MORPHOGENS IN THE WIRING OF THE NERVOUS SYSTEM, 2nd Edition

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MORPHOGENS IN THE WIRING OF THE NERVOUS SYSTEM, 2nd Edition

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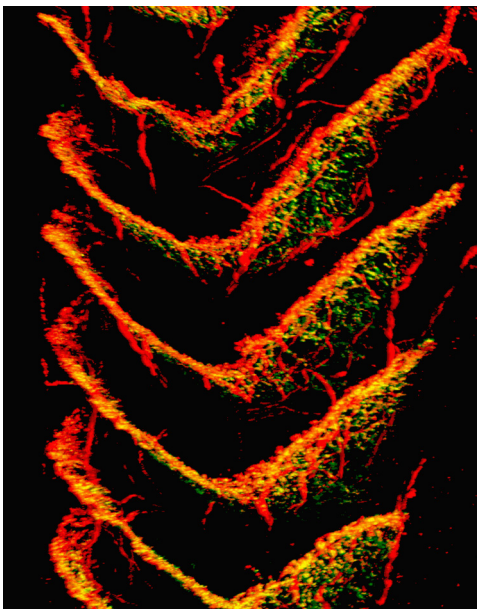

Peripheral neuronal connectivity of a frog tadpole.
The neuromuscular junctions of Xenopus tropicalis embryos (stage NF50) were stained using antibodies against synaptic vesicles and neurofilaments to label the pre-synaptic apparatus (red) along with alpha-bungarotoxin (green) to label the post-synaptic domain. z-stack images were acquired in a Zeiss LSM780 confocal microscopy (CMA Bio-Bio, Universidad de Concepción, Concepción, Chile), and subsequently reconstructed using Zen software for 3D projection.
Orientation: anterior (up), posterior (down), dorsal (left), ventral (right).

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Neuronal function relies on the establishment of proper connections between neurons and their target cells during development. This basic statement involves several cellular processes, such as neuronal differentiation, the polarized outgrowth of axons and dendrites from differentiated neurons, and the pathfinding of axons towards target cells. The subsequent recognition of complementary synaptic partners finally triggers the formation, maturation, and maintenance of functional synapses. Morphogens are secreted signaling molecules commanding

tissue patterning and cell identity during early embryonic development. Remarkably, growing evidence over the last years arising from different invertebrate and vertebrate model organisms has shown that, after cell fate has been established, morphogens also control the precise wiring and function in the developing and mature nervous system. Accordingly, dysfunctions of the signaling pathways activated by these molecules contribute to synaptic disassembly and altered function in diseases affecting the nervous system.

We consider it timely to bring together cumulative evidence pointing to crucial roles for signaling activated by different morphogens in the establishment of precise contacts between neurons and their synaptic partners. Therefore, this research topic issue combines review and research articles aimed to cover the functional relevance of such morphogens on the different steps involved in synaptic assembly and function. Diverse model systems of physiological or pathological conditions have been included, as well as different cellular, biochemical and molecular approaches. Altogether, they contribute in different and complementary ways to build a holistic view of the roles that early development morphogens play during the assembly, maintenance and/or regeneration of functional synapses.

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EDITORIAL
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Editorial: Morphogens in the Wiring of the Nervous System

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Keywords: morphogen, Wnt proteins, BMP, Shh, nervous system

The Editorial on the Research Topic

Morphogens in the Wiring of the Nervous System

Morphogens are secreted signaling molecules that play instructive roles to regulate tissue patterning and cell identity in a concentration-dependent fashion during early embryonic development. Four principal mammalian morphogens include retinoic acid, as well as members of the Wingless-int (Wnt) and transforming growth factor beta (TGF-β)/bone morphogenic protein (BMP) and sonic hedgehog (Shh) families. Morphogen gradients control a plethora of developmental events related to specification and differentiation, cell and tissue polarity, growth control and regeneration.

In the nervous system, neuronal differentiation, the polarized outgrowth of neuronal projections, the pathfinding of axons toward their target cells, and the recognition of pre- and post-synaptic partner cells are fundamental requisites to allow the assembly of functional synapses. During the last decades, a growing body of evidence gathered from invertebrate and vertebrate model organisms has shown that the same morphogens classically known to orchestrate early embryonic development are also involved in the precise wiring of the nervous system.

The aim of this research topic is to highlight the fundamental roles that morphogens play during the establishment of synaptic connectivity. Hence, we have brought together 12 original research articles and eight reviews. They are mainly focused on Wnt (Aviles et al.; Bernis et al.; Berwick and Harvey; Dickins and Salinas; Pinto et al.; Rosso and Inestrosa; Silva-Alvarez et al.; Varela-Nallar and Inestrosa; Aviles et al.; Varela-Nallar et al.), BMP (Gamez et al.; Pinto et al.; Osses and Henriquez), and Shh (Aviles et al.; Reinchisi et al.) signaling pathways. Research also covers the function of signaling cascades activated by other types of morphogens, including the fibroblast growth factors (FGF; Lee and Umemori; Paradiso et al.), the hepatocyte growth factor (HGF) (Bhardwaj et al.), and netrin (Yamagishi et al.). In addition, researchers have contributed with the emerging roles of new molecules, such as the thyroid hormone (Dezonne et al.), SCO-spondin (Vera et al.), and vitamin C (Pastor et al.). Articles are focused on a wide variety of cellular processes involved in the establishment of neuronal connectivity, such as neurogenesis, neuronal specification, and maturation (Berwick and Harvey; Dezonne et al.; Gamez et al.; Pastor et al.; Reinchisi et al.; Rosso and Inestrosa; Varela-Nallar and Inestrosa; Vera et al.; Varela-Nallar et al.; Yamagishi et al.), axonal outgrowth, polarization, and guidance (Aviles et al.; Bernis et al.; Bhardwaj et al.; Pinto et al.; Aviles et al.; Osses and Henriquez), and synapse formation (Dickins and Salinas; Rosso and Inestrosa; Aviles et al.; Osses and Henriquez), either in physiological contexts or in models of diseases affecting the normal function of the nervous system, including epilepsy (Lee and Umemori; Paradiso et al.), Alzheimer's disease (Silva-Alvarez et al.; Varela-Nallar et al.), and amyotrophic lateral sclerosis (Pinto et al.).

We are confident that this integrative research topic emphasizes the central and pleiotropic roles played by morphogens during neural development. We therefore hope that the original articles and reviews presented here will inspire future directions of research focusing on the diversity of

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cell signaling mechanisms controlling the assembly, maintenance and regeneration of the nervous system.

AUTHOR CONTRIBUTIONS

JH and NO wrote, edited and revised the manuscript.

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REVIEW ARTICLE
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Wnts in action: from synapse formation to synaptic maintenance

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A proper balance between synapse assembly and disassembly is crucial for the formation of functional neuronal circuits and synaptic plasticity in the adult brain. During development, synaptogenesis generates a vast excess of synapses, which are subsequently eliminated. Importantly, aberrant synaptic disassembly during development underpins many neurological disorders. Wnt secreted proteins are robust synaptogenic factors that regulate synapse assembly and function in the developing and mature brain. Recent studies show that Wnt blockade with the antagonist Dickkopf-1 (Dkk1) induces the rapid disassembly of synapses in mature neurons. Importantly, Dkk1 mediates synaptic loss induced by Amyloid-ß, a key pathogenic molecule in Alzheimer's disease (AD). These findings provide new insights into the potential contribution of dysfunctional Wnt signaling to synaptic loss observed in neurodegenerative diseases. In this review, we discuss the role of Wnt signaling in vertebrate synaptic assembly, function and maintenance, and consider how dysfunction of Wnt signaling could contribute to synaptic disassembly in neurodegenerative diseases such as AD.

Keywords: synaptogenesis, synapse disassembly, synaptic plasticity, Frizzled, Dvl, LTP, neurodegenerative disease

INTRODUCTION

During early development, Wnts regulate critical cellular processes such as cell proliferation and cell fate, neuronal polarity and migration. In addition, Wnts modulate dendritogenesis, axon guidance and synaptogenesis (Ciani and Salinas, 2005; Ille and Sommer, 2005; Rosso et al., 2005; Salinas and Zou, 2008; Budnik and Salinas, 2011; Park and Shen, 2012; Salinas, 2012). The array of diverse cellular processes regulated by Wnt signaling is achieved through multiple Wnt ligands interacting with numerous receptors and co-receptors that trigger distinct signaling cascades that induce local changes and/or global changes through the modulation of target gene expression (Mikels and Nusse, 2006; Kikuchi et al., 2007; van Amerongen and Nusse, 2009). Additional levels of complexity are conferred by the temporal and spatial expression of secreted regulatory factors, which antagonize or activate specific Wnt pathways, or act as a switch between different Wnt pathways. Several excellent reviews on Wnt signaling pathways and their cellular outcomes are available (Logan and Nusse, 2004; Kohn and Moon, 2005; Gordon and Nusse, 2006; Kikuchi et al., 2007; Angers and Moon, 2009; van Amerongen and Nusse, 2009). Therefore, the different Wnt pathways will not be discussed here.

Studies in the past decade have demonstrated that Wnts are key synaptic organizers that play a critical developmental role in establishing neural circuits. The formation of functional synapses requires the precise assembly of pre- and postsynaptic sites in perfect apposition. This process ultimately depends upon the molecular dialogue between the pre- and postsynaptic sides (**Figure 1**). Indeed, Wnt factors are key players that signal to both pre- and/or postsynaptic sites to promote synapse assembly, morphology and function (Hall et al., 2000; Krylova et al., 2002; Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Henriquez et al., 2008; Farias et al., 2009; Gogolla et al., 2009; Cuitino et al., 2010; Varela-Nallar et al., 2010; Ciani et al., 2011). Wnts, their cognate receptors and several signaling components continue to be expressed in the adult brain, suggesting a role for Wnts in the mature nervous system. Whilst the function of Wnt signaling in the adult is less understood, recent studies indicate a role in synapse maintenance and plasticity (Chen et al., 2006; Gogolla et al., 2009; Cerpa et al., 2011; Ciani et al., 2011; Purro et al., 2012).

Here we discuss the role of Wnt signaling in synapse formation and maintenance. We will first review how Wnts contribute to synapse assembly and function, focusing on the mammalian central nervous system. This prologue is important to highlight the importance of Wnt signaling at the synapse and the consequence of Wnt blockade or Wnt dysfunction on synapse instability observed in certain neurodegenerative diseases.

PRESYNAPTIC REMODELING

As axons enter into their target field, they undergo extensive modeling. Studies in cerebellar mossy fiber axons and dorsal root ganglion cells (DRGs) reveal that Wnt signaling activates a divergent-canonical pathway through Disheveled (Dvl) resulting in the inhibition of Glycogen synthase kinase-3 ß (Gsk3ß) to regulate axonal remodeling (Lucas and Salinas, 1997; Lucas et al., 1998; Krylova et al., 2002; Purro et al., 2008). This pathway is independent of transcription and induces profound changes in growth cone size and axonal microtubule dynamics by affecting Gsk3β-mediated phosphorylation of microtubule-associated

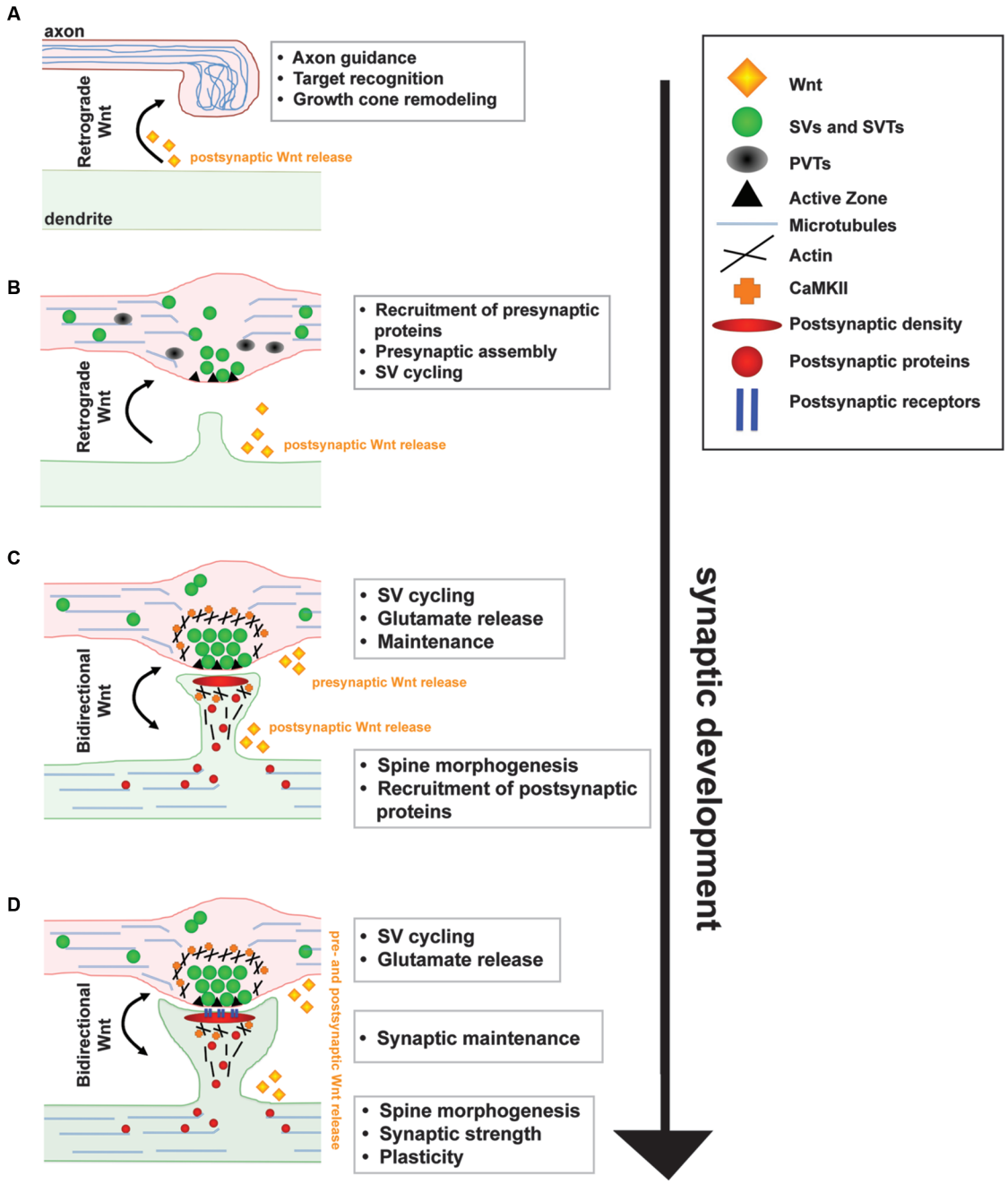

FIGURE 1 | A model for the function of Wnt signaling during synapse development and maintenance. **(A)** Target derived Wnt signals guide incoming axons and induce axon and growth cone remodeling by modulating the cytoskeleton. **(B)** Wnt signals directly to the axon through a divergent canonical pathway to stimulate the recruitment of presynaptic proteins resulting in the formation of synaptic boutons. **(C)** Wnt also signals directly to the postsynaptic dendrite through CaMKII to stimulate spine morphogenesis, postsynaptic protein recruitment and synaptic strength. **(D)** At mature synapses, Wnt signaling regulates synaptic function and maintenance.

proteins, such as MAP1B, and the localization of Adenomatous polyposis coli (APC) (Ciani et al., 2004; Salinas, 2007; Purro et al., 2008). APC, in addition to being a component of the canonical-Wnt signaling destruction complex, is a microtubule plus-end binding protein that captures the distal end of microtubules to the leading edge of the growth cone (Galjart, 2005). During axon remodeling, Wnt3/3a signals through Dvl1 to inhibit Gsk3β resulting in the loss of APC from microtubule plus ends. Wnt-induced APC loss from microtubules results in the loss of directionality of microtubule growth and the subsequent formation of lopped microtubule within growth cones (Purro et al., 2008). These looped microtubules provide a structural mechanism for

capturing molecules required for presynaptic differentiation and the transformation of motile growth cones into presynaptic boutons. Further studies are required to determine how microtubule dynamics and their organization contribute to early stages of presynaptic differentiation and whether Wnts regulate microtubule dynamics, not only at growth cones, but also at the axon shaft to promote the formation of *en passant* synapses.

