyte-lineage cells and produce very few astrocytes in the spinal cord [91]. OPC specification from radial glia in the ventral spinal cord is also directed by sonic hedgehog signalling [92–96], and so the lack of spinal cord oligodendrocytes may be explained by a mechanism similar to that detailed above. However OPCs can be generated from cultured neuroepithelial precursors

derived from *sonic hedgehog* and *smoothened* knockout mice [97, 98], indicating that LRP2 must also interact with other signalling pathways such as basic fibroblast growth factor and insulin-like growth factor I [99], to promote OPC generation from neural stem cells. The decreased number of astrocytes observed in *Lrp2* knockout mice is also interesting. LRP2 is expressed by vimentin-positive cells in the E15 ventral spinal cord [79] that most likely correspond to immature astrocytes [84, 100, 101]. While LRP2 may play a role in regulating the behaviour of astrocytic precursors, it is more likely that the observed phenotype is the result of LRP2 being required for astrocyte specification by radial glia, as this immature glial population is not generated in *Lrp2* knockout mice. Despite these observations that strongly implicate LRP2 in glial cell specification during neural development, the ligands and signalling mechanisms are unknown.

LRP1 appears to fulfill a similar role in regulating glial generation in the brain. LRP1 is expressed by cells within the embryonic ventricular zone and the early postnatal subventricular zone [102]. While the role of LRP1 in regulating neural stem cell function *in vivo* is poorly understood, *in vitro* studies suggest that LRP1 can regulate OPC production. Neural stem cells can be harvested from the cortex of embryonic mice and grown as a suspension culture termed neurospheres. When differentiated, neurospheres generate neurons, astrocytes, and oligodendrocytes. However, neurospheres lacking *Lrp1* generate normal numbers of neurons, but significantly fewer O4-positive oligodendrocytes [102]. These data may reflect a requirement of LRP1 signalling in neural stem cells for OPC specification but could equally result if LRP1 is necessary for the proliferation or differentiation of OPCs (see OPC section below).

3.3. LRPs as Regulators of Neural Stem Cell Proliferation. In the subventricular zone of the adult mouse brain, LRP2 is expressed by ependymal cells underlying the neurogenic niche [78, 103]. The importance of LRP2 expression for neural stem and progenitor cell proliferation was examined in $Lrp2^{267/267}$ mutant mice, which produce a truncated form of LRP2 [104]. $Lrp2^{267/267}$ mice have ~25% fewer proliferating cells in the subventricular zone relative to control mice and a proportional reduction in the number of newborn neurons entering the olfactory bulb [78]. The absence of functional LRP2 from the neurogenic niche was accompanied by increased bone morphogenic proteins 2 and 4, increased phosphorylation of the downstream effectors SMAD1, SMAD5, and SMAD8, and increased activation of the downstream target, inhibitor of DNA binding 3 [78]. It is known that LRP2 can act as an endocytic receptor, sequestering and clearing bone morphogenic protein 4 [75]. However this does not appear to be the mechanism at play here. A ventricular infusion of noggin, the potent bone morphogenic protein 4 antagonist [105], certainly decreases neurogenesis but does so in favour of oligodendrogenesis [106], and this fate-switch is not consistent with the phenotype of the $Lrp2^{267/267}$ mouse [78].

The ability of LRPs to regulate proliferation may be more widespread amongst immature neural cell populations, as LRP1 also regulates the proliferation of cerebellar granular neuron precursors. Cerebellar granular neuron precursors are a temporary cell population that proliferate in the external germinal zone of the developing cerebellum, producing granule neurons from birth until ~P15 in the mouse [107]. This cell population is highly responsive to the promitotic effects of sonic hedgehog [108–110]. However, the effect of sonic hedgehog is negatively regulated by an interaction between LRP1 and protease nexin 1, also known as SERPINE2. Protease nexin 1 complexes with its target proteases and binds to LRP1 on the surface of cultured cerebellar granule neuron precursors [111]. Once endocytosed, protease nexin 1 antagonizes sonic hedgehog signalling, reducing the proliferation of cerebellar granule neurons. This regulation is critical for normal cerebellar development, as the absence of protease nexin 1 *in vivo* delays cerebellar granule neuron precursor differentiation and increases the overall size of the cerebellum [111]. We would predict that conditionally removing *Lrp1* from cerebellar granule neuron precursors would have the same effect.

4. LRPs as Regulators of Neuroblast Function

Neuroblast generation and their subsequent migration into the developing cortex has been well characterised [112]. Postmitotic neuroblasts that are generated in the cortical ventricular zone are destined to form cortical projection neurons [113]. They undergo radial migration out of the germinal zone, moving along the apical processes of radial glia. The final laminar position of a newborn neuron is determined by its birth date, with late-born neuroblasts migrating past early-born neurons, to seed progressively more superficial layers of the cortex [114]. In contrast, cortical interneurons are generated from radial glial cells within the ventricular zones of the medial ganglionic eminence, the caudal ganglionic eminence and the preoptic area, and undergo both radial and tangential migration to populate each of the cortical layers [115–117].

Neuroblasts born in the two neurogenic niches of the adult brain also have vastly different migratory requirements. Those born in the hippocampus are destined to be dentate granule neurons, and send axons from the dentate gyrus to CA3 of the hippocampus [118]. After birth these cells move a very short distance as they mature, migrating from the subgranular zone (the inner lip) of the dentate granule neuron layer to their final position within the layer. On the other hand, neuroblasts born in the subventricular zone migrate tangentially, moving as neuroblast chains through the rostral migratory stream into the olfactory bulb [119]. Upon exiting the rostral migratory stream, the neuroblasts turn and migrate radially and differentiate into granule and periglomerular neurons in the olfactory bulb [83]. This type of chain migration is regulated by signals that modify the actin cytoskeleton including contact-mediated signalling between the neuroblasts and the ensheathing glia [120–123] and the chemorepulsion mediated by slit and netrin [124–126]. Recent evidence suggests that, following neural stem cell specification and neuroblast generation, LDL family members, including LRP1 and LRP2, continue to play a

significant role in regulating the successful maturation and integration of these new cells in the CNS.

4.1. LRP8 and the VLDL Receptor Are Key Regulators of Neuroblast Migration in Development and Adulthood. While this review focuses on LRP1 and LRP2, it is not possible to discuss the role of LDL family members in regulating neuroblast migration without first detailing the importance of the LRP8 and VLDL receptor in cortical development. *Lrp8* and *VLDL receptor* double knockout mice have abnormalities in the layering of the brain, including the ectopic placement of neurons [127, 128], and also exhibit malformation of the cerebellum and spinal cord [127, 129]. LRP8 and the VLDL receptor are high affinity receptors for reelin [130, 131] a large extracellular matrix protein [127, 129, 130]. Mice that lack *reelin* largely phenocopy the distinct cortical lamination defects seen in the *Lrp8* and *VLDL receptor* double knockout mice [127, 129, 130]. Oligomeric reelin binds to LRP8 and the VLDL receptor, activates Src family kinases, and induces phosphorylation of Disabled-1. This signalling pathway enables polarisation, adhesion, stabilisation, process outgrowth, and ultimately neuroblast migration [132–134]. During development reelin is first expressed in the cortical marginal zone by Cajal-Retzius cells [135–137] and later by interneurons [138, 139]. Humans with mutations of the *VLDL receptor* gene have an increased risk of developing schizophrenia, which is thought to result from subtle neuroblast migration defects within the brain [140].

LRP8 and the VLDL receptor can also regulate neuroblast migration when activated by an alternative ligand, thrombospondin-1. Thrombospondin-1 is expressed in the subventricular zone and throughout the rostral migratory stream [141], where it acts on LRP8 and the VLDL receptor to promote neuroblast chain migration. *Thrombospondin-1* knockout mice have defective chain migration, with fewer neuroblasts successfully migrating to the olfactory bulb [141]. This phenotype is also observed in mice lacking LRP8 and VLDL receptor, or Disabled-1, but is not observed in *reelin* knockout mice [142]. However, the successful migration of neurons from the subventricular zone to the olfactory bulb appears to require both ligands. Thrombospondin-1 stabilizes neuroblast chains and increases their length in the subventricular zone and rostral migratory stream, but reelin, produced by mitral cells in the olfactory bulb, is a higher affinity ligand and subsequently directs neuroblast dissociation, allowing them to transition to radial migration [143]. Of the two ligands, only reelin activates the proteasomal degradation of Disabled-1, which is necessary for neuroblast dissociation [141].

There is no evidence that reelin signalling interacts with LRP1 or LRP2. However, thrombospondins are known to interact with membrane proteins such as integrins, CD47, CD36, proteoglycans, and LRP1. Thrombospondin-1 has been shown to interact with LRP1 in combination with calreticulin to promote the focal adhesion of mature oligodendrocytes [144] and microglia [145] but has not been demonstrated to regulate neuroblast migration.

4.2. LRPs, Neuroblast Migration, and Neuronal Development. LRP2 regulates neuroblast migration indirectly. *In vitro* LRP2 and caveolins are expressed by astrocytes and work together to bind and endocytose albumin [40, 146]. This is significant, as albumin uptake activates the transcription factor sterol regulatory binding element protein 1, inducing expression of stearoyl-coA 9-desaturase-1, the key enzyme required for synthesis of the neurotrophic factor oleic acid [147]. In the lateral periventricular zone of the developing rat brain, oleic acid production regulates neuronal growth, migration, axon generation, and early synaptogenesis [148, 149], with the major neurotrophic effect being mediated by the downstream effectors PAR-α, protein kinase A, and neuro D2 [150]. When *stearoyl-coA 9-desaturase-1* is knocked down in lateral periventricular explant cultures, albumin-mediated neuroblast migration is essentially prevented [148].