PRESYNAPTIC ASSEMBLY

Several Wnts promote the assembly of presynaptic release sites. Wnt7a/b and Wnt3/3a stimulate the accumulation of a number of functionally diverse presynaptic proteins including Synapsin 1, vGlut1 and Bassoon, as well as synaptic vesicles (SVs) (Hall et al., 2000; Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Ciani et al., 2011; **Figure 2**). In contrast, blockade of Wnt signaling by secreted frizzled-related proteins (Sfrps) or Dickkofp-1 (Dkk1) inhibits the ability of Wnt7a/b to induce presynaptic protein clustering (Ahmad-Annuar et al., 2006; Davis et al., 2008). Critically, mice deficient in Wnt7a and/or Dvl1 display significant defects in presynaptic protein clustering (Hall et al., 2000; Ahmad-Annuar et al., 2006). Time-course analyses reveal that Wnt7a/b induces synaptic protein clustering within 15 min demonstrating a rapid synaptogenic effect (Ahmad-Annuar et al., 2006; Ciani et al., 2011). Furthermore, Wnts rapidly increase the number of functionally active presynaptic sites without affecting total levels of synaptic proteins (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Varela-Nallar et al., 2009). These findings suggest that Wnts induce presynaptic assembly by promoting the recruitment of existing synaptic proteins and SVs.

Signaling through the seven transmembrane Frizzled (Fz) receptors promotes presynaptic organization. Wnt3a and Wnt7a bind to the Fz1 and Fz5 receptors, respectively, to stimulate presynaptic assembly (Varela-Nallar et al., 2009; Sahores et al., 2010). Both receptors localize at synapses and stimulate the clustering of active zone components and SVs. Moreover, loss of Fz1 or Fz5 function by using the respective soluble cysteine-reach domains (CRD) or shRNA-mediated knockdown of Fz5 blocks Wnt-mediated presynaptic assembly (Varela-Nallar et al., 2009; Sahores et al., 2010). In addition to Fz receptors, Wnts can regulate presynaptic assembly by binding to other receptors. For example, Wnt5a binds to Ror tyrosine kinase receptors to stimulate Synaptophysin clustering in cultured hippocampal neurons (Paganoni et al., 2010). Further studies are required to determine whether different Wnt isoforms selectively bind with specific receptors to regulate distinct aspects during synapse assembly.

Wnt signals via a divergent canonical pathway that is independent of transcription, to stimulate presynaptic assembly. A number of findings support this conclusion. Firstly, blockade of canonical-Wnt signaling at the receptor level by the secreted antagonist Dkk1 prevents Wnt-induced presynaptic differentiation (Davis et al., 2008). Secondly, expression of Dvl1, which localizes to presynaptic sites, is sufficient to induce clustering of presynaptic proteins and to promote the formation of functional neurotransmitter release sites (Ahmad-Annuar et al., 2006). In contrast, neurons from *Dvl1* null mice exhibit fewer neurotransmitter release sites and respond poorly to exogenous Wnt7a/b (Ahmad-Annuar et al., 2006).

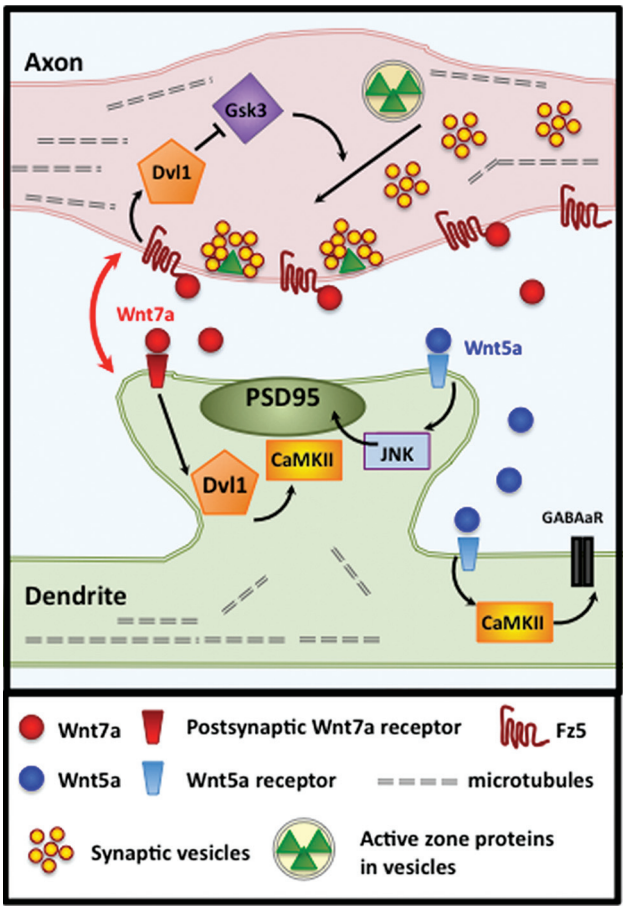

FIGURE 2 | Wnt7a signals bi-directionally at vertebrate synapses. At hippocampal synapses, coordinated bidirectional Wnt7a signaling regulates synapse assembly, development and maintenance. In the axon, Wnt7a binds with Fz5 to activate a divergent-canonical pathway through Dvl1 and Gsk3β that promotes the recruitment of synaptic components, including SV and active zone proteins. In postsynaptic dendrites, Wnt7a promotes spine morphogenesis, PSD-95 recruitment and synaptic strength by signaling via Dvl1 and CaMKII. The postsynaptic Wnt receptor has yet to be determined. Wnt5a signals postsynaptically to promote PSD95 clustering via JNK activation and $GABA_A$ receptor clustering via CaMKII. The postsynaptic Wnt5a receptor has yet to be identified.

In the canonical-Wnt pathway, activation of Dvl1 inhibits the serine/threonine kinase Gsk3β, which phosphorylates and targets β-catenin for degradation. Gsk3β is expressed presynaptically and its pharmacological inhibition mimics Wnt-induced clustering of synaptic components (Lucas and Salinas, 1997; Hall et al., 2000; Ahmad-Annuar et al., 2006; Davis et al., 2008). Together these findings support a role for the canonical Wnt signaling pathway in presynaptic assembly. However, blockade of transcription by RNA polymerase inhibition does not affect Wnt mediated presynaptic assembly (EM Dickins and PC Salinas, unpublished results) neither axonal remodeling, a process that precedes presynaptic assembly (Purro et al., 2008). These results suggest that Wnt might signal locally to regulate Dvl1 and Gsk3β to promote presynaptic assembly.

How does Wnt signaling promote the assembly of synaptic boutons? Previous studies have shown that a divergent-canonical Wnt pathway regulates microtubule dynamics in the axon shaft and the growth cone to induce axonal branching, growth cone spreading, and changes in bouton morphology; such effects depend upon profound changes in microtubule organization (Lucas and Salinas, 1997; Hall et al., 2000; Purro et al., 2008). Thus, local regulation of microtubule dynamics by Wnts could provide a possible mechanism for directed delivery of synaptic components to future synaptic sites. While this model is consistent with the changes observed in the formation of terminal boutons, most synapses in the central nervous system are *en passant*. Therefore, it remains to be determined how *en passant* boutons become assembled. Interestingly, Wnts seem to promote microtubule unbundling along the axon shaft in some neurons suggesting that changes in microtubule organization might contribute to the initial recruitment of synaptic components to future synaptic boutons. Further studies are required to determine the mechanisms by which Wnt signaling stimulates the rapid recruitment of presynaptic proteins.

POSTSYNAPTIC ASSEMBLY

Wnts also signal to dendrites to promote the recruitment of postsynaptic components (Cerpa et al., 2008; Henriquez et al., 2008; Farias et al., 2009; Cuitino et al., 2010; Ciani et al., 2011; Jensen et al., 2012; **Figures 1**, **2**). Interestingly, different Wnt isoforms specifically regulate the assembly of excitatory and/or inhibitory synapses. Wnt7a exclusively stimulates the formation of excitatory synapses, without affecting inhibitory synapses (Ciani et al., 2011). Wnt7a stimulates PSD95 recruitment and the apposition of excitatory pre- and postsynaptic markers (Ciani et al., 2011). Wnt7a promotes excitatory synapse formation by inducing the formation and growth of dendritic spines, structures that primarily receive excitatory inputs. Conversely, *Wnt7a; Dvl1* mutant mice exhibit significant deficits in spine number and morphology in the Cornus Ammonis (CA) CA1 and CA3 regions of the hippocampus (Ciani et al., 2011). These mice also exhibit reduced frequency and amplitude of alpha amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated miniature excitatory postsynaptic currents (mEPSCs). Although the mechanism by which Wnt7a increases spine number remains to be elucidated, spine growth is induced through Dvl1 and local activation of CaMKII at dendritic spines (Ciani et al., 2011).

In contrast to Wnt7a, Wnt5a acts as a pan-synaptogenic factor that stimulates PSD95 clustering at excitatory synapses via JNK activation (Farias et al., 2009) and γ-aminobutyric acid type A ($GABA_A$) receptor clustering at inhibitory synapses through CaMKII signaling (Cuitino et al., 2010; **Figure 2**). Whilst some of the signaling mechanisms by which Wnts promote postsynaptic assembly have been identified, many questions remain unanswered. For instance, the identity of the postsynaptic receptors for Wnts and the mechanisms that regulate the formation of different types of synapses are currently unknown.

The precise role of Wnt5a in synaptic assembly remains poorly understood. Wnt5a has been reported to stimulate spine formation via calcium signaling in cultured hippocampal neurons (Varela-Nallar et al., 2010). However, these results have not been consistently recapitulated in brain slices (Cerpa et al., 2011), and another group reported that Wnt5a inhibits excitatory presynaptic assembly (Davis et al., 2008). The reason for these apparent opposing results is unclear at present. Possible explanations could be the age and/or type of neuronal preparation studied, different sources of Wnt5a (recombinant versus conditioned media from Wnt5a-expressing cells), the concentration used or the exposure time. Further work is required to gain insight into the function of Wnt5a as a positive or negative regulator of excitatory synaptogenesis, and how Wnt5a stimulates postsynaptic differentiation of both excitatory and inhibitory synapses, whereas Wnt7a only promotes excitatory synapse formation.

BIDIRECTIONAL WNT SIGNALING

A number of studies have shown that Wnts can signal in an anterograde and retrograde manner. For example, at the neuromuscular junction (NMJ), Wnt release from motoneurons regulates postsynaptic differentiation on muscle cells (Krylova et al., 2002; Packard et al., 2002; Henriquez et al., 2008; Jensen et al., 2012). In contrast, in the cerebellum, Wnt7a/b is released from postsynaptic cells to regulate presynaptic assembly and remodeling (Hall et al., 2000). In the hippocampus, several Wnts are expressed but the exact source of Wnts at the mossy fiber-granule cell (MF-GC) and Cornus Ammonis (CA3-CA1) synapse remains poorly understood. Recent studies suggest that Wnt7a/b protein is present in the Dentate Gyrus (DG), CA3 and CA1 neurons (Gogolla et al., 2009; Ciani et al., 2011). However, where and how Wnts are secreted to regulate synapse formation and function remains to be elucidated.

Coordinated bidirectional signaling contributes to Wnt mediated synaptic assembly. At the *Drosophila* NMJ, the Wnt family member Wingless (Wg) signals to both sides of the synapse (Packard et al., 2002; Ataman et al., 2008). Similarly, at hippocampal synapses, Wnt7a acts bidirectionally on axons and dendrites suggesting a conserved role for bidirectional Wnt signaling in synapse assembly between vertebrates and invertebrates. However, pre- and postsynaptic assembly is not concurrent. Time-course analysis shows that Wnt7a induces clustering of the presynaptic protein vesicular glutamate transporter1 (vGlut1) within 15 min, whereas clustering of the postsynaptic scaffold protein PSD95 takes longer (Ciani et al., 2011). These results suggest that the presynaptic terminal responds faster to Wnt7a than the postsynaptic side. It is currently unclear whether Wnt7a acts through different receptors to trigger different signaling cascades to coordinate the assembly at both sides of the synapse.

SYNAPTIC FUNCTION

The initial stages of synaptic differentiation occur within the first few hours after the establishment of the axo-dendritic contact. However, the development of a nascent synapse into a functional synapse involves the recruitment of hundreds of proteins, morphological changes and establishment of functional electrophysiological properties (Zhang and Benson, 2001; Knott et al., 2006; Nagerl et al., 2007).

Wnt signaling participates in presynaptic function by promoting the formation of more SV recycling sites and increasing neurotransmitter release (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Varela-Nallar et al., 2009). In hippocampal neurons, the intracellular activation of the canonical-Wnt signaling by

expression of Wnt signaling components or by bath application of Wnt3a or Wnt7a increases the frequency of spontaneous and mEPSCs by a divergent-canonical pathway that mobilizes calcium and is independent of transcription (Beaumont et al., 2007; Cerpa et al., 2008; Avila et al., 2010). Further analyses of SV cycling dynamics showed that Wnt7a specifically enhances SV exocytosis to facilitate neurotransmitter release (Cerpa et al., 2008). Importantly, electrophysiological studies in the cerebellum of *Wnt7a; Dvl1* mutant mice reveal defects in the frequency of mEPSCs, without apparent changes at the structure of active zones as determined by electron microscopy (Ahmad-Annuar et al., 2006). These results suggest that Wnt signaling may be required for neurotransmitter release. Consistent with this hypothesis, Dvl1 has been shown to bind directly to Synaptotagmin (Kishida et al., 2007), a key protein in neurotransmission. Dvl1 appears to regulate SV exo- and endocytosis in PC12 cells (Kishida et al., 2007). Together these studies demonstrate that Wnt signaling contributes to the assembly of functionally active presynaptic sites. However, the role of Wnt signaling in neurotransmitter release remains to be fully demonstrated.

On dendrites, Wnt7a increases the growth and maturation of spines manifested by increased size and PSD95 content (Ciani et al., 2011). In hippocampal neurons, Wnt7a increases spine size by almost 50% within 3 hrs, and by 65% within 16 hrs indicating a rapid and progressive spine growth. Consistent with an increased spine head size, Wnt7a signaling regulates synaptic strength as determined by defects in evoked excitatory postsynaptic currents (EPSCs) in *Wnt7a; Dvl1* mutant mice. Postsynaptic activation of the Wnt pathway by expression of Dvl1 also stimulates spine growth, increases the amount of PSD95 within dendritic spines and the number of spines containing PSD95 (Ciani et al., 2011). Several findings demonstrate that Wnt7a through Dvl1 and CaMKII modulates spine growth and synaptic strength by rapidly activating CaMKII within dendritic spines (Ciani et al., 2011; **Figure 2**). Given the role of CaMKII in the structural and functional plasticity of synapses, these findings raise the interesting possibility that Wnt7a signaling participates in postsynaptic plasticity in the adult brain.

Wnt signaling also modulates inhibitory synapse formation and function (**Figure 2**). Wnt5a increases the insertion and clustering of $GABA_A$ receptors in cultured hippocampal neurons (Cuitino et al., 2010). Moreover, evoked recordings demonstrate that Wnt5a rapidly increases the amplitude of GABA-mediated currents without affecting the pair pulse index suggesting the Wnt5a might act postsynaptically. This postsynaptic effect is blocked by KN93 suggesting that Ca2+/calmodulin dependent kinase (CaMKs) are involved (Cuitino et al., 2010). Although it remains to be determined whether Wnt5a is necessary for the postsynaptic function of inhibitory synapses, these results demonstrate that different Wnts can promote excitatory and/or inhibitory synapse function.