Once neuroblasts stop migrating, their journey is far from over. The immature neurons extend an axonal process to commence formation of the circuitry of the nervous system. The extending axons are tipped with a growth cone, which navigates the extracellular matrix, guiding the axon to its target cell to ultimately form a synapse [151]. A growth cone comprises membranous, receptor-rich, fan-shaped lamellipodia that extend along finger-like projections known as filopodia. The growth cone cytoskeleton is comprised of closely interacting microtubules and filamentous and globular actin [152–154]. Bundles of filamentous actin give structure to the filopodia, as does the cross-linked filamentous actin along the lamellipodial leading edge [152, 154, 155]. Microtubules are arranged as parallel bundles along the axon and splay outwards within the growth cone, providing structure and transport for proteins and organelles [156].

Growth cones are fitted with an elaborate suite of receptors that allow for the simultaneous integration of a multitude of chemotactic cues [157]. Binding of a chemotactic factor to its specific receptor/s on the growth cone membrane induces an intracellular signalling cascade which manipulates the cytoskeletal elements and dictates whether the response of the growth cone culminates in turning, extension, stasis, retraction, collapse, or bifurcation [154]. Well-defined receptors for chemotactic signals include the Eph family of receptor tyrosine kinases, Neuropilin, Roundabout, Deleted in Colorectal Cancer, L1, and Plexins (reviewed in [158]).

LRP1 and LRP2 are highly expressed on the growth cones of developing neurons *in vitro* and have been shown to signal in a codependent manner to promote chemotactic axon guidance within developmental neurons *in vitro* [159]. Together, LRP1 and LRP2 act as chemotactic receptors for a variety of ligands, including metallothioneins and tissue-type plasminogen activator [159]. Metallothioneins are small, highly conserved, inducible heavy metal binding proteins that are avid scavengers of reactive oxygen species [160]. Metallothioneins I and II are widely expressed in the nervous system and elsewhere. They differ by only a few amino acids and appear to have redundant functions. Metallothionein III is highly expressed in the brain, while metallothionein IV appears to be absent from the nervous system [161]. In

cultured growth cones from sensory neurons, the activation of LRP1 and LRP2 by metallothionein II stimulated chemoattraction, resulting in growth cones turning towards the source of metallothionein II [159]. Metallothionein III had the opposite effect and induced chemorepulsion. Other LRP1 ligands, such as α2-macroglobulin, and tissue-type plasminogen activator also induced chemorepulsion [159]. The opposing responses induced by different LRP1 ligands are thought to result from differential activation of downstream signaling pathways, with metallothionein II activating Ca^{2+}/calmodulin-dependent protein kinase and other receptors such as the tropomyosin-related kinase A receptor in complex signaling hubs (see Figure 2(d)).

Various LRP ligands have also been shown to alter neurite outgrowth. For example, metallothionein I/II signalling has been shown to transiently activate Akt and ERK, which belong to the mitogen-activated protein kinase and the phosphoinositide-3 kinase/Akt intracellular signalling pathways [162]. Myelin associated glycoprotein, an established chemorepulsive molecule, is known to interact with LRP1 [163] to inhibit axonal outgrowth and induce growth cone collapse [164, 165]. *In vitro* experiments have demonstrated that myelin associated glycoprotein and LRP1 form a complex with the p75 neurotrophin receptor, to activate RhoA [163], a potent mediator of growth cone collapse and axon retraction [166]. Additionally apolipoprotein E-containing lipoproteins are secreted by astrocytes and have been shown to bind LRP1 on the surface of immature neurons to promote neurite outgrowth generally, without having an effect on directionality [167]. The complexity of LRP signaling interactions in immature neurons remains to be fully deciphered but appear to be context- and ligand-dependent [168].

Mice in which *Lrp1* is selectively deleted from neurons exhibit prominent tremor and dystonia, behavioural abnormalities, hyperactivity, motor dysfunction, age-dependent dendritic spine degeneration, synapse loss, neuroinflammation, memory loss, eventual neurodegeneration, and premature death [169–171], clearly demonstrating that LRP1 is crucial to neuronal function. LRP1 is also found postsynaptically, where it can interact with NMDA receptors *in vitro*, via the intracellular scaffold postsynaptic density protein 95 [169, 172]. LRP1 is able to influence the activity of NMDA receptors and regulate their distribution and internalisation [168, 173, 174], as well as the NMDA-induced internalisation of the AMPA receptor subunit GluR1 [174]. The very nature of this LRP1/NMDA receptor relationship suggests that LRP1 plays an integral role in neurotransmitter-induced calcium signalling, particularly in synaptic plasticity [173, 174].

LRP8 also regulates synaptic plasticity [128, 175]. LRP8 activation, by the addition of reelin to primary mouse cortical neuron cultures, triggers its proteolytic cleavage by γ-secretase. The liberated intracellular domain translocates to the nucleus, along with phosphorylated CREB to enhance the transcription of genes associated with learning and memory [176]. Furthermore, the ability of neurons to produce ATP for synaptic transmission may be tied to LRP1, as cultured neurons lacking *Lrp1* have reduced expression of the glutamate transporters GLUT3 and GLUT4 [177].

5. LRP1 and LRP2 as Regulators of Oligodendrocyte Progenitor Cell Function

OPCs, also known as NG2 glia, are a proliferative, immature cell type found in the developing and adult CNS [178, 179]. OPCs can be identified by their expression of specific proteins such as the NG2 proteoglycan [180] and PDGFRα [181]. During the early stages of embryonic development, OPCs are produced from radial glia in the neuroepithelium of the developing brain and spinal cord [182, 183]. In the mouse spinal cord, OPC generation commences from the ventral pMN domain at E12.5 [182, 184]. The pMN domain is named for its role in generating spinal cord motor neurons and is defined by the expression of two transcription factors, OLIG1 and OLIG2 [185], both of which are highly expressed by OPCs and necessary for their generation and subsequent differentiation [186, 187]. *Olig1/2* expression by pMN domain neural stem cells is induced by a gradient of ventrally secreted sonic hedgehog, suggesting that specification of this domain would also be LRP1/2-dependant. In the absence of *Olig1/2*, stem cells in the pMN domain instead form V2 interneurons and astrocytes [188]. Shortly after their birth, OPCs differentiate into myelinating oligodendrocytes in the spinal cord grey and white matter [183, 189]. It is estimated that approximately 85% of all spinal cord oligodendrocytes originate from the pMN domain, but other domains such as the P3 domain [184] and more dorsal domains [190, 191] also produce OPCs, just slightly later in response to different spatiotemporal cues.

Like spinal cord OPCs, forebrain OPCs have multiple origins. They are generated and migrate in three distinct waves [192]. The initial wave commences in the medial ganglionic eminence and the anterior entopeduncular area at E12.5 in mice. The OPCs migrate from their ventral origins to populate all regions of the developing brain, including the developing cortex [96]. The next wave of OPCs is initiated at E15.5 from the lateral- and caudal-ganglionic eminence, followed by the third and final wave from the cortical neuroepithelium [192]. OPCs derived from the initial wave are lost shortly after birth [192] and the function performed by these temporary OPCs and the signals rendering them susceptible to developmental removal are still unknown. By P13, ~80% of oligodendrocyte-lineage cells in the corpus callosum originate from the cortical neuroepithelium, and the remainder originate from the lateral ganglionic eminence [191]. All OPCs that populate the optic nerve arise from the preoptic area [193].

LRP1 may be a critical regulator of OPC behaviour, as recent microarray [194] and RNA sequencing [195] data indicate that *Lrp1* mRNA is highly expressed by OPCs in the early postnatal mouse brain. However expression of this gene is rapidly downregulated upon differentiation and is barely detectable in oligodendrocytes. The role of LRP2 in regulating this lineage is more clearly established.

5.1. LRP2 Regulates OPC Proliferation and Migration during Development. One of the signalling molecules regulating OPC proliferation and migration is sonic hedgehog [196, 197], and LRP2 appears to regulate OPC proliferation and

migration by modulating sonic hedgehog availability and contributing to the generation of a concentration gradient. In the developing mouse optic nerve, LRP2 is highly expressed by astrocytes [198]. However, LRP2 expression is not homogeneous, being highest in the caudal optic nerve at E14.5, but then changing to be highest in the rostral optic nerve at E16.5. Blocking LRP2 signalling by optic nerve astrocytes leads to a significant reduction in OPC proliferation and migration [198]. *In vitro* studies suggest that the LRP2-mediated uptake and release of sonic hedgehog by astrocytes promotes OPC proliferation and act as a chemoattractant directing their migration [198]. The temporal regulation of LRP2 expression in the caudal versus rostral regions of the optic nerve would be predicted to "trap" sonic hedgehog in the region being populated by OPCs at that time. The expression pattern of LRP2 in the postnatal optic nerve has not been characterised. However as LRP2 is expressed by mature oligodendrocytes in the postnatal spinal cord [199], it might also be upregulated by optic nerve OPCs upon differentiation.

5.2. How Might LRP1 Influence OPC Behaviour? When examining LRP1 function in other cell types, there are a number of mechanisms by which LRP1 could feasibly influence OPC behaviour. For example OPC processes share some structural similarities with the growth cones of developing neurons [200, 201]. In particular growth cones comprise specialised cell membrane extensions called lamellipodia and filopodia, which also extend from the cellular processes of OPCs [200]. LRP1 signalling mediates the chemoattraction and chemorepulsion of growth cones *in vitro*, [159], so perhaps LRP1 could regulate OPC process guidance or even OPC migration. LRP1 is expressed by Schwann cells *in vivo* and regulates the migration and adhesion of immature Schwann cells *in vitro* by the activation and repression of two small Rho GTPases, Rac1 and RhoA, respectively [202]. Rac1 activation stimulates the formation of peripheral lamellae by actin remodelling in the leading process [203]. *Lrp1* knockdown decreases Rac1 activation and increases RhoA activation, which in turn increases cell adhesion and prevents migration [202]. This is of particular interest, as OPCs take on a bipolar morphology when migrating [201], and their movement has been attributed to the NG2-dependent regulation of small Rho GTPases and polarity complex proteins [204].