NEURONAL ACTIVITY REGULATES WNT LEVELS AND FRIZZLED RECEPTOR TRAFFICKING

A number of studies have shown that neuronal activity stimulates the expression and/or release of Wnts in the developing and mature nervous system (Yu and Malenka, 2003; Chen et al., 2006; Wayman et al., 2006; Gogolla et al., 2009; Sahores et al., 2010). Depolarization of young hippocampal neurons stimulates Wnt release, which in turn regulates dendritogenesis (Yu and Malenka, 2003; Wayman et al., 2006). In cultured hippocampal neurons, activation of N-methyl-D-aspartate receptors (NMDARs) through tetanic stimulation induces Wnt3a release resulting in the activation of the canonical Wnt pathway (Chen et al., 2006). Wnt2 is also regulated by activity through a mechanism that requires NMDAR activation and cAMP response element-binding protein (CREB)-mediated transcription to promote dendritic arborization of cultured hippocampal neurons (Wayman et al., 2006). Moreover, *in vivo* studies show that environmental enrichment (EE) stimulates Wnt7a/b expression in the adult mouse hippocampus during the remodeling of mossy fiber terminals that contact CA3 dendrites. Importantly, blockade of endogenous Wnts by local microinjection of the Wnt antagonist Sfrp1 suppresses activity-dependent presynaptic remodeling and synaptogenesis in the CA3 region (Gogolla et al., 2009). Collectively, these studies demonstrate that neuronal activity promotes the expression and/or release of Wnt ligands to regulate dendritic development and adult synaptic remodeling.

Activity-dependent Wnt release induces Fz receptor surface localization, which in turn promotes synapse assembly (**Figure 3**). In cultured hippocampal neurons, Fz5, a receptor for Wnt7a, translocates to the cell surface and to synaptic sites following high frequency stimulation (HFS; Sahores et al., 2010), a protocol that induces long-term potentiation (LTP). Importantly, the Wnt antagonists Sfrps or the CRD of Fz5 that sequesters endogenous Wnts that bind to the Fz5 receptor, block the synaptic localization of Fz5 during HFS. These findings suggest that neuronal activity promotes the expression and/or release of Wnt factors, which

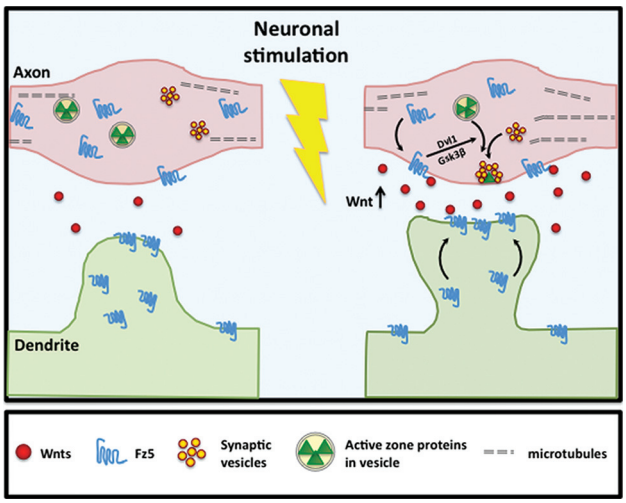

FIGURE 3 | Neuronal activity stimulates Fz5 mobilization to synapse cell surface. Neuronal activity stimulates Wnt expression and/or release. Activity-dependent Wnt release subsequently induces Fz5 cell surface expression at synaptic sites. In hippocampal neurons, activity-dependent Fz5 mobilization through Wnt signaling is required for activity-mediated synapse formation.

are required for Fz5 trafficking to the cell surface and to the synapse. Importantly, Wnt blockade also prevents HFS-mediated synapse assembly (Sahores et al., 2010). These findings demonstrate that Wnt-Fz5 signaling is required for activity-mediated synapse formation. It remains to be identified which Wnt isoforms are modulated by activity to promote Fz5 trafficking to the synapse.

WNT SIGNALING AND FUNCTIONAL PLASTICITY OF MATURE SYNAPSES

Synaptic plasticity refers to enduring changes in synaptic strength in response to different patterns of neuronal activity. Long-term changes to presynaptic neurotransmitter release and/or postsynaptic signal transduction through receptor availability and downstream effectors mediate synaptic plasticity. Synaptic plasticity mediates changes in sensory, motor and somatosensory inputs, as well as learning and memory. The patterns of expression of Wnts strongly suggest that they regulate these processes in the brain.

Wnts are released by activity-dependent mechanisms (Yu and Malenka, 2003; Chen et al., 2006; Wayman et al., 2006; Gogolla et al., 2009). Furthermore, Wnts and many of their signaling components continue to be expressed in the adult brain (Coyle-Rink et al., 2002; Shimogori et al., 2004; De Ferrari et al., 2007; Gogolla et al., 2009). Increasing evidence now suggests that sustained Wnt signaling may contribute to synaptic plasticity (Chen et al., 2006; Beaumont et al., 2007; Ataman et al., 2008; Cerpa et al., 2008; Gogolla et al., 2009; Varela-Nallar et al., 2010; Jensen et al., 2012). Enhanced sensory experience increases Wnt7a/b levels in the adult hippocampus, which act as retrograde signals inducing profound presynaptic plasticity-related modeling of CA3-CA1 mossy fiber synapses (Gogolla et al., 2009). Importantly, local application of the Wnt antagonist Sfrp1 by canulla injection during enhanced sensory experience prevents experience-dependent presynaptic remodeling and terminal complexity (Gogolla et al., 2009). Moreover, Wnt signaling acutely enhances the frequency and amplitude of spontaneous and evoked EPSCs in mature hippocampal neurons (Beaumont et al., 2007; Varela-Nallar et al., 2010; Ciani et al., 2011). These findings suggest that Wnts may contribute to activity-dependent plasticity. Indeed, a role for Wnt in LTP has been suggested. In acute hippocampal brain slices activation of the canonical-Wnt signaling by exogenous Wnt3a or lithium weakly facilitates LTP, whereas specific antibodies against Wnt3a or the secreted extracellular domain of Fz8 decreases LTP (Chen et al., 2006). More recently, Wnt5a has also been shown to facilitate LTP at CA3-CA1 synapses (Cerpa et al., 2011). These studies strongly suggest that Wnts modulate LTP. However, loss-of-function studies are required to fully demonstrate whether Wnt signaling is required for LTP.

WNT SIGNALING IN SYNAPTIC MAINTENANCE

In vivo imaging demonstrates that 70–90% of synapses in the mature brain are stable and persist for extended periods of time, perhaps for the lifespan of the animal (Trachtenberg et al., 2002; Meyer et al., 2003; Alvarez and Sabatini, 2007; Bhatt et al., 2009; Holtmaat and Svoboda, 2009). Synaptic function and maintenance do not only determine stimulus transmission in the brain, but also the stability of axonal and dendritic arbors (Wu and Cline, 1998; Rajan et al., 1999; Niell et al., 2004; Hu et al., 2005; Meyer and Smith, 2006; Ruthazer et al., 2006; Chen et al., 2010). During development, motile "exploratory" axonal branches form preferentially at nascent presynaptic sites; in contrast stable presynaptic sites inhibit dynamic axon behaviors and growth, and are associated with stable axonal branches (Meyer and Smith, 2006). These studies are supported by the finding of a direct correlation between synaptic maintenance and local axon stability, which is enhanced by neuronal activity (Ruthazer et al., 2006). The relationship between synaptic and axonal maintenance is equally reflected in dendritic arbor structure, where loss of synapses is associated with dendritic retraction (Sfakianos et al., 2007; Lin and Koleske, 2010). Together, the findings suggest that axon and dendrite stability and the preservation of neuronal networks are determined by synaptic maintenance.

In contrast to stable synaptic structure, synaptic proteins are highly labile with half-lives of just days or even less (Huh and Wenthold, 1999; Ehlers, 2003). Synaptic proteins shuttle in and out of synapses at a remarkable rate through lateral diffusion or exo- and endocytosis (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Wenthold et al., 2003; Collingridge et al., 2004; Cognet et al., 2006; Gray et al., 2006; Ehlers et al., 2007; Kielland et al., 2009). Synaptic proteins such as Ras, Shank, PSD-95, Bassoon and Synaptophysin are redistributed between adjacent synapses over a time scale of minutes and hours (Kim and Sheng, 2004; Gray et al., 2006; Tsuriel et al., 2006, 2009). Furthermore, highly motile SVs are recycled between multiple presynaptic sites (Darcy et al., 2006; Staras et al., 2010). These studies raise a fundamental question; if synaptic components are only transiently located to synaptic sites, how is the long-term integrity of the synapse maintained?

Synaptogenic factors are ideally suited to modulate synaptic stability. Indeed, Wnt signaling has now been demonstrated to regulate synaptic maintenance in mature neurons. Time-lapse imaging experiments demonstrate that Wnt signaling blockade by

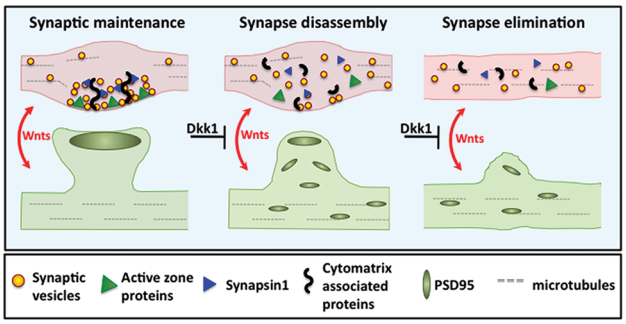

FIGURE 4 | Wnt blockade by Dkk1 induces synapse disassembly. Left panel: Wnt signaling maintains pre- and postsynaptic stability. Middle panel: Blockade of Wnt signaling by Dkk1 induces dispersal of synaptic proteins. During Wnt blockade, functionally diverse synaptic components such as SVs, Synapsin1 and active zone proteins disperse from presynaptic sites. Postsynaptically, PSD95 also disperses. Right panel: Synapse disassembly eventually leads to synapse elimination.

the secreted antagonist Dkk1 eliminates previously stable presynaptic sites within 20 min (Purro et al., 2012). Dkk1 rapidly induces the delocalization of pre- and postsynaptic components in mature and stable hippocampal synapses (**Figure 4**). This effect is accompanied by a reduced number of SV recycling sites. Importantly, ultrastructural analyses of remaining synapses reveal smaller active zones and postsynaptic densities, suggesting coordinated pre- and postsynaptic shrinkage and disassembly by Wnt blockade with Dkk1. These synaptic changes occur in the absence of cell death or decreased cell viability (Purro et al., 2012), suggesting that Dkk1 directly affects the synapse. These findings provide evidence that endogenous Wnt signaling is required for synaptic maintenance.

Synaptic activity is the key stimulus for maintaining the molecular composition of synapses. Several synaptic proteins and signaling molecules are synthesized in an activity dependent manner (Steward and Schuman, 2001; West et al., 2001). Activity-dependent mechanisms also regulate the trafficking of synaptic proteins to active synaptic sites (Ehlers et al., 2007; Kielland et al., 2009). As discussed above, neuronal activity regulates the expression and release of Wnts. Moreover, Wnt blockade induces rapid synapse disassembly (Purro et al., 2012). We therefore propose that neuronal activity promotes the stability of synapses by modulating the levels of Wnts. Conversely, at inactive synapses limited levels of Wnts could render these synapses more prone to destabilization and disassembly.

Mounting evidence suggests that in several neurodegenerative diseases, the loss of synapses correlates well with cognitive decline and motor impairment before cell death is evident. These findings highlight the importance for understanding the cellular and molecular mechanisms that control the stability of synapses while permitting plastic changes to occur.

DYSFUNCTIONAL WNT SIGNALING IN NEURODEGENERATIVE DISORDERS

Dysfunctional Wnt signaling is linked to a number of neurological disorders including autism, schizophrenia, bipolar disorders, Alzheimer's disease (AD) and Parkinson's disease (De Ferrari and Moon, 2006; Inestrosa and Arenas, 2010; Okerlund and Cheyette, 2011; Scott et al., 2013). A common feature underlying these neurological disorders is aberrant synapse function and/or synapse degeneration. Synapse disassembly often precedes and might even trigger the widespread and catastrophic cell death that hallmarks many neurodegenerative disorders (Selkoe, 2002; Luo and O'Leary, 2005; Saxena and Caroni, 2007; Shankar et al., 2007; Stevens et al., 2007; Rosen and Stevens, 2010; Kessels et al., 2013). Biopsies of human AD brains reveal a ∼15–35% loss of synapses per neuron within 2–4 years of clinical AD onset (Terry et al., 1991). Importantly, the degree of cognitive decline correlates with loss of Synaptophysin-labeled presynaptic sites (Masliah et al., 2001). These findings are consistent with the results obtained from transgenic mouse models of AD, where spine loss is best correlated with cognitive impairment than with the deposits of Amyloid plaques (Knobloch and Mansuy, 2008). Synapse loss in the AD brain correlates with the accumulation of soluble Amyloid-β (Aβ) prior to the onset of neuronal cell death, suggesting that soluble Aβ contributes to synaptic defects (Hsia et al., 1999; Selkoe, 2002; Lacor et al., 2007; Shankar et al., 2007). Indeed, Aß modulates synaptic molecules resulting in synaptic dysfunction (Cisse et al., 2011). However, the molecular mechanisms by which soluble Aβ promotes synapse loss are poorly understood. Together the findings strongly suggest that synapse disassembly is an early event in AD.

A potential role for Wnt signaling in the pathogenesis of AD has been suggested by a number of recent studies (De Ferrari and Moon, 2006; Caraci et al., 2008; Inestrosa and Arenas, 2010; Purro et al., 2012). Exogenous Wnt5a ameliorates Aβ-induced cell death in hippocampal neurons (De Ferrari et al., 2003; Cerpa et al., 2010). In addition, Wnt3a rescues Aβ-mediated loss of neurogenesis and neural differentiation in embryonic hippocampal progenitor cells *in vitro* (Shruster et al., 2011). Soluble Aβ binds to Fz5 and blocks canonical Wnt signaling in NB2A cells (Magdesian et al., 2008). Increased Gsk3β activity, which inhibits Wnt signaling, induces the hyperphosphorylation of Tau and adversely affects Amyloid precursor protein (APP) processing (Mudher et al., 2001; Hooper et al., 2008). However, the first clear link between Wnt signaling and AD came from a genome-wide association study demonstrating that a genetic variant of LRP6, the receptor for Dkk1, is strongly associated with late-onset AD (De Ferrari et al., 2007). This variant of LRP6 exhibits decreased level of Wnt signaling. Together these results suggest that decreased levels of Wnt signaling could contribute to Aβ-mediated neuropathogenesis.

Further links between dysfunction of Wnt signaling and AD comes from studies on the Wnt antagonist Dkk1. Dkk1 expression is elevated in human postmortem AD brains and in mouse models of AD (Caricasole et al., 2004; Rosi et al., 2010). In hippocampal brain slices, acute exposure to Aβ oligomers rapidly increases Dkk1 mRNA and Dkk1 protein levels in the CA1, CA3 and DG (within 3 hrs), with a concomitant loss of synapses (Purro et al., 2012). Crucially, blockade of Dkk1 with specific neutralizing antibodies suppresses the ability of Aβ to stimulate synapse loss (Purro et al., 2012). These studies demonstrate that Dkk1 is required for Aβ-induced synapse elimination. We propose that dysfunction in Wnt signaling contributes to Aß-mediated synaptic toxicity and the subsequence cognitive decline observed in dementia (**Figure 5**).

How does Aβ induce Dkk1 expression? A recent report suggests that increased soluble Aβ induces the intracellular accumulation of the cell survival factor clusterin, possibly by preventing its neural secretion (Killick et al., 2012). It was posited that intracellular clusterin accumulation activates p53 signaling, which stimulates Dkk1 expression. Consistent with this idea, knockdown of clusterin prevents Aβ toxicity and upregulation of Dkk1 in cultured hippocampal neurons. Moreover, pharmacological inhibition of the p53 pathway blocks Aβ induction of Dkk1 (Killick et al., 2012). Thus, p53 activation contributes to Aβ-mediated Dkk1 induction. It would be critical to establish whether p53 also contributes to Dkk1 increased levels during synaptic disassembly induced by Aβ *in vivo*.

Recent studies have shown that endogenous Dkk1 expression becomes progressively elevated in the aged brain and may be associated with general cognitive decline as well as susceptibility

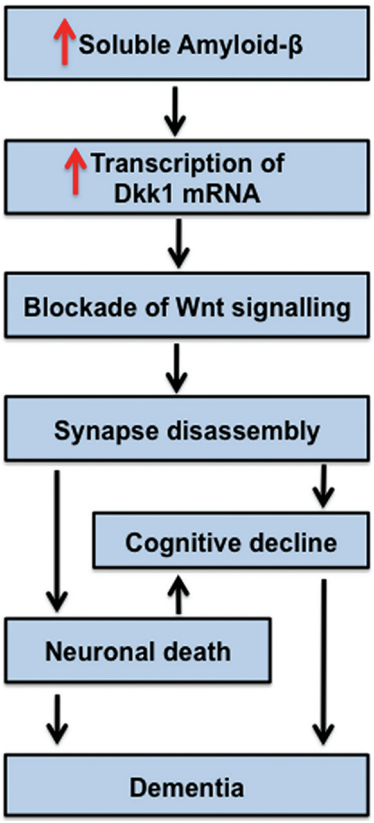

FIGURE 5 | Model for Dkk1 in the progression of AD. Increased levels of soluble Amyloid-β stimulate the transcription of Dkk1, which blocks endogenous Wnts important to the stability of synapses. Thus, Dkk1 induces synapse disassembly by the dispersal of synaptic components. Loss of synapses correlates with cognitive decline. We propose that Dkk1-induced synapse elimination is an early event in the progression of AD. The notion that synapse disassembly precedes cell death is in accordance with current models for the progression of AD.

to age related pathological neurodegenerative disorder (Scott et al., 2013; Seib et al., 2013). Indeed, Dkk1 knockdown increases neurogenesis in the adult hippocampus and this effect has been correlated with enhanced working spatial memory and memory consolidation (Seib et al., 2013). However, it remains to be examined whether changes in spatial memory are due to changes in synapse integrity.

CONCLUDING REMARKS

The delicate balance between synapse assembly, disassembly and maintenance are highly regulated processes controlled by a complex molecular dialogue between pre- and postsynaptic neurons. Importantly, neuronal activity, which has profound effects on these processes, contributes to the expression and release of Wnt factors, which in turn modulate this trans-synaptic dialogue. Indeed, several studies have demonstrated that Wnts contribute to activity-mediated synapse formation during early neuronal circuit development and during experience-dependent plasticity.

Wnt-mediated synaptic maintenance is now emerging in relation to neurodegeneration. Indeed, the Wnt antagonist Dkk1, which has strong synaptic disassembly activity, is now linked to AD. Importantly, Dkk1 mediates the effect of Aβ on synaptic disassembly. Future *in vivo* studies will provide key evidence for the contribution of dysfunction in Wnt signaling to synaptic degeneration in AD.

It is an exciting time to study Wnt signaling in the nervous system as we begin to unravel the mechanisms by which Wnts modulate experience-mediated plasticity. Moreover, the potential role of Wnts in synaptic maintenance opens new avenues to develop therapeutic strategies for the treatment of neurodegenerative diseases at early stages. Finally, the finding that Dkk1 is elevated in the AD brain has sparked enthusiasm that this molecule could be used as a biomarker at early stages of AD or other neurodegenerative diseases.

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Sonic hedgehog and Wnt: antagonists in morphogenesis but collaborators in axon guidance

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As indicated by their name, morphogens were first identified for their role in the formation of tissues early in development. Secreted from a source, they spread through the tissue to form gradients by which they affect the differentiation of precursor cells in a concentration-dependent manner. In this context, the antagonistic roles of the morphogens of the Wnt family and Sonic hedgehog (Shh) in the specification of cell types along the dorso-ventral axis of the neural tube have been studied in detail. However, more recently, morphogens have been demonstrated to act well beyond the early stages of nervous system development, as additional roles of morphogen gradients in vertebrate neural circuit formation have been identified. Both Wnt and Shh affect neural circuit formation at several stages by their influence on neurite extension, axon pathfinding and synapse formation. In this review, we will summarize the mechanisms of morphogen function during axon guidance in the vertebrate nervous system.

Keywords: neural circuit, Frizzled, Ryk, Smoothened, spinal cord, morphogen, attraction, repulsion

INTRODUCTION

Morphogens, defined as secreted molecules that act in a concentration gradient to affect the differentiation of precursor cells, are also involved in the establishment of neural connections. Both Sonic hedgehog (Shh) and Wnts play important roles in different cellular events during neural development (Wilson and Stoeckli, 2012). Shh activity is triggered by its binding to the Patched (Ptc) receptor and the consequent derepression of Smoothened (Smo), leading to translocation of GliA to the nucleus, inducing the transcription of target genes. Wnts regulate transcriptional activity by binding to a receptor complex formed by a Frizzled (Fz) family member and Lrp5/6. This in turn leads to the inhibition of GSK3β and the accumulation of β-catenin, which can enter the nucleus and act together with Tcf/Lef transcription factors to regulate target gene expression. In addition to this so-called canonical pathway, Wnt ligands are able to activate alternative signaling pathways, such as the planar cell polarity (PCP) pathway and the calcium pathway (see Section Molecular mechanisms of Wnt-mediated axon guidance). The PCP pathway is not only involved in tissue polarity but also affects axon guidance and cell migration.

During morphogenesis, Shh and Wnts act antagonistically in the patterning of the neural tube (**Figure 1**). Shh has a ventralizing activity (Dessaud et al., 2008), which is antagonized by Wnt signaling (Ulloa and Martí, 2010). At the molecular level, Gli3, a signaling molecule downstream of Shh, appears to be the link, as Gli3 expression is induced by Wnts (Ulloa and Martí, 2010).

Roles for Shh and Wnts later in neural development have been described more recently. After an initial report on Wnt's role in commissure formation in *Drosophila* (Yoshikawa et al., 2003), Wnts were also implicated in axon guidance in vertebrates (Lyuksyutova et al., 2003; see below). At about the same time, Shh was shown to be involved in vertebrate axon guidance (Charron et al., 2003; Bourikas et al., 2005; see below). So far, such a role for Shh has not been found in invertebrates. In addition, both Wnts and Shh were found to affect synaptogenesis (Salinas and Zou, 2008; Harwell et al., 2012). Morphogen signaling in these late stages of neural development is relatively poorly understood, as it is more complex than canonical signaling.

In this review, we will discuss the role of morphogens in neural circuit formation by concentrating on axon guidance. In many areas of the developing nervous system, Wnts and Shh are expressed in overlapping areas. In contrast to their effects in early development, where they were found to antagonize each other, Shh and Wnts often collaborate in axon guidance, although the effect on a navigating growth cone may still be antagonistic. Some of the molecular mechanisms underlying these signaling activities are beginning to be elucidated.

Shh AND Wnts CONTRIBUTE TO AXON GUIDANCE OF MANY DIFFERENT NEURONAL POPULATIONS

dI1 COMMISSURAL AXONS OF THE SPINAL CORD

Commissural axons in the developing spinal cord have provided an accessible, informative *in vivo* model to investigate the molecular mechanisms of axon guidance (Chédotal, 2011). During development, dorsally-located dI1 commissural neurons project their axons ventrally toward and across the ventral midline at the floorplate, forming axon commissures that enable bilateral neural communication (**Figure 2**). After crossing the midline, the axons make an abrupt 90° turn and extend rostrally in close contact with the contralateral floorplate border. Despite the apparent simplicity of this trajectory, the navigating axons must make many complex pathfinding decisions *en route* to their target. Initially, they perceive the floorplate as attractive, but upon arrival at this intermediate target, they must switch their response to repulsion in order to move on. Upon exiting the floorplate, dI1 axons make

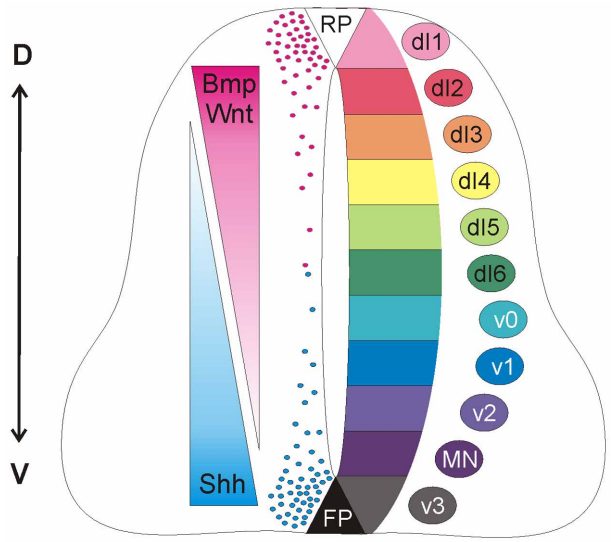

FIGURE 1 | Antagonistic activities of Shh and Wnt/BMP pattern the developing spinal cord. Counteracting gradients of Shh, secreted from the floorplate (FP), and Wnt/BMP, derived from the roof plate (RP) induce the concentration-dependent differentiation of precursor cells along the dorso-ventral (D-V) axis. During morphogenesis, Shh and Wnts have antagonistic functions. Shh promotes the formation of Gli activator forms, while Wnts directly induce the expression of Gli3, which acts as a transcriptional repressor in the absence of Shh (see Dessaud et al., 2008, for details). In turn, the specific combinations of transcription factors induced by Shh and Wnts generate a cell identity code that specifies the neural progenitor subtypes. As these cells exit the cell cycle, they distribute laterally in a specific order along the dorso-ventral axis (dI1-v3).

a sharp rostral turn into the longitudinal axis to continue their post-crossing trajectory. As an intermediate target for these axons, the floorplate is a major source of attractive and repulsive, long-range and short-range cues. The correct interpretation of these signals by the navigating axons is only possible by precise spatiotemporal control over cellular signaling pathways (Stein and Tessier-Lavigne, 2001; Zisman et al., 2007; Yoon et al., 2009).

The initial ventral projection of commissural axons is determined in part by Netrin1, a long-range, floorplate-derived chemoattractant (Serafini et al., 1996). Commissural axons express the Netrin1 receptor DCC (deleted in colon cancer) (Keino-Masu et al., 1996), and accordingly, mice mutant for either *Netrin1* or *DCC* display severe axon guidance defects, in which many commissural axons fail to invade the ventral spinal cord and are unable to cross the midline (Fazeli et al., 1997). However, not all commissural axons are affected in *Netrin1* mutant mice, suggesting that an additional chemoattractive mechanism acts in parallel to Netrin1-DCC to guide axons toward the floorplate.

Indeed, Charron et al. (2003) demonstrated that in *Netrin*-deficient mice Shh attracts commissural axons ventrally (**Figure 2A**). *In vitro*, the *Netrin1*-deficient floorplate was still able to elicit commissural growth cone turning, an effect that was mimicked by the presence of Shh-expressing COS cells. Shh also acted directly as a chemoattractant on isolated *Xenopus* spinal axons. Thus, Shh is a chemoattractant for commissural axons in collaboration with Netrin-1, although its effect is normally masked by the stronger effect of Netrin-1. When Smo was inhibited by cyclopamine, or when Smo was conditionally inactivated in the dorsal spinal cord, the chemoattractant effects of Shh were blocked *in vitro* and commissural axons projected abnormally toward the floorplate *in vivo*. Thus, Shh acts via Smo as an axonal chemoattractant in the dorsoventral axis of the spinal cord.

Although the attractive guidance effects of Shh were transduced via Smo, Smo does not bind Shh directly. Thus, the next step was to identify the cell surface receptor/s responsible for Shh binding. Two structurally related candidates, Cdon (Cell adhesion molecule-related/downregulated by Oncogenes) and Boc (Brother of Cdon) were tested for their ability to bind Shh and mediate its attractive axon guidance effects (Okada et al., 2006). While both receptors bound specifically to Shh, only Boc was expressed by differentiating commissural neurons. In Boc knockout mice, commissural axons were misdirected and invaded the motor columns as they approached the floorplate, a phenotype which resembled that previously described following disruption of Smo. Additionally, RNA-interference-mediated knockdown of Boc impaired the turning response of rat commissural axons toward an ectopic source of Shh *in vitro*. Taken together, these data suggested that Boc was an essential receptor for Shh in attractive commissural axon guidance.

In addition to its role as an attractant of pre-crossing axons toward the floorplate in the dorsoventral axis (Charron et al., 2003), Shh also acts as a repulsive guidance cue for post-crossing axons, directing them into the longitudinal axis toward the brain (Bourikas et al., 2005; Yam et al., 2012). In chicken, this rapid switch from attractant to repellant is due to a change in the growth cone receptors that detect and transduce the Shh signal (**Figure 2B**). When Shh was reduced in a spatiotemporally-controlled manner by *in ovo* electroporation of dsRNA, post-crossing commissural axons either stalled at the floorplate exit site, or even turned caudally instead of rostrally. Consistent with its decreasing posterior^{high} to anterior^{low} expression pattern in the chick floorplate, Shh can directly repel post-crossing commissural axons from spinal cord explants (Bourikas et al., 2005). In line with the finding that neither Smo nor Ptc mRNAs were expressed in commissural neurons after their axons reached the midline, Hedgehog-interacting protein (Hhip) was identified as the Shh receptor mediating repulsion. Hhip was transiently upregulated at the time when the commissural axons turn into the longitudinal axis (Bourikas et al., 2005). A recent report confirms that Shh also acts as a longitudinal repellent in mammals (Yam et al., 2012), although the role of Hhip could not be confirmed in the mouse.