LRP1 also has the potential to influence OPC migration by acting as a coreceptor for PDGFRα signalling, in a similar way that it promotes fibroblast migration by cosignalling with PDGFRβ. When PDGFBB binds to PDGFRβ on the surface of cultured mouse embryonic fibroblasts, it induces migration. However this involves the association of LRP1 with PDGFRβ [205, 206]. The two receptors are internalised and colocalize in the endosomal compartment, where the kinase domain of PDGFRβ phosphorylates the distal NPXY motif of LRP1 [65, 205, 207]. Once phosphorylated, LRP1 has an increased affinity for the intracellular domain for SHP-2 [206, 208], outcompeting PDGFRβ for this interaction, and preventing further activation of downstream signalling pathways [206]. While OPCs do not express PDGFRβ, they express high levels of the related receptor, PDGFRα, which is also internalised following ligand binding [209], suggesting an association with an unidentified endocytic receptor which we propose could be LRP1. PDGFAA is known to bind to PDGFRα on the surface of OPCs and activate a phosphorylation cascade involving the Fyn tyrosine kinase and cyclin-dependant kinase 5 [210], a known regulator of the actin cytoskeleton in neurons [211]. By interacting with PDGFRα it is feasible that LRP1 could promote not only OPC migration but also proliferation and cell survival [181, 210, 212, 213]. While the signalling mechanism is likely to be different, a role for LRP1 in regulating cell survival is not unprecedented, as LRP1 has been shown to protect Schwann cells against TNFα-induced cell death in a sciatic nerve crush injury model *in vivo* and *in vitro* [214].

LRP1 could equally influence OPC migration by regulating lipid availability within the cell, as the establishment of cell polarity and movement of the leading edge during migration is dependent on the availability of cholesterol [215, 216]. Most lipid-carrying proteins cannot cross the blood brain barrier and therefore must be generated within the CNS. Apolipoprotein E is secreted by astrocytes and functions as an effective lipid transport protein and can bind LRP1 [217, 218]. Lipoproteins form noncovalent aggregates with triglycerides, phospholipids, and cholesterol esters before they bind to specific receptors and are internalised and utilized by the cell [219]. Upon binding of apolipoprotein E to LRP1, the complex is internalised where its lipid content is discharged, making it available to the cell [220], before apolipoprotein E is resecreted [221]. Once internalised, lipoproteins may be utilized by OPCs for a number of functions.

LRP1-mediated lipid uptake may alternatively allow OPCs to sustain their postsynaptic connections with neurons. Forebrain neuron-specific *Lrp1* gene knockout mice have severe deficiencies in lipid metabolism and show synapse loss [171]. The presynaptic use of cholesterol by neurons is high, due to the requirements of lipid-rich neurotransmitter vesicles [222]. However, the postsynaptic cell also utilizes cholesterol for receptor recycling in and out of the postsynaptic membrane. Therefore, cholesterol uptake into OPCs may be critical for the formation of axon-OPC synapses and maintenance of the OPC postsynaptic density.

6. Conclusions and Outlook

Our knowledge of LRP1 and LRP2 processing and trafficking has come a long way in the past decade. Without even considering the possibility that cleaved forms of these proteins may regulate gene transcription or perform dominant negative signalling functions, a growing number of studies clearly indicate that LRP1 and LRP2 perform a diverse range of cellular functions in neural stem and progenitor cell populations. The generation of conditional knockout mice has now made it possible to perform the detailed studies that will be necessary to understand the role of LRP1 and LRP2 in each immature cell type, across a variety of developmental stages. This is particularly critical now that we understand that LRP1 and LRP2 can influence the balance of growth factor and morphogen signalling, making them critical spatial and temporal regulators of neural development.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] M. S. Brown and J. L. Goldstein, "A receptor-mediated pathway for cholesterol homeostasis," *Science*, vol. 232, no. 4746, pp. 34–47, 1986.

[2] R. J. Binder, D. K. Han, and P. K. Srivastava, "CD91: a receptor for heat shock protein gp96," *Nature Immunology*, vol. 1, no. 2, pp. 151–155, 2000.

[3] C.-X. Liu, S. Musco, N. M. Lisitsina, S. Y. Yaklichkin, and N. A. Lisitsyn, "Genomic organization of a new candidate tumor suppressor gene, LRP1B," *Genomics*, vol. 69, no. 2, pp. 271–274, 2000.

[4] P. Marschang, J. Brich, E. J. Weeber et al., "Normal development and fertility of knockout mice lacking the tumor suppressor gene LRP1b suggest functional compensation by LRP1," *Molecular and Cellular Biology*, vol. 24, no. 9, pp. 3782–3793, 2004.

[5] A. Saito, S. Pietromonaco, A. Loo et al., "Complete cloning and sequencing of rat Gp330 megalin, a distinctive member of the low-density-lipoprotein receptor gene family," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 21, pp. 9725–9729, 1994.

[6] P. J. Hey, R. C. J. Twells, M. S. Phillips et al., "Cloning of a novel member of the low-density lipoprotein receptor family," *Gene*, vol. 216, no. 1, pp. 103–111, 1998.

[7] S. D. Brown, R. C. J. Twells, P. J. Hey et al., "Isolation and characterization of LRP6, a novel member of the low density lipoprotein receptor gene family," *Biochemical and Biophysical Research Communications*, vol. 248, no. 3, pp. 879–888, 1998.

[8] D. R. Riddell, D. V. Vinogradov, A. K. Stannard, N. Chadwick, and J. S. Owen, "Identification and characterization of LRP8 (apoER2) in human blood platelets," *Journal of Lipid Research*, vol. 40, no. 10, pp. 1925–1930, 1999.

[9] N. L. Daly, M. J. Scanlon, J. T. Djordjevic, P. A. Kroon, and R. Smith, "Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 14, pp. 6334–6338, 1995.

[10] V. Llorente-Cortés, P. Costales, J. B. Bernués, S. Camino-Lopez, and L. Badimon, "Sterol regulatory element-binding protein-2 negatively regulates low density lipoprotein receptor-related protein transcription," *Journal of Molecular Biology*, vol. 359, no. 4, pp. 950–960, 2006.

[11] J. Castellano, R. Aledo, J. Sendra et al., "Hypoxia stimulates low-density lipoprotein receptor-related protein-1 expression through hypoxia-inducible factor-1α in human vascular smooth muscle cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 6, pp. 1411–1420, 2011.

[12] T. R. Grana, J. Lamarre, and B. E. Kalisch, "Nerve growth factor-mediated regulation of low density lipoprotein receptor-related protein promoter activation," *Cellular and Molecular Neurobiology*, vol. 33, no. 2, pp. 269–282, 2013.

[13] Y. Yamanaka, M. Faghihi, M. Magistri, O. Alvarez-Garcia, M. Lotz, and C. Wahlestedt, "Antisense RNA controls LRP1 sense transcript expression through interaction with a chromatin-associated protein, HMGB2," *Cell Reports*, vol. 11, no. 6, pp. 967–976, 2015.

[14] G. Rudenko, L. Henry, K. Henderson et al., "Structure of the LDL receptor extracellular domain at endosomal pH," *Science*, vol. 298, no. 5602, pp. 2353–2358, 2002.

[15] T. E. Willnow, S. A. Armstrong, R. E. Hammer, and J. Herz, "Functional expression of low density lipoprotein receptor-related protein is controlled by receptor-associated protein in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 10, pp. 4537–4541, 1995.

[16] G. J. Bu and M. P. Marzolo, "Role of RAP in the biogenesis of lipoprotein receptors," *Trends in Cardiovascular Medicine*, vol. 10, no. 4, pp. 148–155, 2000.

[17] J. E. Croy, W. D. Shin, M. F. Knauer, D. J. Knauer, and E. A. Komives, "All three LDL receptor homology regions of the LDL receptor-related protein bind multiple ligands," *Biochemistry*, vol. 42, no. 44, pp. 13049–13057, 2003.

[18] S. M. Reekmans, T. Pflanzner, P. L. S. M. Gordts et al., "Inactivation of the proximal NPXY motif impairs early steps in LRP1 biosynthesis," *Cellular and Molecular Life Sciences*, vol. 67, no. 1, pp. 135–145, 2010.

[19] D. Lee, J. D. Walsh, I. Mikhailenko et al., "RAP uses a histidine switch to regulate its interaction with LRP in the ER and Golgi," *Molecular Cell*, vol. 22, no. 3, pp. 423–430, 2006.

[20] G. Bu, H. J. Geuze, G. J. Strous, and A. L. Schwartz, "39-kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein," *The EMBO Journal*, vol. 14, no. 10, pp. 2269–2280, 1995.

[21] T. E. Willnow, J. M. Moehring, N. M. Inocencio, T. J. Moehring, and J. Herz, "The low-density-lipoprotein receptor-related protein (LRP) is processed by furin in vivo and in vitro," *Biochemical Journal*, vol. 313, no. 1, pp. 71–76, 1996.

[22] R.-P. Czekay, R. A. Orlando, L. Woodward, M. Lundstrom, and M. G. Farquhar, "Endocytic trafficking of megalin/RAP complexes: dissociation of the complexes in late endosomes," *Molecular Biology of the Cell*, vol. 8, no. 3, pp. 517–532, 1997.

[23] C. A. F. von Arnim, A. Kinoshita, I. D. Peltan et al., "The low density lipoprotein receptor-related protein (LRP) is a novel beta-secretase (BACE1) substrate," *The Journal of Biological Chemistry*, vol. 280, no. 18, pp. 17777–17785, 2005.

[24] C. Selvais, L. D'Auria, D. Tyteca et al., "Cell cholesterol modulates metalloproteinase-dependent shedding of low-density lipoprotein receptor-related protein-1 (LRP-1) and clearance function," *The FASEB Journal*, vol. 25, no. 8, pp. 2770–2781, 2011.

[25] K. A. Quinn, V. J. Pye, Y.-P. Dai, C. N. Chesterman, and D. A. Owensby, "Characterization of the soluble form of the low density lipoprotein receptor-related protein (LRP)," *Experimental Cell Research*, vol. 251, no. 2, pp. 433–441, 1999.

[26] K. A. Quinn, P. G. Grimsley, Y.-P. Dai, M. Tapner, C. N. Chesterman, and D. A. Owensby, "Soluble low density lipoprotein receptor-related protein (LRP) circulates in human plasma," *The Journal of Biological Chemistry*, vol. 272, no. 38, pp. 23946–23951, 1997.

[27] Q. Liu, J. Zhang, H. Tran et al., "LRP1 shedding in human brain: roles of ADAM10 and ADAM17," *Molecular Neurodegeneration*, vol. 4, no. 1, article 17, 2009.

[28] C. Spuch, D. Antequera, C. Pascual et al., "Soluble megalin is reduced in cerebrospinal fluid samples of Alzheimer's disease patients," *Frontiers in Cellular Neuroscience*, vol. 9, article 134, 2015.

[29] M. Shah, O. Y. Baterina Jr., V. Taupin, and M. Farquhar, "ARH directs megalin to the endocytic recycling compartment to regulate its proteolysis and gene expression," *The Journal of Cell Biology*, vol. 202, no. 1, pp. 113–127, 2013.

[30] D. Biemesderfer, "Regulated intramembrane proteolysis of megalin: linking urinary protein and gene regulation in proximal tubule?" *Kidney International*, vol. 69, no. 10, pp. 1717–1721, 2006.

[31] P. May, Y. Krishna Reddy, and J. Herz, "Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain," *The Journal of Biological Chemistry*, vol. 277, no. 21, pp. 18736–18743, 2002.

[32] W.-J. Chen, J. L. Goldstein, and M. S. Brown, "NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor," *The Journal of Biological Chemistry*, vol. 265, no. 6, pp. 3116–3123, 1990.

[33] Y. Li, M. P. Marzolo, P. van Kerkhof, G. J. Strous, and G. Bu, "The YXXL motif, but not the two NPXY motifs, serves as the dominant endocytosis signal for low density lipoprotein receptor-related protein," *Journal of Biological Chemistry*, vol. 275, no. 22, pp. 17187–17194, 2000.

[34] Y. Li, P. van Kerkhof, M. P. Marzolo, G. J. Strous, and G. Bu, "Identification of a major cyclic AMP-dependent protein kinase A phosphorylation site within the cytoplasmic tail of the low-density lipoprotein receptor-related protein: implication for receptor-mediated endocytosis," *Molecular and Cellular Biology*, vol. 21, no. 4, pp. 1185–1195, 2001.

[35] A. V. Oleinikov, J. Zhao, and S. P. Makker, "Cytosolic adaptor protein Dab2 is an intracellular ligand of endocytic receptor gp600/megalin," *Biochemical Journal*, vol. 347, no. 3, pp. 613–621, 2000.

[36] J. Nagai, E. I. Christensen, S. M. Morris, T. E. Willnow, J. A. Cooper, and R. Nielsen, "Mutually dependent localization of megalin and Dab2 in the renal proximal tubule," *American Journal of Physiology—Renal Physiology*, vol. 289, no. 3, pp. F569–F576, 2005.

[37] L. M. Traub, "Tickets to ride: Selecting cargo for clathrin-regulated internalization," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 9, pp. 583–596, 2009.

[38] D. Chetrit, L. Barzilay, G. Horn, T. Bielik, N. I. Smorodinsky, and M. Ehrlich, "Negative regulation of the endocytic adaptor disabled-2 (Dab2) in mitosis," *The Journal of Biological Chemistry*, vol. 286, no. 7, pp. 5392–5403, 2011.

[39] N. A. Wolff, W.-K. Lee, M. Abouhamed, and F. Thévenod, "Role of ARF6 in internalization of metal-binding proteins, metallothionein and transferrin, and cadmium-metallothionein toxicity in kidney proximal tubule cells," *Toxicology and Applied Pharmacology*, vol. 230, no. 1, pp. 78–85, 2008.

[40] A. Bento-Abreu, A. Velasco, E. Polo-Hernández et al., "Albumin endocytosis via megalin in astrocytes is caveola- and Dab-1 dependent and is required for the synthesis of the neurotrophic factor oleic acid," *Journal of Neurochemistry*, vol. 111, no. 1, pp. 49–60, 2009.

[41] J. F. Aranda, A. Canfran-Duque, L. Goedeke, Y. Suarez, and C. Fernandez-Hernando, "The miR-199-dynamin regulatory axis controls receptor-mediated endocytosis," *Journal of Cell Science*, vol. 128, no. 17, pp. 3197–3209, 2015.

[42] H. Jeon, W. Meng, J. Takagi, M. J. Eck, T. A. Springer, and S. C. Blacklow, "Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair," *Nature Structural & Molecular Biology*, vol. 8, no. 6, pp. 499–504, 2001.

[43] M. Donoso, J. Cancino, J. Lee et al., "Polarized traffic of LRP1 involves AP1B and SNX17 operating on Y-dependent sorting motifs in different pathways," *Molecular Biology of the Cell*, vol. 20, no. 1, pp. 481–497, 2009.

[44] P. Farfán, J. Lee, J. Larios, P. Sotelo, G. Bu, and M.-P. Marzolo, "A sorting nexin 17-binding domain within the LRP1 cytoplasmic tail mediates receptor recycling through the basolateral sorting endosome," *Traffic*, vol. 14, no. 7, pp. 823–838, 2013.

[45] K. W. S. Ko, R. S. McLeod, R. K. Avramoglu et al., "Mutation at the processing site of chicken low density lipoprotein receptor-related protein impairs efficient endoplasmic reticulum exit, but proteolytic cleavage is not essential for its endocytic functions," *The Journal of Biological Chemistry*, vol. 273, no. 43, pp. 27779–27785, 1998.

[46] M. Nagai, T. Meerloo, T. Takeda, and M. G. Farquhar, "The adaptor protein ARH escorts megalin to and through endosomes," *Molecular Biology of the Cell*, vol. 14, no. 12, pp. 4984–4996, 2003.

[47] S. Lehtonen, M. Shah, R. Nielsen et al., "The endocytic adaptor protein ARH associates with motor and centrosomal proteins and is involved in centrosome assembly and cytokinesis," *Molecular Biology of the Cell*, vol. 19, no. 7, pp. 2949–2961, 2008.

[48] M. I. Yuseff, P. Farfán, G. Bu, and M.-P. Marzolo, "A cytoplasmic PPPSP motif determines megalin's phosphorylation and regulates receptor's recycling and surface expression," *Traffic*, vol. 8, no. 9, pp. 1215–1230, 2007.

[49] C. Spuch, S. Ortolano, and C. Navarro, "LRP-1 and LRP-2 receptors function in the membrane neuron. Trafficking mechanisms and proteolytic processing in alzheimer's disease," *Frontiers in Physiology*, vol. 3, article 269, 2012.

[50] J. Herz and D. K. Strickland, "LRP: a multifunctional scavenger and signaling receptor," *Journal of Clinical Investigation*, vol. 108, no. 6, pp. 779–784, 2001.

[51] M.-P. Marzolo and P. Farfán, "New insights into the roles of megalin/LRP2 and the regulation of its functional expression," *Biological Research*, vol. 44, no. 1, pp. 89–105, 2011.

[52] T. E. Willnow, J. L. Goldstein, K. Orth, M. S. Brown, and J. Herz, "Low density lipoprotein receptor-related protein and gp330 bind similar ligands, including plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance," *The Journal of Biological Chemistry*, vol. 267, no. 36, pp. 26172–26180, 1992.

[53] G. Bu, E. A. Maksymovitch, and A. L. Schwartz, "Receptor-mediated endocytosis of tissue-type plasminogen activator by low density lipoprotein receptor-related protein on human hepatoma HepG2 cells," *The Journal of Biological Chemistry*, vol. 268, no. 17, pp. 13002–13009, 1993.

[54] S. R. Grobmyer, A. Kuo, M. Orishimo, S. S. Okada, D. B. Cines, and E. S. Barnathan, "Determinants of binding and internalization of tissue-type plasminogen activator by human vascular smooth muscle and endothelial cells," *The Journal of Biological Chemistry*, vol. 268, no. 18, pp. 13291–13300, 1993.

[55] S. Lin, J. Racz, M. F. Tai et al., "A role for low density lipoprotein receptor-related protein 1 in the cellular uptake of tissue plasminogen activator in the lungs," *Pharmaceutical Research*, vol. 33, no. 1, pp. 72–82, 2016.

[56] M. Ambjørn, J. W. Asmussen, M. Lindstam et al., "Metallothionein and a peptide modeled after metallothionein, EmtinB, induce neuronal differentiation and survival through binding to receptors of the low-density lipoprotein receptor family," *Journal of Neurochemistry*, vol. 104, no. 1, pp. 21–37, 2008.

[57] J. A. Hanover, M. C. Willingham, and I. Pastan, "Receptor-mediated endocytosis of α_2-macroglobulin: solubilization and partial purification of the fibroblast α_2-macroglobulin receptor," *Annals of the New York Academy of Sciences*, vol. 421, pp. 410–423, 1983.

[58] P. Marynen, F. Van Leuven, J.-J. Cassiman, and H. Van den Berghe, "Solubilization and affinity purification of the α2-macroglobulin receptor from human fibroblasts," *The Journal of Biological Chemistry*, vol. 259, no. 11, pp. 7075–7079, 1984.

[59] C. J. Parkyn, E. G. M. Vermeulen, R. C. Mootoosamy et al., "LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein," *Journal of Cell Science*, vol. 121, no. 6, pp. 773–783, 2008.

[60] M. M. Sousa, L. Berglund, and M. J. Saraiva, "Transthyretin in high density lipoproteins: association with apolipoprotein A-I," *Journal of Lipid Research*, vol. 41, no. 1, pp. 58–65, 2000.

[61] A. Nykjaer, D. Dragun, D. Walther et al., "An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D_3," *Cell*, vol. 96, no. 4, pp. 507–515, 1999.

[62] T. E. Willnow, A. Christ, and A. Hammes, "Endocytic receptor-mediated control of morphogen signaling," *Development*, vol. 139, no. 23, pp. 4311–4319, 2012.

[63] M. Trommsdorff, J.-P. Borg, B. Margolis, and J. Herz, "Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein," *The Journal of Biological Chemistry*, vol. 273, no. 50, pp. 33556–33560, 1998.