Experiments with mouse spinal cord explants from the Zou laboratory also implicated Wnt ligands in post-crossing commissural axon guidance (Lyuksyutova et al., 2003). According to the anterior^{high} to posterior^{low} gradient of *Wnt4* mRNA expression in the floorplate along the mouse spinal cord, Wnt4 was shown to attract post-crossing commissural axons (**Figure 2C**). Mice lacking the Wnt receptor Frizzled3 (Fz3) exhibited randomization of commissural axons after crossing the floorplate, suggesting

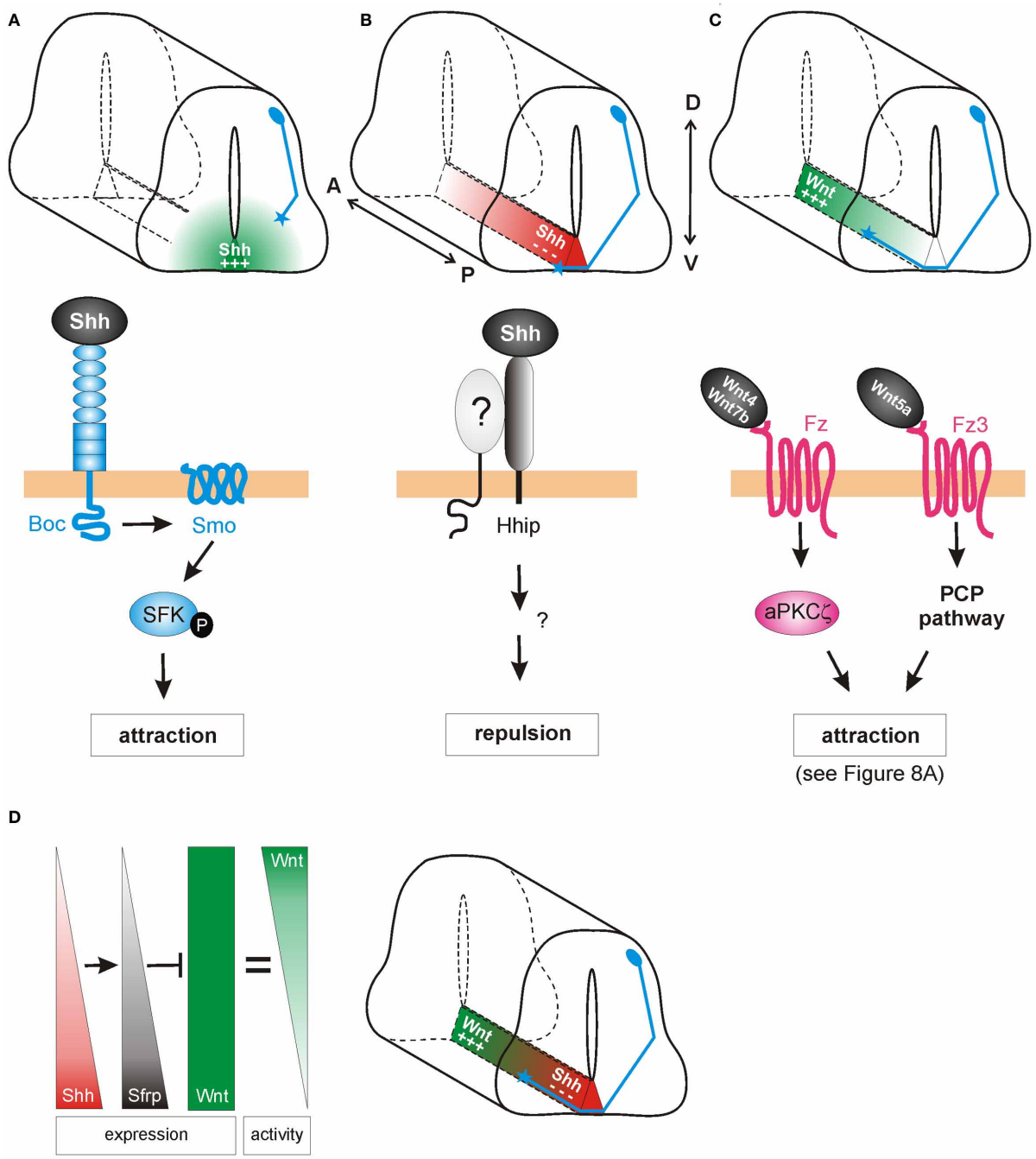

FIGURE 2 | Shh and Wnts guide commissural axons in the vertebrate spinal cord. (A) Pre-crossing commissural axons (blue) are attracted ventrally toward the midline by an increasing gradient of Shh produced in the floorplate (green). The attractive effect of Shh is mediated by Smoothened (Smo) and Brother of CDO (Boc) in a transcription-independent manner. Instead, the activation of Src family kinases (SFK) induces cytoskeletal rearrangements in the growth cone. **(B)** The response of commissural axons to Shh switches from attraction to repulsion when axons reach the midline. Post-crossing commissural axons are pushed anteriorly by a posterior^{high} to anterior^{low} gradient of Shh (red). The repellent activity of Shh is mediated by Hedgehog-interacting protein (Hhip), a receptor that is transiently upregulated on commissural axons at the time of their turning into the longitudinal axis. An additional signaling co-receptor may also be involved. **(C)** An anterior^{high} to posterior^{low} gradient of Wnt activity works in parallel to Shh repulsion to attract post-crossing commissural axons anteriorly. Depending on the species, Wnt4, Wnt5a, and Wnt7a are attractants for post-crossing commissural axons via non-canonical pathways. In mouse, Fz3, in response to Wnt4/Wnt7b, activates a complex containing an atypical protein kinase C (aPKCζ). In response to Wnt5a, the PCP pathway is activated. See text for more details. **(D)** In chick, Shh was shown to shape Wnt activity indirectly. Wnt5a and Wnt7a are expressed uniformly along the longitudinal axis. In addition to its direct effect on post-crossing commissural axons, Shh induces the expression of the Wnt antagonist Sfrp1 in a posterior^{high} to anterior^{low} gradient in the floorplate. The antagonistic activity of Sfrp in turn regulates the activity of Wnts in the floorplate, such that an attractive "activity gradient" of Wnt is formed. Thus, Shh (red) and Wnt (green) gradients collaborate to guide post-crossing commissural axons anteriorly.

that Wnt4 might act via the Fz3 receptor to attract these axons (Lyuksyutova et al., 2003). However, the genetic or biochemical interaction between Wnt4 and Fz3 has, to date, not been shown. More recently, post-crossing commissural axons were shown to respond to a Wnt activity gradient *in vivo*, in the chicken spinal cord (Domanitskaya et al., 2010). In the chicken, Wnt5a and Wnt7a were shown to act as attractive cues *in vivo*, as assessed by *in ovo* RNA interference. In contrast to the mouse, *Wnt5a* and *Wnt7a* are not expressed in a gradient along the A-P axis of the lumbar spinal cord in chicken. Rather, a Wnt activity gradient (anterior^{high} to posterior^{low}) is shaped by the graded expression (anterior^{low} to posterior^{high}) of the Wnt antagonist, Secreted frizzled-related protein (Sfrp) (**Figure 2D**; Domanitskaya et al., 2010).

Intriguingly, Sfrp is a transcriptional target of Shh not only in mesodermal tissue (Lee et al., 2000) but also in the developing neural tube. Ectopic expression of Shh in the chicken spinal cord induced both *Sfrp1* and *Sfrp2* (Domanitskaya et al., 2010), suggesting that during normal development, the graded expression (anterior^{low} to posterior^{high}) of *Sfrps* in the floorplate was induced by the corresponding gradient of Shh. Interestingly, these Sfrps did not act directly as guidance cues, but rather indirectly by preventing Wnts from binding to their Fz receptors and thus by shaping a Wnt activity gradient. Together, these findings identified a close link between the two guidance cues for post-crossing commissural axons in chicken: Shh not only repels post-crossing commissural axons directly (Bourikas et al., 2005), but also influences Wnt activity indirectly by inducing the graded expression of the Wnt antagonists Sfrp1 and Sfrp2, which in turn shape the functional gradient of Wnt activity (**Figure 2D**). Whether Shh induces Sfrps in the mouse floorplate (a mechanism that could sharpen the existing Wnt4 expression gradient) is unknown. The molecular pathway by which Shh induces Sfrps also requires investigation, as do the signaling mechanisms downstream of Wnt in post-crossing commissural axon guidance in the chicken.

RETINAL GANGLION CELL AXONS AND TOPOGRAPHIC MAPPING

Another well-studied model for neural circuit formation is the visual system (Erskine and Herrera, 2007; Petros et al., 2008). During development, retinal ganglion cells (RGCs) of the eye project their axons through quite diverse microenvironments *en route* to their targets in the brain. Initially, RGC axons all grow to the optic disc at the center of the retina, where they exit the eye and course within the optic nerve toward the midline, where they must decide to grow either ipsilaterally or contralaterally. By crossing the midline, RGC axons form the optic chiasm. Post-chiasmic RGC axons then travel along the lateral surface of the neuroepithelium in the diencephalon, forming the optic tract. Finally, the axons turn caudally toward their final target, the optic tectum. Growing evidence indicates that Shh is an important guidance cue for RGC axons at several stages along this route. Interestingly, Shh displays a dual activity in this system. Depending on its concentration, Shh either promotes or prevents RGC axon growth (Kolpak et al., 2005, 2009; Gordon et al., 2010). Upon reaching the tectum, RGC axons rely on Wnt signaling in order to correctly identify their targets (Schmitt et al., 2006) (**Figure 3**).

The initial guidance of RGC axons within the retina toward the optic disc is regulated by several classical cues, including Slit1 (Jin et al., 2003), Netrin1 (Deiner et al., 1997), and EphBs (Birgbauer et al., 2000). Shh acts in addition to these cues to influence RGC axon projection inside the retina. Kolpak et al. (2005) identified Shh as a factor secreted from the central retina that positively affected RGC axon growth. When RGC axons were exposed to low concentrations of Shh *in vitro*, they displayed increased outgrowth. This effect was blocked by the addition of cyclopamine,

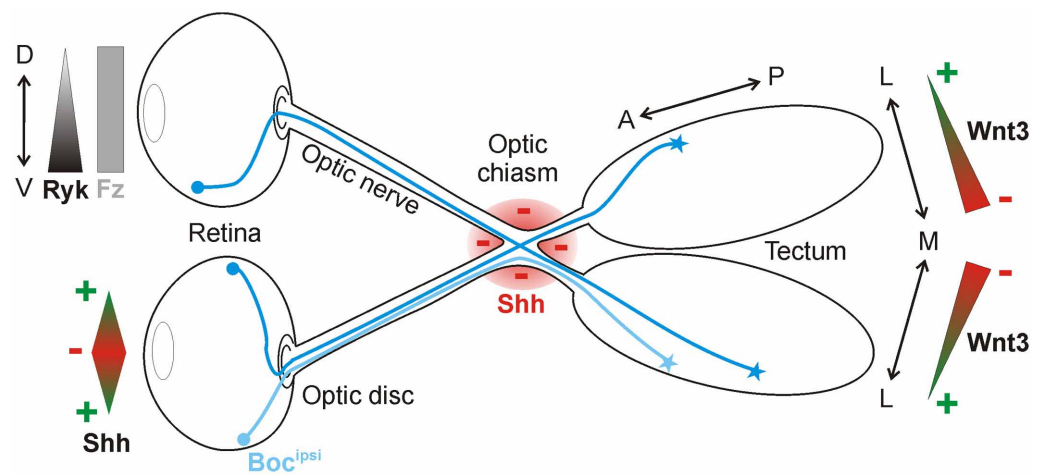

FIGURE 3 | Shh and Wnt3 are axon guidance cues in the visual system. In the retina, Shh is bifunctional: low concentrations promote RGC axon outgrowth toward the optic disc, whereas high concentrations push axons into the optic nerve. Shh is also expressed along the border of the optic chiasm (red), where it acts as a repellant for RGC axons in the optic nerve. Ipsilaterally projecting RGC axons express Boc (Boc^{ipsi}), which mediates Shh repulsion at the chiasm. Wnt3 is expressed in a medial^{high}-to-lateral^{low} gradient in the tectum. At high concentrations, Wnt3 inhibits the growth of both dorsal and ventral RGC axons via Ryk as a receptor. At low concentrations, Wnt3 stimulates the growth of dorsal RGC axons to the lateral tectum in a Fz-dependent manner.

suggesting that Shh promoted axon outgrowth via Smo. When faced with a choice of substrate in stripe assay experiments, RGC axons preferred to grow on stripes containing a low concentration of Shh. These positive outgrowth responses to Shh occurred rapidly, suggesting that Shh was acting directly on the growth cone, via a transcription-independent pathway.

In the same study, Kolpak et al. (2005) found that high concentrations of Shh instead had negative outgrowth effects on RGC axons, as assessed in their co-culture and stripe assay experiments. Like the positive effects, the concentration-dependent negative effects of Shh were mediated by Smo, since they were blocked by cyclopamine. Taken together, the experiments suggested that the precise level of Shh protein expression inside the retina is critical for the projection of RGC axons toward the optic disc.

Shh has also been reported as a repellant molecule for RGCs further along in their trajectory. After leaving the eye through the optic disc, RGC axons approach the optic chiasm and prepare to innervate either the ipsilateral or contralateral side of the brain. Shh is expressed along the border of the optic chiasm, defining a barrier at the ventral midline that could guide the projection of RGC axons (**Figure 3**). In agreement with this idea, ectopic expression of Shh at the midline prevents RGC axons from crossing (Trousse et al., 2001). Conversely, the injection of E13.5 mouse embryos with a hybridoma producing a Shh-blocking antibody causes aberrant projection of RGC axons at the optic chiasm by E18.5 (Sanchez-Camacho and Bovolenta, 2008). However, in that study, the blockade of Shh signaling for five days could have led to changes in midline patterning, leaving open the possibility that the effects observed were not due to a direct guidance effect of Shh but rather to patterning defects. *In vitro* experiments do however support the notion that Shh acts directly as a chemorepellant for RGC axons, since axons from retinal explants were reduced in number and length following the addition of exogenous recombinant Shh. Time-lapse analysis revealed that axons from retinal explants rapidly retracted in the presence of Shh, in a concentration-dependent manner (Kolpak et al., 2005).

A recent report suggests that Boc is the receptor responsible for the repulsion of ipsilateral RGC axons from Shh at the chiasm (Fabre et al., 2010). Interestingly, this finding indicates that Boc-mediated transduction of the Shh signal leads to opposite axon guidance effects in commissural neurons (attraction; see Section dI1 commissural axons of the spinal cord) versus RGC neurons (repulsion). The molecular mechanisms underlying these differential, Boc-mediated effects of Shh are yet to be elucidated. Additionally, Smo has also been implicated in the guidance of RGC axons *in vivo* (Sanchez-Camacho and Bovolenta, 2008). When a Smo inhibitor was electroporated into contralaterally-projecting RGC neurons, the axons displayed growth and guidance defects at the midline. These experiments suggested that Shh signaling functions cell-autonomously to control the pathfinding of RGC axons (Sanchez-Camacho and Bovolenta, 2008). However, Boc is not expressed in the contralaterally-projecting RGCs (Fabre et al., 2010), thus the identity of the Shh receptor mediating this effect of Shh on contralateral RGC axons remains to be determined. It is also unknown whether the ipsilaterally-projecting RGC axons require Smo for their response to Shh.

In *Xenopus* embryos, Shh has also been demonstrated to guide RGC axons within the optic tract, after they have crossed the midline (Gordon et al., 2010). *Xshh* was expressed adjacent to the ventral optic tract during RGC axon extension, and the RGCs were found to co-express Ptc and Smo. Bath application of cyclopamine caused abnormal phenotypes, including defasciculation and widening of the ventral optic tract, axonal misprojection into the neuroepithelium and targeting errors in the tectum. Conversely, ectopic activation of Shh signaling by implanting beads soaked in N-Shh caused a deflection of RGC axons away from their normal pathway, or a cessation of axonal extension. Together, these findings indicate that Shh signaling is required to define the path of retinal axons in the optic tract.

Since *Xshh* mRNA expression was not detected near the optic tectum, it is not clear how Shh could be influencing axonal targeting in the tectum directly. Shh was suggested to either diffuse over long distances from ventrally located sources (Gordon et al., 2010) or it may come from the RGC axons themselves. RGC axons have been reported to express Shh (Traiffort et al., 2001; Sanchez-Camacho and Bovolenta, 2008). However, there is no experimental evidence for either of these hypotheses. Moreover, an effect of Shh on patterning of the tectum or on the differentiation and maturation of the RGCs has not been taken into account. Additional experiments will be required to definitively demonstrate that Shh is directly required for axonal targeting in the tectum.

A patterning effect of Shh is not so far-fetched, as a recent study in zebrafish embryos suggests that indeed Shh guides RGC axons within the eye via an indirect mechanism, by patterning the optic stalk (Stacher Hörndli and Chien, 2012). The authors used transplantation studies to functionally test for the cell autonomy of Shh pathway components during intraretinal RGC axon guidance. When retinal precursor cells (RPCs) from wildtype donors were transplanted into hosts lacking *shh* or *smo*, RGC axons were misguided. Conversely, RPCs from donors lacking *shh* or *smo* projected normally when transplanted into wildtype hosts. These findings indicated that both Smo and Shh were acting non-cell-autonomously in intraretinal axon pathfinding, suggesting that Shh was required to indirectly pattern the eye rather than as a direct guidance cue.