[64] P. Boucher, P. Liu, M. Gotthardt, T. Hiesberger, R. G. W. Anderson, and J. Herz, "Platelet-derived growth factor mediates tyrosine phosphorylation of the cytoplasmic domain of the low density lipoprotein receptor-related protein in caveolae," *The Journal of Biological Chemistry*, vol. 277, no. 18, pp. 15507–15513, 2002.

[65] E. Loukinova, S. Ranganathan, S. Kuznetsov et al., "Platelet-derived growth factor (PDGF)-induced tyrosine phosphorylation of the low density lipoprotein receptor-related protein (LRP). Evidence for integrated co-receptor function between LRP and the PDGF," *The Journal of Biological Chemistry*, vol. 277, no. 18, pp. 15499–15506, 2002.

[66] Y. Shi, E. Mantuano, G. Inoue, W. M. Campana, and S. L. Gonias, "Ligand binding to LRP1 transactivates trk receptors by a src family kinase-dependent pathway," *Science Signaling*, vol. 2, no. 68, article ra18, 2009.

[67] C. U. Pietrzik, I.-S. Yoon, S. Jaeger, T. Busse, S. Weggen, and E. H. Koo, "FE65 constitutes the functional link between the low-density lipoprotein receptor-related protein and the amyloid precursor protein," *Journal of Neuroscience*, vol. 24, no. 17, pp. 4259–4265, 2004.

[68] E. Woldt, R. L. Matz, J. Terrand et al., "Differential signaling by adaptor molecules LRP1 and ShcA regulates adipogenesis by the insulin-like growth factor-1 receptor," *The Journal of Biological Chemistry*, vol. 286, no. 19, pp. 16775–16782, 2011.

[69] J. Herz, D. E. Clouthier, and R. E. Hammer, "LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation," *Cell*, vol. 71, no. 3, pp. 411–421, 1992.

[70] T. E. Willnow, J. Hilpert, S. A. Armstrong et al., "Defective forebrain development in mice lacking gp330/megalin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 16, pp. 8460–8464, 1996.

[71] S. Kantarci, L. Al-Gazali, R. Hill et al., "Mutations in LRP2, coding for the multi-ligand receptor megalin, cause Donnai-Barrow and Facio oculo acousticorenal (FOAR) syndromes," *European Journal of Human Genetics*, vol. 15, supplement 1, p. 3, 2007.

[72] B. B. Wolf, M. B. S. Lopes, S. R. VandenBerg, and S. L. Gonias, "Characterization and immunohistochemical localization of α2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain," *American Journal of Pathology*, vol. 141, no. 1, pp. 37–42, 1992.

[73] G. Bu, E. A. Maksymovitch, J. M. Nerbonne, and A. L. Schwartz, "Expression and function of the low-density-lipoprotein receptor-related protein (LRP) in mammalian central neurons," *The Journal of Biological Chemistry*, vol. 269, no. 28, pp. 18521–18528, 1994.

[74] A. Gaultier, X. Wu, N. Le Moan et al., "Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis," *Journal of Cell Science*, vol. 122, part 8, pp. 1155–1162, 2009.

[75] R. Spoelgen, A. Hammes, U. Anzenberger et al., "LRP2/megalin is required for patterning of the ventral telencephalon," *Development*, vol. 132, no. 2, pp. 405–414, 2005.

[76] E. Assémat, F. Châtelet, J. Chandellier et al., "Overlapping expression patterns of the multiligand endocytic receptors cubilin and megalin in the CNS, sensory organs and developing epithelia of the rodent embryo," *Gene Expression Patterns*, vol. 6, no. 1, pp. 69–78, 2005.

[77] J. T. Chun, L. Wang, G. M. Pasinetti, C. E. Finch, and B. V. Zlokovic, "Glycoprotein 330/megalin (LRP-2) has low prevalence as mRNA and protein in brain microvessels and choroid plexus," *Experimental Neurology*, vol. 157, no. 1, pp. 194–201, 1999.

[78] C. R. Gajera, H. Emich, O. Lioubinski et al., "LRP2 in ependymal cells regulates BMP signaling in the adult neurogenic niche," *Journal of Cell Science*, vol. 123, no. 11, pp. 1922–1930, 2010.

[79] G. Wicher, M. Larsson, L. Rask, and H. Aldskogius, "Low-density lipoprotein receptor-related protein (LRP)-2/megalin is transiently expressed in a subpopulation of neural progenitors in the embryonic mouse spinal cord," *Journal of Comparative Neurology*, vol. 492, no. 2, pp. 123–131, 2005.

[80] A. A. Pollen, T. J. Nowakowski, J. Chen et al., "Molecular identity of human outer radial glia during cortical development," *Cell*, vol. 163, no. 1, pp. 55–67, 2015.

[81] N. Kessaris, N. Pringle, and W. D. Richardson, "Specification of CNS glia from neural stem cells in the embryonic neuroepithelium," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 363, no. 1489, pp. 71–85, 2008.

[82] K. M. Young, M. Fogarty, N. Kessaris, and W. D. Richardson, "Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb," *The Journal of Neuroscience*, vol. 27, no. 31, pp. 8286–8296, 2007.

[83] M. O'Rourke, R. Gasperini, and K. M. Young, "Adult myelination: wrapping up neuronal plasticity," *Neural Regeneration Research*, vol. 9, no. 13, pp. 1261–1264, 2014.

[84] K. M. Young, T. Mitsumori, N. Pringle, M. Grist, N. Kessaris, and W. D. Richardson, "An *Fgfr3-iCreER*^{T2} transgenic mouse line for studies of neural stem cells and astrocytes," *Glia*, vol. 58, no. 8, pp. 943–953, 2010.

[85] M. I. Capurro, W. Shi, and J. Filmus, "LRP1 mediates the Shh-induced endocytosis of the GPC3-Shh complex," *Journal of Cell Science*, vol. 125, part 14, pp. 3380–3389, 2012.

[86] R. A. McCarthy, J. L. Barth, M. R. Chintalapudi et al., "Megalin functions as an endocytic sonic hedgehog receptor," *The Journal of Biological Chemistry*, vol. 277, no. 28, pp. 25660–25667, 2002.

[87] C. R. Morales, J. Zeng, M. El Alfy et al., "Epithelial trafficking of Sonic hedgehog by megalin," *Journal of Histochemistry and Cytochemistry*, vol. 54, no. 10, pp. 1115–1127, 2006.

[88] Z. Choudhry, A. A. Rikani, A. M. Choudhry et al., "Sonic hedgehog signalling pathway: a complex network," *Annals of Neurosciences*, vol. 21, no. 1, pp. 1–4, 2014.

[89] A. Christ, A. Christa, E. Kur et al., "LRP2 is an auxiliary SHH receptor required to condition the forebrain ventral midline for inductive signals," *Developmental Cell*, vol. 22, no. 2, pp. 268–278, 2012.

[90] M. Fuccillo, A. L. Joyner, and G. Fishell, "Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development," *Nature Reviews Neuroscience*, vol. 7, no. 10, pp. 772–783, 2006.

[91] G. Wicher and H. Aldskogius, "Megalin deficiency induces critical changes in mouse spinal cord development," *NeuroReport*, vol. 19, no. 5, pp. 559–563, 2008.

[92] C. Poncet, C. Soula, F. Trousse et al., "Induction of oligodendrocyte progenitors in the trunk neural tube by ventralizing signals: effects of notochord and floor plate grafts, and of sonic hedgehog," *Mechanisms of Development*, vol. 60, no. 1, pp. 13–32, 1996.

[93] N. P. Pringle, W.-P. Yu, S. Guthrie et al., "Determination of neuroepithelial cell fate: induction of the oligodendrocyte lineage by ventral midline cells and sonic hedgehog," *Developmental Biology*, vol. 177, no. 1, pp. 30–42, 1996.

[94] D. M. Orentas, J. E. Hayes, K. L. Dyer, and R. H. Miller, "Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors," *Development*, vol. 126, no. 11, pp. 2419–2429, 1999.

[95] S. Nery, H. Wichterle, and G. Fishell, "Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain," *Development*, vol. 128, no. 4, pp. 527–540, 2001.

[96] N. Tekki-Kessaris, R. Woodruff, A. C. Hall et al., "Hedgehog-dependent oligodendrocyte lineage specification in the telencephalon," *Development*, vol. 128, no. 13, pp. 2545–2554, 2001.

[97] J. Cai, Y. Qi, X. Hu et al., "Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling," *Neuron*, vol. 45, no. 1, pp. 41–53, 2005.

[98] S. Chandran, H. Kato, D. Gerreli, A. Compston, C. N. Svendsen, and N. D. Allen, "FGF-dependent generation of oligodendrocytes by a hedgehog-independent pathway," *Development*, vol. 130, no. 26, pp. 6599–6609, 2003.

[99] M. Bolós, S. Fernandez, and I. Torres-Aleman, "Oral administration of a GSK3 inhibitor increases brain insulin-like growth factor I levels," *The Journal of Biological Chemistry*, vol. 285, no. 23, pp. 17693–17700, 2010.

[100] N. P. Pringle, W.-P. Yu, M. Howell, J. S. Colvin, D. M. Ornitz, and W. D. Richardson, "Fgfr3 expression by astrocytes and their precursors: evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains," *Development*, vol. 130, no. 1, pp. 93–102, 2003.

[101] Y. Y. Wu, Y. Liu, E. M. Levine, and M. S. Rao, "Hes1 but not Hes5 regulates an astrocyte versus oligodendrocyte fate choice in glial restricted precursors," *Developmental Dynamics*, vol. 226, no. 4, pp. 675–689, 2003.

[102] E. Hennen, D. Safina, U. Haussmann et al., "A LewisX glycoprotein screen identifies the low density lipoprotein receptor-related protein 1 (LRP1) as a modulator of oligodendrogenesis in mice," *The Journal of Biological Chemistry*, vol. 288, no. 23, pp. 16538–16545, 2013.