To confirm this result, Stacher Hörndli and Chien (2012) applied pharmacological inhibitors of the Shh pathway during specific stages of embryonic development. While early application of the Smo antagonist SANT75 caused severe intraretinal pathfinding errors in most embryos, SANT75 treatment at the onset of RGC differentiation generated a significantly weaker phenotype, with most embryos displaying normal RGC pathfinding. Thus, Shh signaling was required during optic vesicle patterning, but not during RGC axon projection out of the eye. Indeed, the expression of several optic stalk markers was reduced or absent in *shh* and *smo* mutants, including Pax2, *netrin1*, *chemokine ligand 12a* (*cxcl12a*), and its homolog *cxcl12b*.

Unlike in mouse (Deiner et al., 1997), the loss of netrin1 by morpholino injection did not affect intraretinal RGC axon pathfinding in zebrafish. Rather, Stacher Hörndli and Chien (2012) examined whether the downregulation of chemokine signaling at the optic disc might be responsible for the Shh-induced

RGC phenotype. Strikingly, *cxcl12a* mutants exhibited highly penetrant intraretinal RGC pathfinding errors, which resembled the phenotypes observed in Shh pathway mutants. Ectopic expression of Cxcl12a and additional transplantation experiments revealed that Cxcl12a was a direct chemoattractant for RGC axons. Additionally, the Shh and chemokine pathways interacted genetically to mediate RGC axon guidance inside the eye. Taken together, this study provided evidence that in zebrafish, Shh does not directly guide RGC axons toward the optic disc. Rather, Shh is required earlier in development to correctly pattern the eye and induce the expression of chemokines. In turn, Cxcl12a acts as an attractant for RGC axons inside the eye (Stacher Hörndli and Chien, 2012). This role in optic stalk patterning was consistent with a previous report that Shh signaling indirectly regulates axon pathfinding at the zebrafish midline by determining the expression of Slit guidance molecules, which in turn govern the positioning of midline glia (Barresi et al., 2005).

In contrast to Shh, where an effect of RGC axon targeting in the tectum has not yet been convincingly demonstrated, Wnts clearly influence topographic map formation in the visual system (**Figure 3**). In the chicken optic tectum, the morphogen Wnt3 is expressed in a medial^{high} to lateral^{low} gradient at the stage when RGC axons arrive (Schmitt et al., 2006). Wnt3 affects mediolateral maps due to a biphasic effect. At low concentrations, Wnt3 inhibits the growth of ventral RGC axons, whereas at high concentrations, Wnt3 inhibits the growth of both dorsal and ventral RGC axons. The Wnt receptor Ryk, which is expressed in the RGCs in a ventral^{high} to dorsal^{low} gradient, mediates RGC axon growth inhibition. However, Wnt3 not only inhibits the growth of axons, it also stimulates the growth of dorsal RGC axons to the lateral tectum at low concentrations. In this case, the growth-promoting effect of Wnt3 is mediated by Fz receptors (Schmitt et al., 2006). All in all, Wnt3 is a lateral mapping force that counterbalances the medial mapping force exerted by EphrinB1-EphB signaling (Hindges et al., 2002).

DESCENDING NERVE PATHWAYS

Axons of projection neurons from the brain travel in descending tracts within the white matter of the spinal cord. Descending axons arise from various locations in the brain and synapse primarily on spinal interneurons, thus enabling the regulation of posture, movement, pain and sensation. Both Shh and Wnt are implicated in the repulsive guidance of descending nerve tracts (**Figure 4**).

Serotonergic axons from the caudal raphe nuclei (CRN) descend ipsilaterally and project to the caudal-most regions of the spinal cord, forming the raphe-spinal tract (RST). Along their trajectory, these serotonergic axons are specifically restricted to the ventral and ventrolateral funiculus. In contrast to what was observed in the posterior spinal cord in chick (Bourikas et al., 2005) and rat (Yam et al., 2012), Song et al. (2012) reported a decreasing anterior^{high} to posterior^{low} gradient of Shh in the mouse spinal cord at the cervical and thoracic levels. The authors hypothesized that floorplate-derived Shh diffusing away from its source would form gradients not only in the dorsoventral (D-V) and anteroposterior (A-P) axes, but also in the mediolateral (M-L) axis during guidance of serotonergic RST axons. These gradients of repulsive Shh would not only direct axons posteriorly down the spinal cord, but would also restrict the RST axons to the ventral and ventrolateral funiculus (**Figure 4A**).

Using conventional *in vitro* assays, Song and colleagues reported that Shh-expressing cells strongly repelled the outgrowth of axons emanating from CRN explants (Song et al., 2012). Extending these results, the authors developed an A-P graded guidance assay, in which CRN explants were placed at different positions along the A-P axis of dissected ventral spinal cords, which were expected to differentially secrete diffusible factors according to their A-P expression patterns. In this assay, CRN axons were consistently repelled at all levels along the A-P axis, but the effects were stronger when the CRN explants were cultured in proximity to the anterior spinal cord. This finding was

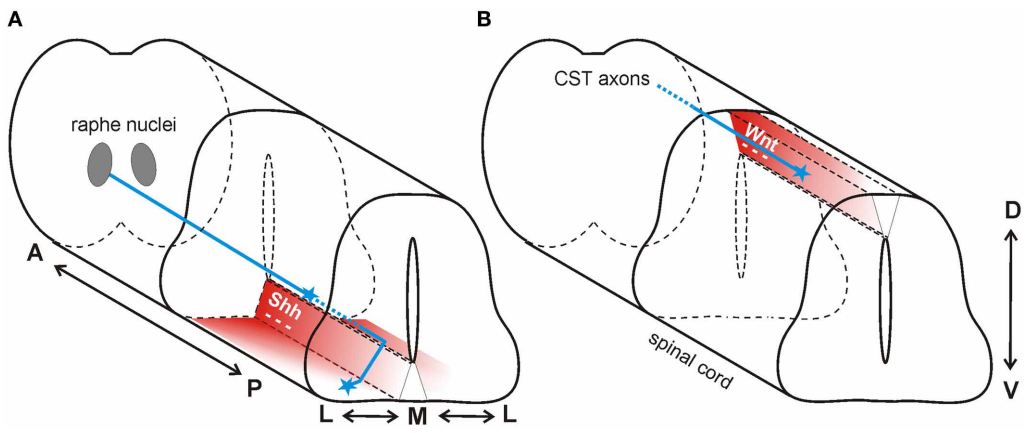

FIGURE 4 | Wnt and Shh are repellants for descending axon tracts of the brain. (A) In mouse, Shh repels axons of the raphe spinal tract (blue) multidirectionally. In the anterior spinal cord, Shh (red) is expressed in an anterior^{high} to posterior^{low} (A-P) gradient, and in a medial^{high} to lateral^{low} (M-L) gradient. Thus, RST axons are pushed to grow both posteriorly and laterally. **(B)** In mouse, a decreasing anterior^{high} to posterior^{low} Wnt gradient (red) in the roofplate of the dorsal spinal cord repels descending corticospinal tract axons (blue) posteriorly.

consistent with a diffusible axon guidance cue from the ventral spinal cord establishing a high-to-low repulsive gradient that would direct RST axons toward the caudal spinal cord. Shh was demonstrated to act in such a manner, since function-blocking antibodies restored the growth of CRN axons in these co-cultures. Thus, serotonergic RST axons, like dI1 commissural axons of the lumbar spinal cord (Bourikas et al., 2005), are guided along the longitudinal axis by a repulsive gradient of Shh.

Next, Song et al. (2012) asked whether a gradient of repulsive Shh in the mediolateral axis of the ventral spinal cord could act similarly, thereby restricting RST axons to the ventral and ventro-lateral funiculus. To test this idea, CRN explants were co-cultured next to neural tube slices taken from different medial to lateral locations. Again, axons displayed differential responsiveness in this *in vitro* assay, with medial neural tube slices suppressing outgrowth more strongly than lateral slices. This graded response was abolished by the addition of Shh pathway blockers.

In vivo experiments supported the findings of the *in vitro* assays. Song et al. (2012) examined RST axon pathfinding in embryos in which Shh signaling was perturbed by different means by analyzing *Shh* hypomorphic mice, mice with conditional inactivation of *Smo*, and mice that were electroporated *in utero* with a Shh-insensitive version of Ptc. In all case, neural tube patterning was unaffected, but the formation of the RST was abnormal. The phenotypes in the three conditions were not identical, however. While interference with Ptc and Smo led to stalling of the RST axons, a failure to innervate the posterior spinal cord and abnormal invasion of the medial neural tube, the *Shh* hypomorphs displayed a reduced number of axons extending into the spinal cord. Over-activation of the Shh pathway (by the expression of a constitutively active version of Smo) caused RST axons to loop back and deflect to the brainstem, suggesting that increased Shh-Smo repulsion pushed the descending axons away from the cervical spinal cord. Taken together, these findings showed that Shh guides serotonergic RST axons multidirectionally. Shh not only directs the posterior growth of these axons in the longitudinal axis, but is also required to position the descending tracts in their appropriate mediolateral position in the ventral spinal cord. In addition to identifying a new Shh-responsive population of axons, this study revealed a novel, efficient mechanism of axon guidance along several axes: simultaneous, multidirectional guidance by a single molecule.

In addition to the Shh gradient mentioned above for the guidance of serotonergic axons of the raphe nucleus, there is also an anterior^{high} to posterior^{low} expression gradient of *Wnt1* and *Wnt5a* at cervical and thoracic levels of the mouse spinal cord (Liu et al., 2005). In contrast to the attractive effect of Wnt4 in the ventral neural tube, Wnt1 and Wnt5a had a repulsive effect on descending corticospinal tract axons (**Figure 4B**). Axons extending from cortical explants derived from postnatal day 0 (P0) brains were repelled by Wnt1 and Wnt5a in a Ryk-dependent manner (Liu et al., 2005). *In vivo* perturbation of Ryk by injection of function-blocking antibodies into the spinal cord produced abnormal guidance of CST axons, consistent with its role as a mediator of a repulsive cue *in vitro* (Liu et al., 2005).

DOPAMINERGIC NEURONS OF THE MIDBRAIN AND BRAINSTEM

Midbrain dopaminergic neurons (mDNs) are a functionally diverse population, which is reflected by a structural heterogeneity in their axonal projections. In mammals, mDN axons project rostrally toward the forebrain, where they expand both dorsoventrally and mediolaterally. The axons from the medially located ventral tegmental area (VTA) project along a ventromedial course and primarily target more medial target tissues in the forebrain, while the more lateral axons originating from the substantia nigra (SN) project dorsolaterally and target lateral forebrain regions. Several classical guidance cues have been implicated in mDN pathfinding, including Netrins (Lin et al., 2005), Slits (Bagri et al., 2002), Ephrins (Sieber et al., 2004), and Semaphorins (Yamauchi et al., 2009). In addition, Shh was recently identified as a chemoattractant for mDN axons both *in vitro* and *in vivo* (Hammond et al., 2009). On the other hand, Wnts may either attract or repel mDN axons (Fenstermaker et al., 2010).

Shh is expressed in the ventral midline (floorplate) of the midbrain during the time that mDN axons traverse rostrally (E12.5-E15.5 in mouse). As in other regions along the neural tube, Shh forms a ventral to dorsal gradient within this area (Ericson et al., 1995). mDN axons express Ptc and Smo, but interestingly, they do not express other components of the canonical Shh pathway, such as Gli1, Gli2, or Gli3, during this time (Hammond et al., 2009). Explants containing mDN neurons projected axons toward a source of Shh, an effect that could be blocked by addition of the Smo antagonist, cyclopamine. These results suggested that Shh was an attractive cue for mDN axons. Consistent with this idea, Smo was also required for mDN axon guidance in the intact CNS. Hammond et al. (2009) took advantage of conditional mutant mice (*Nestin-Smo* ko mice) in which Smo is specifically and completely inactivated in the central nervous system by E11.5. In these embryos, the patterning and specification of the embryonic midbrain (including mDNs) and their forebrain targets are unaltered, since canonical Shh signaling is no longer required for these processes after E10 (Hynes et al., 1995; Blaess et al., 2006). Thus, the requirement for Shh signaling during the guidance of mDN axons could be assessed independently of its earlier roles in tissue patterning. In the conditional Smo knockout mouse, the lateral mDN projected axons normally toward their rostral targets, whereas projections from the medial mDN were misdirected. Within this abnormal population, the ventral-most fibers were more severely affected, suggesting a role for Shh signaling specifically in the ventral targeting of medial dopaminergic axons. In support of this, co-culture assays showed that medial, but not lateral, mDN axons were attracted to a Shh source. An open question is how this difference in sensitivity to Shh is achieved at the molecular level, since both populations of mDNs express Smo at similar levels. Therefore, another unidentified modulator of Shh signaling must be differentially expressed in these cell populations.

In the midbrain, Wnt5a is expressed in an anterior^{low} to posterior^{high} gradient whereas Wnt7b is expressed in an anterior^{high} to posterior^{low} gradient. *In vivo*, *Wnt5a*$^{-/-}$ mice exhibit only minor and transient posterior projections of mDN axons. *In vitro*, axons of cultured mDN neurons are repelled by exogenous Wnt5a, whereas these axons are attracted by

exogenous Wnt7b (Fenstermaker et al., 2010). These effects of Wnt ligands on mDN axons were abolished when open-book explants from *Frizzled3*$^{-/-}$ mice were co-cultured *in vitro* with a Wnt source (Fenstermaker et al., 2010). Therefore, Wnt5a repels mDN axons and Wnt7b attracts them, in a Fz3-dependent fashion (see also Section Molecular mechanisms of Wnt-mediated axon guidance). The mild and transient effects observed in the *Wnt5a*$^{-/-}$ mice might therefore be explained by the combinatorial, collaborative effects of Wnt5a and Wnt7b during normal development. The remaining attractive Wnt7b activity may be sufficient to overcome loss of Wnt5a *in vivo*.

MOLECULAR MECHANISMS OF AXON GUIDANCE MEDIATED BY Shh

CHEMOATTRACTION

An intracellular signaling cascade that mediates the chemoattractive guidance response to Shh has been described in rodent commissural neurons (Yam et al., 2009). Previous reports indicated that commissural axons express Smo (Charron et al., 2003; Yam et al., 2009) and Boc (Okada et al., 2006), and that these proteins are required for the attraction of axons toward an increasing gradient of Shh derived from the floorplate (see Section dI1 commissural axons of the spinal cord). The possible contribution of Ptc to the chemoattractive effect of Shh is yet to be studied.

Shh signaling could occur via at least two pathways: (1) Boc/Smo could elicit a canonical Shh signal in the nucleus via Gli-dependent transcriptional changes, or (2) Shh could act locally at the growth cone through an alternative, transcription-independent pathway. In order to distinguish between these possibilities, Yam et al. (2009) used an *in vitro* axon guidance assay for commissural neurons. In a so-called Dunn chamber, dissociated commissural neurons growing on coverslips were exposed to stable gradients of axon guidance molecules. Axon responses to these gradients could be assayed within 1–2 h, thus enabling short-term pathways (i.e., those likely to be transcription-independent) to be identified and analyzed.

Dissociated commissural axons responded very swiftly to a gradient of Shh in this assay. The addition of transcriptional inhibitors had no effect, supporting the idea that the chemoattractive turning response to Shh occurred independently of transcription. Accordingly, the expression of Gli3R, a dominant repressor of Gli-mediated transcription (Persson et al., 2002), did not affect the ability of commissural axons in explants to turn toward a source of Shh.