[103] G. Zheng, D. R. Bachinsky, I. Stamenkovic et al., "Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/alpha 2MR, and the receptor-associated protein (RAP)," *Journal of Histochemistry & Cytochemistry*, vol. 42, no. 4, pp. 531–542, 1994.

[104] K. Zarbalis, S. R. May, Y. Shen, M. Ekker, J. L. R. Rubenstein, and A. S. Peterson, "A focused and efficient genetic screening strategy in the mouse: identification of mutations that disrupt cortical development," *PLoS Biology*, vol. 2, no. 8, pp. 1177–1187, 2004.

[105] D. A. Lim, A. D. Tramontin, J. M. Trevejo, D. G. Herrera, J. M. García-Verdugo, and A. Alvarez-Buylla, "Noggin antagonizes BMP signaling to create a niche for adult neurogenesis," *Neuron*, vol. 28, no. 3, pp. 713–726, 2000.

[106] J. K. Sabo, T. J. Kilpatrick, and H. S. Cate, "Effects of bone morphogenic proteins on neural precursor cells and regulation during central nervous system injury," *NeuroSignals*, vol. 17, no. 4, pp. 255–264, 2009.

[107] H. Marzban, M. R. Del Bigio, J. Alizadeh, S. Ghavami, R. M. Zachariah, and M. Rastegar, "Cellular commitment in the developing cerebellum," *Frontiers in Cellular Neuroscience*, vol. 8, article 450, 2015.

[108] N. Dahmane and A. Ruiz i Altaba, "Sonic hedgehog regulates the growth and patterning of the cerebellum," *Development*, vol. 126, no. 14, pp. 3089–3100, 1999.

[109] R. J. Wechsler-Reya and M. P. Scott, "Control of neuronal precursor proliferation in the cerebellum by sonic hedgehog," *Neuron*, vol. 22, no. 1, pp. 103–114, 1999.

[110] P. Haldipur, U. Bharti, S. Govindan et al., "Expression of sonic hedgehog during cell proliferation in the human cerebellum," *Stem Cells and Development*, vol. 21, no. 7, pp. 1059–1068, 2012.

[111] C. Vaillant, O. Michos, S. Orlicki et al., "Protease nexin 1 and its receptor LRP modulate SHH signalling during cerebellar development," *Development*, vol. 134, no. 9, pp. 1745–1754, 2007.

[112] J. Stiles and T. L. Jernigan, "The basics of brain development," *Neuropsychology Review*, vol. 20, no. 4, pp. 327–348, 2010.

[113] J. G. Parnavelas, "The origin and migration of cortical neurones: new vistas," *Trends in Neurosciences*, vol. 23, no. 3, pp. 126–131, 2000.

[114] A. Kriegstein, S. Noctor, and V. Martínez-Cerdeño, "Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion," *Nature Reviews Neuroscience*, vol. 7, no. 11, pp. 883–890, 2006.

[115] A. A. Lavdas, M. Grigoriou, V. Pachnis, and J. G. Parnavelas, "The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex," *Journal of Neuroscience*, vol. 19, no. 18, pp. 7881–7888, 1999.

[116] K. Letinic, R. Zoncu, and P. Rakic, "Origin of GABAergic neurons in the human neocortex," *Nature*, vol. 417, no. 6889, pp. 645–649, 2002.

[117] S. Nery, G. Fishell, and J. G. Corbin, "The caudal ganglionic eminence is a source of distinct cortical and subcortical cell populations," *Nature Neuroscience*, vol. 5, no. 12, pp. 1279–1287, 2002.

[118] N. B. Hastings and E. Gould, "Rapid extension of axons into the CA3 region by adult-generated granule cells (vol 413, pg 146, 1999)," *Journal of Comparative Neurology*, vol. 415, no. 1, p. 144, 1999.

[119] F. Doetsch, I. Caillé, D. A. Lim, J. M. Garcia-Verdugo, and A. Alvarez-Buylla, "Subventricular zone astrocytes are neural stem cells in the adult mammalian brain," *Cell*, vol. 97, no. 6, pp. 703–716, 1999.

[120] S. Alberti, S. M. Krause, O. Kretz et al., "Neuronal migration in the murine rostral migratory stream requires serum response factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 17, pp. 6148–6153, 2005.

[121] H. Tomasiewicz, K. Ono, D. L. Yee et al., "Genetic deletion of a neural cell-adhesion molecule variant (N-CAM-180) produces distinct defects in the central-nervous-system," *Neuron*, vol. 11, no. 6, pp. 1163–1174, 1993.

[122] G. Chazal, P. Durbec, A. Jankovski, G. Rougon, and H. Cremer, "Consequences of neural cell adhesion molecule deficiency on cell migration in the rostral migratory stream of the mouse," *The Journal of Neuroscience*, vol. 20, no. 4, pp. 1446–1457, 2000.

[123] H. Y. Hu, H. Tomasiewicz, T. Magnuson, and U. Rutishauser, "The role of polysialic acid in migration of olfactory bulb interneuron precursors in the subventricular zone," *Neuron*, vol. 16, no. 4, pp. 735–743, 1996.

[124] K. T. Nguyen-Ba-Charvet, N. Picard-Riera, M. Tessier-Lavigne, A. B.-V. Evercooren, C. Sotelo, and A. Chédotal, "Multiple roles for slits in the control of cell migration in the rostral migratory stream," *The Journal of Neuroscience*, vol. 24, no. 6, pp. 1497–1506, 2004.

[125] W. Wu, K. Wong, J.-H. Chen et al., "Directional guidance of neuronal migration in the olfactory system by the protein Slit," *Nature*, vol. 400, no. 6742, pp. 331–336, 1999.

[126] S.-I. Murase and A. F. Horwitz, "Deleted in colorectal carcinoma and differentially expressed integrins mediate the directional migration of neural precursors in the rostral migratory stream," *Journal of Neuroscience*, vol. 22, no. 9, pp. 3568–3579, 2002.

[127] M. Trommsdorff, M. Gotthardt, T. Hiesberger et al., "Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2," *Cell*, vol. 97, no. 6, pp. 689–701, 1999.

[128] E. J. Weeber, U. Beffert, C. Jones et al., "Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning," *The Journal of Biological Chemistry*, vol. 277, no. 42, pp. 39944–39952, 2002.

[129] J. Su, M. A. Klemm, A. M. Josephson, and M. A. Fox, "Contributions of VLDLR and LRP8 in the establishment of retinogeniculate projections," *Neural Development*, vol. 8, no. 1, article 11, 2013.

[130] G. D'Arcangelo, K. Nakajima, T. Miyata, M. Ogawa, K. Mikoshiba, and T. Curran, "Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody," *The Journal of Neuroscience*, vol. 17, no. 1, pp. 23–31, 1997.

[131] T. Hiesberger, M. Trommsdorff, B. W. Howell et al., "Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation," *Neuron*, vol. 24, no. 2, pp. 481–489, 1999.

[132] S. Niu, A. Renfro, C. C. Quattrocchi, M. Sheldon, and G. D'Arcangelo, "Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dab1 pathway," *Neuron*, vol. 41, no. 1, pp. 71–84, 2004.

[133] A. J. Nichols and E. C. Olson, "Reelin promotes neuronal orientation and dendritogenesis during preplate splitting," *Cerebral Cortex*, vol. 20, no. 9, pp. 2213–2223, 2010.

[134] K. Sekine, T. Kawauchi, K.-I. Kubo et al., "Reelin controls neuronal positioning by promoting cell-matrix adhesion via inside-out activation of integrin $\alpha5\beta1$," *Neuron*, vol. 76, no. 2, pp. 353–369, 2012.

[135] M. Frotscher, "Cajal-Retzius cells, Reelin, and the formation of layers," *Current Opinion in Neurobiology*, vol. 8, no. 5, pp. 570–575, 1998.

[136] M. Marín-Padilla, "Cajal-Retzius cells and the development of the neocortex," *Trends in Neurosciences*, vol. 21, no. 2, pp. 64–71, 1998.

[137] E. Soriano and J. A. del Río, "The cells of cajal-retzius: still a mystery one century after," *Neuron*, vol. 46, no. 3, pp. 389–394, 2005.

[138] S. Alcántara, M. Ruiz, G. D'Arcangelo et al., "Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse," *Journal of Neuroscience*, vol. 18, no. 19, pp. 7779–7799, 1998.

[139] A. Drakew, M. Frotscher, T. Deller, M. Ogawa, and B. Heimrich, "Developmental distribution of a reeler gene-related antigen in the rat hippocampal formation visualized by CR-50 immunocytochemistry," *Neuroscience*, vol. 82, no. 4, pp. 1079–1086, 1997.

[140] S. I. Deutsch, J. A. Burket, and E. Katz, "Does subtle disturbance of neuronal migration contribute to schizophrenia and other neurodevelopmental disorders? Potential genetic mechanisms with possible treatment implications," *European Neuropsychopharmacology*, vol. 20, no. 5, pp. 281–287, 2010.

[141] S. M. Blake, V. Strasser, N. Andrade et al., "Thrombospondin-1 binds to ApoER2 and VLDL receptor and functions in postnatal neuronal migration," *The EMBO Journal*, vol. 27, no. 22, pp. 3069–3080, 2008.

[142] N. Andrade, V. Komnenovic, S. M. Blake et al., "ApoER2/VLDL receptor and Dab1 in the rostral migratory stream function in postnatal neuronal migration independently of Reelin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 20, pp. 8508–8513, 2007.

[143] I. Hack, M. Bancila, K. Loulier, P. Carroll, and H. Cremer, "Reelin is a detachment signal in tangential chain-migration during postnatal neurogenesis," *Nature Neuroscience*, vol. 5, no. 10, pp. 939–945, 2002.

[144] T. I. Gudz, T. E. Schneider, T. A. Haas, and W. B. Macklin, "Myelin proteolipid protein forms a complex with integrins and may participate in integrin receptor signaling in oligodendrocytes," *Journal of Neuroscience*, vol. 22, no. 17, pp. 7398–7407, 2002.

[145] F. Le Marrec-Croq, A. Bocquet-Garcon, J. Vizioli et al., "Calreticulin contributes to C1q-dependent recruitment of microglia in the leech Hirudo medicinalis following a CNS injury," *Medical Science Monitor*, vol. 20, pp. 644–653, 2014.

[146] A. Bento-Abreu, A. Velasco, E. Polo-Hernández, P. L. Pérez-Reyes, A. Tabernero, and J. M. Medina, "Megalin is a receptor for albumin in astrocytes and is required for the synthesis of the neurotrophic factor oleic acid," *Journal of Neurochemistry*, vol. 106, no. 3, pp. 1149–1159, 2008.

[147] A. Tabernero, A. Velasco, B. Granda, E. M. Lavado, and J. M. Medina, "Transcytosis of albumin in astrocytes activates the

sterol regulatory element-binding protein-1, which promotes the synthesis of the neurotrophic factor oleic acid," *The Journal of Biological Chemistry*, vol. 277, no. 6, pp. 4240–4246, 2002.

[148] E. Polo-Hernández, V. Tello, Á. A. Arroyo et al., "Oleic acid synthesized by stearoyl-CoA desaturase (SCD-1) in the lateral periventricular zone of the developing rat brain mediates neuronal growth, migration and the arrangement of prospective synapses," *Brain Research C*, vol. 1570, pp. 13–25, 2014.

[149] E. Polo-Hernández, F. de Castro, A. G. García-García, A. Tabernero, and J. M. Medina, "Oleic acid synthesized in the periventricular zone promotes axonogenesis in the striatum during brain development," *Journal of Neurochemistry*, vol. 114, no. 6, pp. 1756–1766, 2010.

[150] B. Granda, A. Tabernero, V. Tello, and J. M. Medina, "Oleic acid induces GAP-43 expression through a protein kinase C-mediated mechanism that is independent of NGF but synergistic with NT-3 and NT-4/5," *Brain Research*, vol. 988, no. 1-2, pp. 1–8, 2003.

[151] M. Caudy and D. Bentley, "Pioneer growth cone morphologies reveal proximal increases in substrate affinity within leg segments of grasshopper embryos," *The Journal of Neuroscience*, vol. 6, no. 2, pp. 364–379, 1986.

[152] A. K. Lewis and P. C. Bridgman, "Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity," *The Journal of Cell Biology*, vol. 119, no. 5, pp. 1219–1243, 1992.

[153] D. Bentley and T. P. O'Connor, "Cytoskeletal events in growth cone steering," *Current Opinion in Neurobiology*, vol. 4, no. 1, pp. 43–48, 1994.

[154] D. M. Suter and P. Forscher, "Substrate-cytoskeletal coupling as a mechanism for the regulation of growth cone motility and guidance," *Journal of Neurobiology*, vol. 44, no. 2, pp. 97–113, 2000.

[155] C. A. Korey and D. Van Vactor, "From the growth cone surface to the cytoskeleton: one journey, many paths," *Journal of Neurobiology*, vol. 44, no. 2, pp. 184–193, 2000.

[156] M. E. Dailey and P. C. Bridgman, "Dynamics of the endoplasmic reticulum and other membranous organelles in growth cones of cultured neurons," *Journal of Neuroscience*, vol. 9, no. 6, pp. 1897–1909, 1989.

[157] M. Tessier-Lavigne and C. S. Goodman, "The molecular biology of axon guidance," *Science*, vol. 274, no. 5290, pp. 1123–1133, 1996.

[158] B. J. Dickson, "Molecular mechanisms of axon guidance," *Science*, vol. 298, no. 5600, pp. 1959–1964, 2002.

[159] L. M. Landowski, M. Pavez, L. S. Brown et al., "Low-density lipoprotein receptor-related proteins in a novel mechanism of axon guidance and peripheral nerve regeneration," *The Journal of Biological Chemistry*, vol. 291, no. 3, pp. 1092–1102, 2016.

[160] P. J. Thornalley and M. Vašák, "Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals," *Biochimica et Biophysica Acta (BBA)—Protein Structure and Molecular Enzymology*, vol. 827, no. 1, pp. 36–44, 1985.

[161] J. Hidalgo, M. Aschner, P. Zatta, and M. Vašák, "Roles of the metallothionein family of proteins in the central nervous system," *Brain Research Bulletin*, vol. 55, no. 2, pp. 133–145, 2001.

[162] J. Y. K. Leung, W. R. Bennett, R. P. Herbert et al., "Metallothionein promotes regenerative axonal sprouting of dorsal root ganglion neurons after physical axotomy," *Cellular and Molecular Life Sciences*, vol. 69, no. 5, pp. 809–817, 2012.

[163] T. L. Stiles, T. L. Dickendesher, A. Gaultier et al., "LDL receptor-related protein-1 is a sialic-acid-independent receptor for myelin-associated glycoprotein that functions in neurite outgrowth inhibition by MAG and CNS myelin," *Journal of Cell Science*, vol. 126, part 1, pp. 209–220, 2013.

[164] J. R. Henley, K.-H. Huang, D. Wang, and M.-M. Poo, "Calcium mediates bidirectional growth cone turning induced by myelin-associated glycoprotein," *Neuron*, vol. 44, no. 6, pp. 909–916, 2004.

[165] J. H. Hines, M. Abu-Rub, and J. R. Henley, "Asymmetric endocytosis and remodeling of β_1-integrin adhesions during growth cone chemorepulsion by MAG," *Nature Neuroscience*, vol. 13, no. 7, pp. 829–837, 2010.

[166] K. Y. Wu, U. Hengst, L. J. Cox et al., "Local translation of RhoA regulates growth cone collapse," *Nature*, vol. 436, no. 7053, pp. 1020–1024, 2005.

[167] M. Nakato, M. Matsuo, N. Kono et al., "Neurite outgrowth stimulation by n-3 and n-6 PUFAs of phospholipids in apoE-containing lipoproteins secreted from glial cells," *Journal of Lipid Research*, vol. 56, no. 10, pp. 1880–1890, 2015.

[168] E. Mantuano, M. S. Lam, and S. L. Gonias, "LRP1 assembles unique co-receptor systems to initiate cell signaling in response to tissue-type plasminogen activator and Myelin-associated glycoprotein," *The Journal of Biological Chemistry*, vol. 288, no. 47, pp. 34009–34018, 2013.

[169] P. May, A. Rohlmann, H. H. Bock et al., "Neuronal LRP1 functionally associates with postsynaptic proteins and is required for normal motor function in mice," *Molecular and Cellular Biology*, vol. 24, no. 20, pp. 8872–8883, 2004.

[170] M. Mulder, P. J. Jansen, B. J. A. Janssen et al., "Low-density lipoprotein receptor-knockout mice display impaired spatial memory associated with a decreased synaptic density in the hippocampus," *Neurobiology of Disease*, vol. 16, no. 1, pp. 212–219, 2004.

[171] Q. Liu, J. Trotter, J. Zhang et al., "Neuronal LRP1 knockout in adult mice leads to impaired brain lipid metabolism and progressive, age-dependent synapse loss and neurodegeneration," *Journal of Neuroscience*, vol. 30, no. 50, pp. 17068–17078, 2010.

[172] M. Gotthardt, M. Trommsdorff, M. F. Nevitt et al., "Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction," *The Journal of Biological Chemistry*, vol. 275, no. 33, pp. 25616–25624, 2000.

[173] W. Maier, M. Bednorz, S. Meister et al., "LRP1 is critical for the surface distribution and internalization of the NR2B NMDA receptor subtype," *Molecular Neurodegeneration*, vol. 8, no. 1, article 25, 2013.

[174] C. Nakajima, A. Kulik, M. Frotscher et al., "Low density lipoprotein receptor-related protein 1 (LRP1) modulates *N*-methyl-D-aspartate (NMDA) receptor-dependent intracellular signaling and NMDA-induced regulation of postsynaptic protein complexes," *The Journal of Biological Chemistry*, vol. 288, no. 30, pp. 21909–21923, 2013.

[175] S. Qiu, L. F. Zhao, K. M. Korwek, and E. J. Weeber, "Differential reelin-induced enhancement of NMDA and AMPA receptor activity in the adult hippocampus," *Journal of Neuroscience*, vol. 26, no. 50, pp. 12943–12955, 2006.

[176] F. Telese, Q. Ma, P. M. Perez et al., "LRP8-Reelin-regulated neuronal enhancer signature underlying learning and memory formation," *Neuron*, vol. 86, no. 3, pp. 696–710, 2015.

[177] C. Liu, J. Hu, C. Tsai et al., "Neuronal LRP1 regulates glucose metabolism and insulin signaling in the brain," *The Journal of Neuroscience*, vol. 35, no. 14, pp. 5851–5859, 2015.

[178] L. Dimou, C. Simon, F. Kirchhoff, H. Takebayashi, and M. Götz, "Progeny of Olig2-expressing progenitors in the gray and white matter of the adult mouse cerebral cortex," *Journal of Neuroscience*, vol. 28, no. 41, pp. 10434–10442, 2008.

[179] S. Wang and K. M. Young, "Review: white matter plasticity in adulthood," *Neuroscience*, vol. 276, pp. 148–160, 2014.

[180] A. Nishiyama, X.-H. Lin, N. Giese, C.-H. Heldin, and W. B. Stallcup, "Co-localization of NG2 proteoglycan and PDGF α-receptor on O_2A progenitor cells in the developing rat brain," *Journal of Neuroscience Research*, vol. 43, no. 3, pp. 299–314, 1996.

[181] W. D. Richardson, N. Pringle, M. J. Mosley, B. Westermark, and M. Dubois-Dalcg, "A role for platelet-derived growth factor in normal gliogenesis in the central nervous system," *Cell*, vol. 53, no. 2, pp. 309–319, 1988.

[182] E. Noll and R. H. Miller, "Oligodendrocyte precursors originate at the ventral ventricular zone dorsal to the ventral midline region in the embryonic rat spinal cord," *Development*, vol. 118, no. 2, pp. 563–573, 1993.

[183] N. P. Pringle and W. D. Richardson, "A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage," *Development*, vol. 117, no. 2, pp. 525–533, 1993.