Further experiments revealed that Shh attracts commissural axons by activating Src family kinases (SFKs) in the growth cone, in a Smo- and Boc-dependent manner (Yam et al., 2009). In the presence of a Shh gradient, the SFKs Src and Fyn were rapidly phosphorylated and asymmetrically distributed in the growth cone of commissural neurons. This local, polarized response elicited growth cone turning, since SFKs can modulate cytoskeletal rearrangement and filopodial dynamics (Suter and Forscher, 2001; Robles et al., 2005; Liu et al., 2007). Thus, in attractive commissural axon guidance, Shh signals via a rapid, local, transcription-independent mechanism (**Figure 2A**).

It is currently unknown whether the same pathway is activated in RGC neurons and/or dopaminergic neurons to elicit chemoattraction toward Shh. However, in ipsilateral RGC axons, Boc has been described as the receptor responsible for the repulsion of axons from Shh at the chiasm (Fabre et al., 2010; see Section Retinal ganglion cell axons and topographic mapping), rather than as an attractive guidance receptor. The expression and role of Boc in dopaminergic neurons has not been investigated to date.

CHEMOREPULSION

In commissural axon guidance, the axonal response to floorplate-derived Shh switches from attraction in the dorsoventral axis to repulsion in the longitudinal axis, within just a few hours. In the chick, this switch in responsiveness is due to a change in the expression of Shh receptors on the axons. Pre-crossing commissural axons which express Boc and Smo are attracted to Shh via a non-canonical SFK-mediated pathway (Okada et al., 2006; Yam et al., 2009). Post-crossing axons express Hhip and are repelled by Shh (Bourikas et al., 2005). However, the intracellular signaling mechanisms transduced by Hhip are currently unknown. Hhip is a type-I transmembrane protein (Chuang and McMahon, 1999), but its short cytoplasmic tail seems unlikely to directly induce intracellular signals which influence growth cone turning. Rather, Hhip could be involved as a Shh-binding unit in a co-receptor complex with an unidentified signaling receptor (**Figure 2B**).

In mouse, a recent report suggests that the switch in responsiveness to Shh that occurs at the midline is due to a cell-intrinsic timer mechanism, mediated by 14-3-3 proteins (Yam et al., 2012). Using again their Dunn chamber assay, Yam et al. (2012) confirmed the direct repulsive effect of Shh on post-crossing commissural axon turning *in vitro*, as demonstrated earlier (Bourikas et al., 2005). Interestingly, the response of the axons to a Shh gradient changed according to the number of days they had been in culture. While commissural axons at 2 days *in vitro* (DIV) were attracted up a Shh gradient (Yam et al., 2009, 2012), neurons at 3-4 DIV switched their direction of growth, turning away from higher concentrations of Shh. Based on these findings, the authors suggested that the response of commissural neurons to Shh might change over time, via a cell-intrinsic mechanism even in the absence of floorplate contact.

Previous work had implicated 14-3-3 adaptor proteins in repulsive neuronal responses, and they had been localized to growth cones (Kent et al., 2010). Both 14-3-3β and 14-3-3γ were enriched in post-crossing commissural axons, consistent with their ability to mediate anteroposterior guidance responses (Yam et al., 2012). Furthermore, these proteins increased in expression over time *in vitro*, consistent with their potential involvement in the time-dependent change in Shh responsiveness. To test this hypothesis, 14-3-3 proteins were antagonized *in vitro* with pharmacological inhibitors or by electroporation of a plasmid encoding a shRNA construct, and the response of 3DIV commissural axons to a Shh gradient was assayed. A reduction in 14-3-3 activity shifted the repulsive response of 3DIV commissural neurons from repulsion to attraction. *In vivo*, inhibition of 14-3-3 prevented the correct anterior turn in a subset (<35%) of post-crossing commissural axons, an effect that was dependent on PKA activity. Results from the converse gain-of-function experiments were consistent with the idea that 14-3-3 proteins can regulate a

cell-intrinsic, temporal change in turning responses to Shh (Yam et al., 2012). However, there are several outstanding questions arising from this study. For example, it remains to be shown how a gradual upregulation of 14-3-3 proteins can be causally linked to an abrupt switch in responsiveness of the growth cone in the floorplate. It is also unknown how a 14-3-3-dependent change in PKA activity regulates the growth cone responsiveness to Shh, since the levels of cAMP were unchanged in 4DIV growth cones compared to 2DIV growth cones (Yam et al., 2012). Additionally, the growth cone receptor responsible for detecting the longitudinal gradient of Shh in post-crossing axons remains to be identified, although Smo is apparently involved. Finally, although the authors claim that the commissural neurons used in the Dunn chamber assays are floorplate naïve, they were isolated from E13 rats. At this age (equivalent to E11.5 in mouse or HH23-24 in chick), many commissural axons have clearly reached the ventral midline and some have even crossed the floorplate and turned into the longitudinal axis (Bovolenta and Dodd, 1990; Ruiz de Almodovar et al., 2011). Therefore, it is possible that many of the isolated neurons used in these experiments had already been under the influence of a floorplate-derived cue.

Another pathway mediating the negative guidance effects of Shh has recently been described in chick RGC axons (Guo et al., 2012). Here, the authors investigated the involvement of protein kinase C (PKC) family members in Shh-mediated repulsive axon guidance (**Figure 5A**). The PKC family of serine/threonine kinases is comprised of several members that are sub-categorized as conventional (α, βI, βII, γ), novel (δ, ε, η, θ), or atypical (ζ, λ), based on their second-messenger requirements (Steinberg, 2008). Conventional PKCs require diacylglycerols (DAG) and Ca^{2+} for their activation, while novel PKCs require DAG only and atypical PKCs require neither. PKCs were previously implicated in canonical Shh signaling (Riobo et al., 2006) and in repulsive axon guidance (Kolpak et al., 2009), but the specific activities of different family members and their substrates was not well understood. Using pharmacological inhibitors in RGC cultures, Guo et al. (2012) showed that PKCα played a specific role in RGC growth cone collapse in response to Shh. Additionally, Shh rapidly activated PKCα, and led to a Smo-dependent increase in Ca^{2+} in the growth cone.

Next, the authors sought a downstream target of PKCα that might mediate the repulsive guidance effects of Shh. One predicted cytoplasmic target was integrin-linked kinase (ILK), an important scaffolding protein that can link cell adhesion and growth factor signaling to the actin cytoskeleton (Hannigan et al., 2005) and had previously been implicated in neurite outgrowth (Ishii et al., 2001) and neuronal polarity determination (Guo et al., 2007). Indeed, Shh stimulation significantly increased the levels of phosphorylated ILK in RGC axons, an effect that was inhibited by pharmacological blockade of PKCα. Disruption of PKCα or ILK by the expression of mutant constructs or pharmacological inhibitors also caused a significant reduction in both Shh-induced macropinocytosis (a clathrin-independent endocytosis pathway mediating growth cone collapse; Kolpak et al., 2009) and repulsive axon turning (Guo et al., 2012). Taken together, the results indicated that that PKCα and ILK were required for the negative axon guidance effects of Shh.

Finally, Guo et al. (2012) assessed the roles of PKCα and ILK in Shh-mediated RGC axon guidance *in vivo*. The optic vesicles of chicken embryos were injected with RCAS viruses expressing GFP alone, or GFP fused with dominant-negative PKCα, or ILK-DM (a double-mutant ILK construct that could not be phosphorylated by PKCα). Indeed, the expression of DN-PKCα or ILK-DM resulted in misprojection of RGC axons into the ipsilateral optic tract and contralateral optic nerve at the optic chiasm, consistent with a disruption in Shh-mediated signaling at the chiasm. Interestingly, neither DN-PKCα nor ILK-DM affected the projection of RGC axons within the retina toward the optic disc (see Section Retinal ganglion cell axons and topographic mapping). This finding was consistent with PKCα and ILK specifically mediating the negative guidance effects of high concentrations of Shh on RGC axons. ILK-DM was less effective than DN-PKCα in inhibiting Shh-induced repulsive effects. This observation suggests that ILK is not the sole effector of PKCα signaling. The identification of other cytoplasmic targets is open to future research.

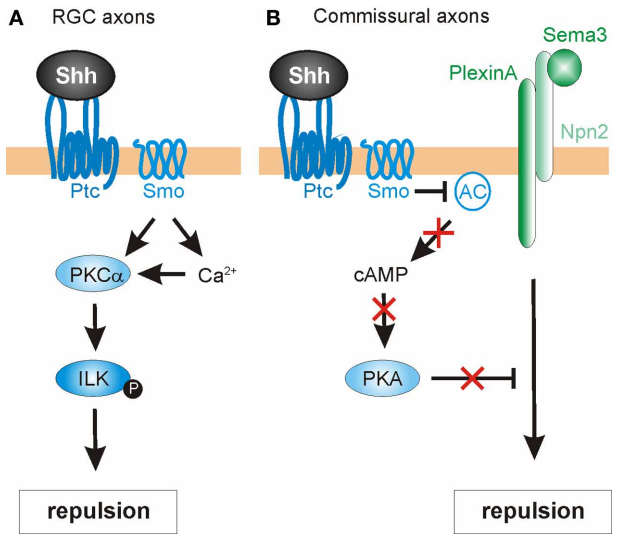

FIGURE 5 | Intracellular mechanisms mediating Shh-induced repulsive axon guidance. (A) In RGC axons, Shh binding to Ptc promotes the Smo-dependent activation of protein kinase Cα (PKCα), which in turn phosphorylates integrin-linked kinase (ILK). ILK promotes repulsive axon turning. **(B)** In commissural neurons, Shh acts through Ptc and Smo to block adenylyl cyclase (AC) activity. This lowers cAMP levels and inhibits protein kinase A (PKA) activity. In turn, this confers growth cone sensitivity to class 3 semaphorins (Sema3), by allowing repulsive signaling downstream of a PlexinA-Npn2 complex.

INDIRECT REGULATION OF AXON GUIDANCE BY MODULATION OF CYCLIC NUCLEOTIDES

In addition to acting directly as an axon guidance cue, Shh may also influence growth cone responsiveness via local intracellular mechanisms. One cytoplasmic, non-canonical target of Shh signaling is the cyclic nucleotides, which can modulate signaling responses to axon guidance cues (Song et al., 1997). The ratio of cAMP/cGMP determines attractive or repulsive axonal

responses (Song et al., 1998), with attraction being favored by $cAMP^{high}/cGMP^{low}$, and $cAMP^{low}/cGMP^{high}$ favoring repulsion. Since Shh can reduce the activity of protein kinase A (PKA), it can therefore influence cyclic nucleotide levels, thus modulating the responsiveness of axons to guidance cues.

This hypothesis is supported from several studies. For example, chick RGC axons not only display a lack of outgrowth following exposure to Shh, but also exhibit a marked reduction in cytoplasmic cAMP (Trousse et al., 2001). More directly, a recent study indicates that Shh modulates cAMP levels in commissural axons as they reach the floorplate (Parra and Zou, 2010) (**Figure 5B**). This acts as a molecular "switch," allowing commissural axons to acquire responsiveness to midline repellants and escape to the contralateral side. The authors found that disruption of Shh signaling (via shRNA-mediated knock down of Smo, function-blocking antibodies or the expression of a dominant-negative form of Ptc) resulted in severe guidance defects in commissural axons. The abnormalities were assessed in open-book preparations, revealing phenotypes that included stalling/knotting in the floorplate, randomized turning, and overshooting of post-crossing axons, and recrossing of the midline. These abnormalities resembled those found in embryos deficient for Neuropilin-2 (Npn2), a receptor component for Sema3B and Sema3F, two secreted class-3 Semaphorins (Zou et al., 2000). Sema3B/3F provide important chemorepulsive signals that, together with the Slits, expel commissural axons out of the midline. Commissural axons only respond to these repellants after reaching the floorplate, suggesting that a floorplate-derived signal, such as Shh, might mediate a switch in guidance responses. Indeed, Parra and Zou (2010) found that pre-crossing commissural axons were only repelled by Sema3B/3F *in vitro* after exposure to Shh. Cyclic nucleotides were shown to be important for this phenomenon. The authors first examined whether cAMP/PKA activity was needed for proper midline axon pathfinding. Indeed, increasing the level of cAMP in the explant cultures by application of the adenylyl cyclase activator forskolin caused similar defects on commissural axons as seen with disrupted Shh signaling. Additionally, forskolin attenuated the Shh-induced repulsive response to Sema3B/3F by pre-crossing commissural axons. Together, these findings suggest that Shh regulates cAMP levels, which in turn modulates the sensitivity of growth cones to Semaphorins (**Figure 5B**). In agreement with this model, PKA activity has previously been shown to be coupled with Semaphorin-PlexinA-mediated repulsion *in vivo* (Terman and Kolodkin, 2004). However, the precise mechanism by which the Shh-mediated cAMP level activates Sema3 signaling in vertebrates is unknown. Taken together, these findings identify Shh not only as a direct chemotropic cue, but also as a midline-derived switch that activates growth cone responses to other axon guidance molecules.

CANONICAL Shh SIGNALING IN AXON GUIDANCE?

The mechanisms described above delineate several non-canonical mechanisms by which Shh guides axons or modulates growth cone responsiveness. However, Shh's transcriptional activity might also contribute to correct axon pathfinding, by regulating the levels and types of guidance receptors and/or modulators

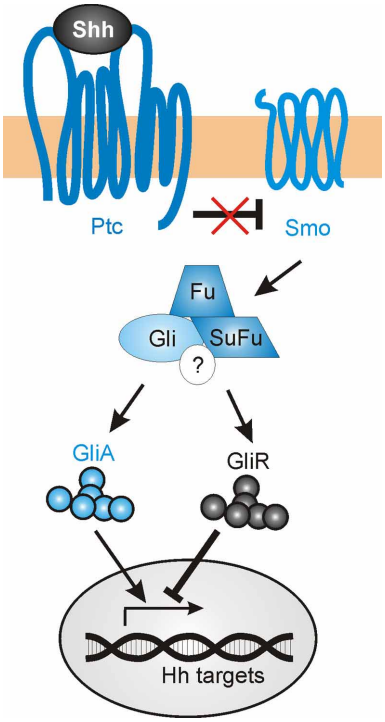

FIGURE 6 | The canonical Shh signaling pathway. In the absence of Shh, Gli transcription factors are proteolytically processed to repressor forms (GliR), which block the transcription of target genes. Shh binding to the 12-pass transmembrane receptor Patched (Ptc) relieves the inhibition of Smoothened (Smo), which in turn promotes the accumulation of the activator forms of Gli (GliA) while suppressing GliR production. The regulation of Gli activity occurs via an intracellular complex composed of Fused (Fu), Suppressor of Fused (SuFu) and possibly other components (?).

of signaling that are expressed (**Figure 6**). The transcriptional activity of Shh has been shown to indirectly regulate axon guidance both in the spinal cord (Domanitskaya et al., 2010; see Section dI1 commissural axons of the spinal cord) and in the retina (Stacher Hörndli and Chien, 2012; see Section Retinal ganglion cell axons and topographic mapping) via the cell-non-autonomous induction of cues (Sfrps and chemokines) that indirectly or directly mediate growth cone behavior. However, the role of Shh canonical signaling in the navigating neurons themselves has not been extensively studied to date, especially in an *in vivo* context in which multiple guidance cues are presented simultaneously.

In a speculative view, navigating axons that approach a Shh source could activate both a non-canonical guidance response, as well as initiating transcriptional activity in the nucleus. As the axons encounter increasing levels of Shh expression, there is a progressive upregulation of Shh-induced genes. Some of these directly affect the signaling response to Shh, including Ptc and Hhip, while others could activate other signaling pathways that mediate axon guidance (such as EphB4, EphrinB2, PlexinA2, or Adamts1) (Chuang and McMahon, 1999; Oliver et al., 2003; Yu et al., 2008). In sum, Shh-induced transcriptional activity could alter the growth cone sensitivity to numerous guidance factors as

axons navigate toward their targets, allowing the axons to respond dynamically to changing environments.