[184] W. D. Richardson, N. Kessaris, and N. Pringle, "Oligodendrocyte wars," *Nature Reviews Neuroscience*, vol. 7, no. 1, pp. 11–18, 2006.

[185] Q. Zhou, S. Wang, and D. J. Anderson, "Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors," *Neuron*, vol. 25, no. 2, pp. 331–343, 2000.

[186] Q. R. Lu, D.-I. Yuk, J. A. Alberta et al., "Sonic hedgehog—regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system," *Neuron*, vol. 25, no. 2, pp. 317–329, 2000.

[187] J. Dai, K. K. Bercury, J. T. Ahrendsen, and W. B. Macklin, "Olig1 function is required for oligodendrocyte differentiation in the mouse brain," *Journal of Neuroscience*, vol. 35, no. 10, pp. 4386–4402, 2015.

[188] Q. Zhou and D. J. Anderson, "The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification," *Cell*, vol. 109, no. 1, pp. 61–73, 2002.

[189] J. Fok-Seang and R. H. Miller, "Distribution and differentiation of $A2B5^+$ glial precursors in the developing rat spinal cord," *Journal of Neuroscience Research*, vol. 37, no. 2, pp. 219–235, 1994.

[190] M. Fogarty, W. D. Richardson, and N. Kessaris, "A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord," *Development*, vol. 132, no. 8, pp. 1951–1959, 2005.

[191] R. B. Tripathi, L. E. Clarke, V. Burzomato et al., "Dorsally and ventrally derived oligodendrocytes have similar electrical properties but myelinate preferred tracts," *The Journal of Neuroscience*, vol. 31, no. 18, pp. 6809–6819, 2011.

[192] N. Kessaris, M. Fogarty, P. Iannarelli, M. Grist, M. Wegner, and W. D. Richardson, "Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage," *Nature Neuroscience*, vol. 9, no. 2, pp. 173–179, 2006.

[193] R. K. Small, P. Riddle, and M. Noble, "Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve," *Nature*, vol. 328, no. 6126, pp. 155–157, 1987.

[194] J. D. Cahoy, B. Emery, A. Kaushal et al., "A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function," *The Journal of Neuroscience*, vol. 28, no. 1, pp. 264–278, 2008.

[195] Y. Zhang, K. Chen, S. A. Sloan et al., "An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex," *Journal of Neuroscience*, vol. 34, no. 36, pp. 11929–11947, 2014.

[196] L. Gao and R. H. Miller, "Specification of optic nerve oligodendrocyte precursors by retinal ganglion cell axons," *The Journal of Neuroscience*, vol. 26, no. 29, pp. 7619–7628, 2006.

[197] K. Murray, V. Calaora, C. Rottkamp, O. Guicherit, and M. Dubois-Dalcq, "Sonic Hedgehog is a potent inducer of rat oligodendrocyte development from cortical precursors in vitro," *Molecular and Cellular Neuroscience*, vol. 19, no. 3, pp. 320–332, 2002.

[198] M. C. Ortega, O. Cases, P. Merchán, R. Kozyraki, D. Clemente, and F. De Castro, "Megalin mediates the influence of sonic hedgehog on oligodendrocyte precursor cell migration and proliferation during development," *Glia*, vol. 60, no. 6, pp. 851–866, 2012.

[199] G. Wicher, M. Larsson, Å. F. Svenningsen, E. Gyllencreutz, L. Rask, and H. Aldskogius, "Low density lipoprotein receptor-related protein-2/megalin is expressed in oligodendrocytes in the mouse spinal cord white matter," *Journal of Neuroscience Research*, vol. 83, no. 5, pp. 864–873, 2006.

[200] C. Schmidt, C. Ohlemeyer, C. Labrakakis, T. Walter, H. Kettenmann, and J. Schnitzer, "Analysis of motile oligodendrocyte precursor cells in vitro and in brain slices," *Glia*, vol. 20, no. 4, pp. 284–298, 1997.

[201] P. B. Simpson and R. C. Armstrong, "Intracellular signals and cytoskeletal elements involved in oligodendrocyte progenitor migration," *Glia*, vol. 26, no. 1, pp. 22–35, 1999.

[202] E. Mantuano, M. Jo, S. L. Gonias, and W. M. Campana, "Low density Lipoprotein Receptor-related Protein (LRP1) regulates Rac1 and RhoA reciprocally to control Schwann cell adhesion and migration," *The Journal of Biological Chemistry*, vol. 285, no. 19, pp. 14259–14266, 2010.

[203] R. Pankov, Y. Endo, S. Even-Ram et al., "A Rac switch regulates random versus directionally persistent cell migration," *Journal of Cell Biology*, vol. 170, no. 5, pp. 793–802, 2005.

[204] F. Binamé, D. Sakry, L. Dimou, V. Jolivel, and J. Trotter, "NG2 regulates directional migration of oligodendrocyte precursor cells via Rho GTPases and polarity complex proteins," *The Journal of Neuroscience*, vol. 33, no. 26, pp. 10858–10874, 2013.

[205] S. C. Muratoglu, I. Mikhailenko, C. Newton, M. Migliorini, and D. K. Strickland, "Low density lipoprotein receptor-related protein 1 (LRP1) forms a signaling complex with platelet-derived growth factor receptor-β in endosomes and regulates activation of the MAPK pathway," *The Journal of Biological Chemistry*, vol. 285, no. 19, pp. 14308–14317, 2010.

[206] J. Craig, I. Mikhailenko, N. Noyes, M. Migliorini, and D. K. Strickland, "The LDL receptor-related protein 1 (LRP1) regulates the PDGF signaling pathway by binding the protein phosphatase SHP-2 and modulating SHP-2- mediated PDGF signaling events," *PLoS ONE*, vol. 8, no. 7, Article ID e70432, 2013.

[207] C. S. Newton, E. Loukinova, I. Mikhailenko et al., "Platelet-derived growth factor receptor-β (PDGFR-β) activation promotes its association with the low density lipoprotein receptor-related protein (LRP): evidence for co-receptor function," *The Journal of Biological Chemistry*, vol. 280, no. 30, pp. 27872–27878, 2005.

[208] L. Rönnstrand, A.-K. Arvidsson, A. Kallin et al., "SHP-2 binds to Tyr763 and Tyr1009 in the PDGF β-receptor and mediates PDGF-induced activation of the Ras/MAP kinase pathway and chemotaxis," *Oncogene*, vol. 18, no. 25, pp. 3696–3702, 1999.

[209] K. Avrov and A. Kazlauskas, "The role of c-Src in platelet-derived growth factor alpha receptor internalization," *Experimental Cell Research*, vol. 291, no. 2, pp. 426–434, 2003.

[210] Y. Miyamoto, J. Yamauchi, and A. Tanoue, "Cdk5 phosphorylation of WAVE2 regulates oligodendrocyte precursor cell migration through nonreceptor tyrosine kinase fyn," *The Journal of Neuroscience*, vol. 28, no. 33, pp. 8326–8337, 2008.

[211] T. Chae, Y. T. Kwon, R. Bronson, P. Dikkes, L. En, and L.-H. Tsai, "Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality," *Neuron*, vol. 18, no. 1, pp. 29–42, 1997.

[212] S. Rosenkranz, K. A. DeMali, J. A. Gelderloos, C. Bazenet, and A. Kazlauskas, "Identification of the receptor-associated signaling enzymes that are required for platelet-derived growth factor-AA-dependent chemotaxis and DNA synthesis," *The Journal of Biological Chemistry*, vol. 274, no. 40, pp. 28335–28343, 1999.

[213] R. D. McKinnon, S. Waldron, and M. E. Kiel, "PDGF α-receptor signal strength controls an RTK rheostat that integrates phosphoinositol 3′-kinase and phospholipase Cγ pathways during oligodendrocyte maturation," *The Journal of Neuroscience*, vol. 25, no. 14, pp. 3499–3508, 2005.

[214] W. M. Campana, X. Li, N. Dragojlovic, J. Janes, A. Gaultier, and S. L. Gonias, "The low-density lipoprotein receptor-related protein is a pro-survival receptor in Schwann cells: possible implications in peripheral nerve injury," *The Journal of Neuroscience*, vol. 26, no. 43, pp. 11197–11207, 2006.

[215] S. Mañes and C. Martínez-A, "Cholesterol domains regulate the actin cytoskeleton at the leading edge of moving cells," *Trends in Cell Biology*, vol. 14, no. 6, pp. 275–278, 2004.

[216] S. Mañes, E. Mira, C. Gómez-Moutón et al., "Membrane raft microdomains mediate front-rear polarity in migrating cells," *The EMBO Journal*, vol. 18, no. 22, pp. 6211–6220, 1999.

[217] J. K. Boyles, R. E. Pitas, E. Wilson, R. W. Mahley, and J. M. Taylor, "Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system," *The Journal of Clinical Investigation*, vol. 76, no. 4, pp. 1501–1513, 1985.

[218] U. Beisiegel, W. Weber, G. Ihrke, J. Herz, and K. K. Stanley, "The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein," *Nature*, vol. 341, no. 6238, pp. 162–164, 1989.

[219] J. D. Morrisett, R. L. Jackson, and A. M. Gotto Jr., "Lipoproteins: structure and function," *Annual Review of Biochemistry*, vol. 44, pp. 183–207, 1975.

[220] T. E. Willnow, A. Hammes, and S. Eaton, "Lipoproteins and their receptors in embryonic development: more than cholesterol clearance," *Development*, vol. 134, no. 18, pp. 3239–3249, 2007.

[221] A. Laatsch, M. Panteli, M. Sornsakrin, B. Hoffzimmer, T. Grewal, and J. Heeren, "Low density lipoprotein receptor-related protein 1 dependent endosomal trapping and recycling of apolipoprotein," *PLoS ONE*, vol. 7, no. 1, Article ID e29385, 2012.

[222] F. W. Pfrieger, "Role of cholesterol in synapse formation and function," *Biochimica et Biophysica Acta (BBA)—Biomembranes*, vol. 1610, no. 2, pp. 271–280, 2003.