MOLECULAR MECHANISMS OF Wnt-MEDIATED AXON GUIDANCE

Wnt ligands are able to activate a variety of receptors on the cell surface (van Amerongen et al., 2008). Initially, Wnt signaling was subdivided into three pathways, the canonical (also known as β-catenin-dependent) pathway, the PCP pathway, and the Wnt/Ca^{2+} pathway (**Figure 7**). Considering the many Wnt functions during post-morphogenesis Wnt signaling has become a complex network of interactors (van Amerongen and Nusse, 2009; Clark et al., 2012; Nusse, 2012; Salinas, 2012). Upon binding of the Wnt ligands to Fz, the intracellular protein Dishevelled (Dvl) is activated and can initiate all three signaling cascades: the canonical (β-catenin-dependent) pathway, the calcium (Ca^{2+}) pathway and the PCP pathway. In the canonical Wnt pathway, stimulation of Dvl leads to inactivation of GSK3β, preventing it from phosphorylating β-catenin. Unphosphorylated β-catenin is no longer degraded by the proteasome and is able to enter the nucleus, where it interacts with the Tcf/Lef transcription factors to activate the transcription of target genes (Macdonald et al., 2007; Nusse, 2012). In the Wnt/Ca^{2+} pathway, Wnt ligands induce an increase in intracellular calcium leading to the activation of PKC and calcium/calmodulin-dependent protein kinase II (CaMKII) (Kohn and Moon, 2005; Semenov et al., 2007). The PCP pathway mediates tissue polarity and is less understood than canonical Wnt signaling in terms of biochemical interactions. It involves the participation of transmembrane proteins such as Flamingo (a.k.a. Celsr), Van Gogh-like proteins (Vangl, a.k.a Strabismus) and Protein tyrosine kinase 7 (PTK7), as well as intracellular proteins such as Prickle, Diego, and Daam. The activation of the PCP pathway leads to stimulation of Rho and Rac GTPases and the consequent rearrangement of the cytoskeleton (Semenov et al., 2007; Wang and Nathans, 2007; Simons and Mlodzik, 2008).

Even though several Wnt ligands and their axon guidance receptors have been identified, little is known about the intracellular cascades that lead the growth cones to respond to a certain cue. Despite the participation of canonical Wnt signaling in *C. elegans* anteroposterior axon guidance (Maro et al., 2009), there is so far no evidence for the involvement of this pathway in vertebrate axon guidance. In fact, mice mutant for Lrp6 (an indispensable co-receptor in the canonical pathway) do not exhibit major pathfinding defects (Lyuksyutova et al., 2003), suggesting that the axon guidance functions of Wnt are not

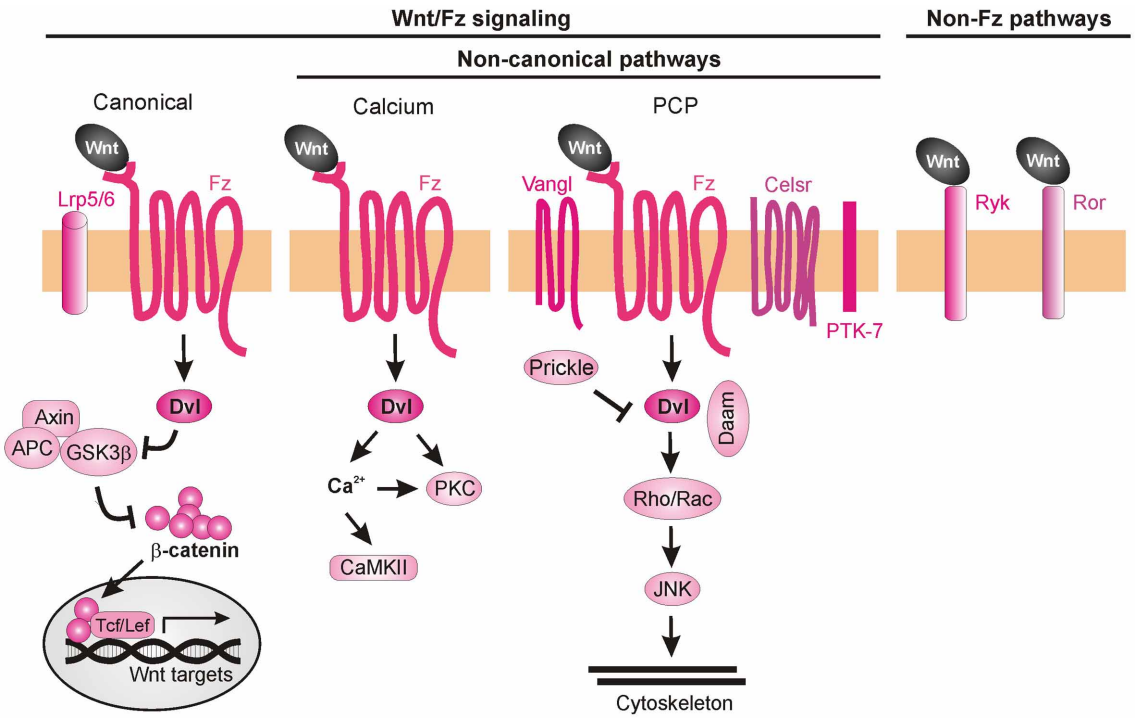

FIGURE 7 | Wnt signaling pathways. Wnt ligands can transduce their signal through at least three Frizzled-dependent (Fz) pathways: the canonical, the calcium, and the PCP pathways, all of which involve the activation of Dishevelled (Dvl). The canonical pathway requires the co-receptors Lrp5/6 to recruit Dvl and inhibit the "destruction complex" composed of Adenomatous polyposis coli (APC), Axin, and Glycogen synthase 3β (GSK3β). Wnt binding leads to the phosphorylation of GSK3β in this complex and, as a consequence, to the accumulation of unphosphorylated β-catenin, which can enter the nucleus and together with the transcription factors Tcf/Lef induce the expression of target genes. Activation of the calcium pathway results in an increase of cytosolic calcium (Ca^{2+}) and the subsequent activation of calcium-dependent kinases (CaMKII). In the PCP pathway, activation of the transmembrane proteins Vangl, Celsr, and PTK7 and the recruitment of the intracellular proteins Prickle and Daam lead to activation of Rho GTPases and JNK, promoting cytoskeleton remodeling. More recently, Fz-independent Wnt signaling has been described. Wnt ligands can bind directly to receptors such as Ryk or Ror. The intracellular signaling cascades activated in these cases are poorly understood.

mediated by canonical signaling events. The non-canonical Wnt signaling pathways that have been implicated in axon guidance are described below.

PKC-PI3K SIGNALING IN COMMISSURAL AXON GUIDANCE

Searching for a role of PKC in post-crossing commissural axon guidance, Wolf et al. (2008) found that a calcium-independent PKC pathway is involved. The general pharmacological inhibition of all PKCs resulted in abnormal turning of post-crossing commissural axons in open-book explants, whereas inhibition of only conventional PKCs did not affect A-P turning (Wolf et al., 2008). Taken together, these data suggest that non-conventional PKC signaling (i.e., calcium-independent) is involved in axon turning. Pan-inhibition of PKCs also perturbed Wnt4-induced axon outgrowth. Treatment of the explants with a specific inhibitor of the atypical PKCζ showed similar guidance defects. The pharmacological studies were confirmed with the expression of a dominant-negative form of PKCζ in rat spinal cords, where a significant increase of post-crossing commissural axons that turned caudally was seen (Wolf et al., 2008). Since PKCζ can be activated by phosphatidylinositol-3-kinase (PI3K) signaling, the authors pharmacologically blocked this pathway using the PI3K inhibitor wortmannin, and found randomization of turning by post-crossing commissural axons. Furthermore, inhibition of PI3Ks by expression of a kinase-defective form of PI3Kγ in the spinal cord caused aberrant phenotypes of post-crossing commissural axons. Interestingly, axons that expressed the wild-type form of the PI3Kγ kinase domain (p110-WTγ) were observed to turn into the longitudinal axis before crossing the floorplate. In addition, overexpression of p110-WTγ in pre-crossing commissural neurons, which are normally not responsive to Wnt4, became sensitive to Wnt4 attraction (Wolf et al., 2008). The effect of p110-WTγ in explants and cultured cells suggests that PI3Kγ acts as a regulator that turns on the responsiveness of commissural axons to Wnt4 after midline crossing (**Figure 8A**).

Ryk-DEPENDENT WNT/CA^{2+} SIGNALING

Besides their role as attractive cues in post-crossing commissural axon guidance, Wnt ligands have also been shown to act as repulsive cues for post-crossing cortical axons that cross the midline in the corpus callosum (Keeble et al., 2006). In *Ryk*$^{-/-}$ mutant mice, callosal axons are able to cross the midline but then fail to project away from the midline area, and thus, form aberrant axonal trajectories on the contralateral side. Keeble et al. (2006) found that *Wnt5a* is expressed around the corpus callosum at the time when cortical axons cross the midline. *In vitro* studies demonstrated that axons from cortical explants acquired sensitivity to Wnt5a at E18, concurrent with midline crossing. Responsiveness to Wnt5a was mediated by Ryk. However, *Ryk* mRNA levels were highest before midline crossing, thus it remains to be shown how Ryk-mediated repulsive signals are restricted to post-crossing callosal axons.

Further studies have shed more light on the roles of Ryk in mediating the responses of cortical axons to Wnt5a. Li et al. (2009) found that the graded application of Wnt5a to hamster cortical explants not only repelled cortical axons but also

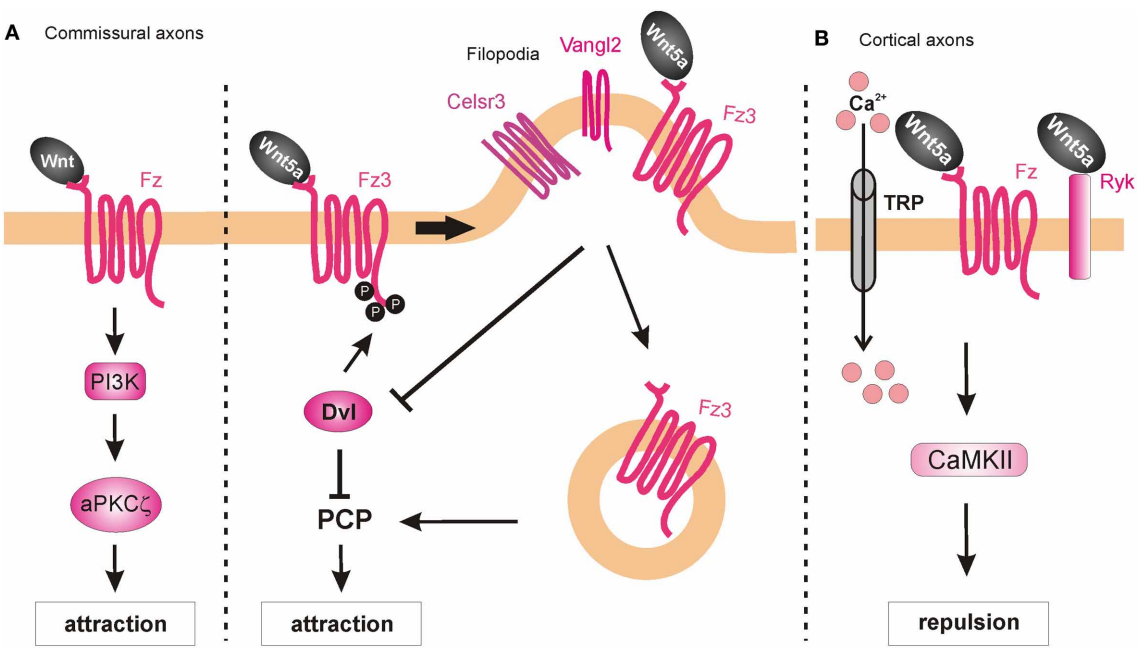

FIGURE 8 | Intracellular mechanisms underlying Wnt-induced axon guidance. (A) Wnts promote commissural axon attraction via the activation of the PI3K-atypical PKCζ signaling and the PCP pathway. The PCP pathway is inhibited through the Dvl-dependent phosphorylation of Fz3, which as a result remains in the plasma membrane. The local expression of Vangl2 in the filopodia of the growth cone prevents the Dvl-induced phosphorylation of Fz3 upon binding of Wnt5a and results in the internalization of Fzd3 and the activation of the PCP pathway. **(B)** Wnt5a repels cortical axons by binding to both Fz and Ryk receptors, which allow the entrance of calcium via the TRP channels. Activation of Ryk alone results in axon growth promotion, whereas co-activation of Ryk and Fz is required for axon repulsion.

increased their rates of outgrowth. Thus, it appeared that Wnt5a could simultaneously activate two different processes in cortical axons: repulsion and outgrowth. These distinct responses were mediated by two different receptor compositions. While axonal outgrowth was carried out via Ryk alone, repulsion required the activities of both Ryk and Fz receptors. Intriguingly, the authors found that both the outgrowth and repulsive responses were dependent on calcium signaling, as cortical axons no longer showed any response to a Wnt5a gradient after chelation of cytoplasmic calcium. However, the specific pharmacological inhibition of IP3 receptors prevented outgrowth but not repulsive turning of cortical neurons in response to Wnt5a, while the blockade of TRP channels inhibited both Wnt5a-induced outgrowth and repulsion (Li et al., 2009). Thus, Wnt5a-induced axon outgrowth requires cytosolic calcium from both intracellular and extracellular sources, while Wnt5a-induced repulsion requires calcium only from extracellular sources. Next, Li et al. (2009) performed pharmacological inhibition of other calcium signaling components that were known for their role in axonal outgrowth. Inhibition of phospholipase C (PLC), which is upstream of IP3, or CaMKII, which is downstream of calcium signaling, prevented Wnt5a-induced outgrowth, thus confirming the role of Wnt/calcium pathway in axon outgrowth. The activity of Wnt/calcium pathway in the growth and guidance of dissociated cortical axons was further confirmed in a cortical slice model of the developing corpus callosum (Hutchins et al., 2011) (**Figure 8B**). Importantly, these studies showed that Fz receptors are able to mediate repulsion in vertebrate axon guidance, consistent with previous reports in *C. elegans* (Pan et al., 2006). Furthermore, the results showed that Ryk can promote axonal outgrowth, in addition to repulsion, in response to Wnt5a. However, it is still unknown how the growth cone can distinguish between the different calcium sources to mediate the distinct outputs.

Wnt/PCP PATHWAY IN AXON GUIDANCE

Recently, it was shown that Ryk can also regulate the PCP pathway by interacting with Vangl2 during vertebrate development (Andre et al., 2012; Macheda et al., 2012). Interestingly, Wnt5a enhances the biochemical interaction between Ryk and Vangl2, and regulates the stability of Vangl2 (Andre et al., 2012). Based on these findings, it would be interesting to determine whether Ryk signals through the PCP pathway to fulfill any of its roles in axon guidance. However, to date, there is no evidence supporting such a mechanism.

On the other hand, a role for the core PCP component Vangl2 in axon guidance has been demonstrated. Vangl2, Fz3, and Celsr3 are expressed in serotonergic and dopaminergic neurons in the midbrain at the time when these neurons extend their axons through the brainstem (Fenstermaker et al., 2010). The PCP pathway is involved in the guidance of serotonergic axons, since mice deficient in Frizzled3, Vangl2 (Loop-tail, Lp), and Celsr3 all show abnormal projections of both ascending and descending serotonergic axons. These mice also show posterior misprojections of dopaminergic axons, which normally project anteriorly. Wnt5a is expressed in local gradients in the brainstem: in the hindbrain, it is expressed in an anterior^{high} to posterior^{low} fashion, but switches to an increasing posterior gradient at the level of the rhombomere 4; while in the midbrain, Wnt5a shows an anterior^{low} to posterior^{high} gradient that is changed at the isthmus. In contrast, Wnt7b is expressed in an anterior^{high} to posterior^{low} gradient in the midbrain. Using open-book explants of the hindbrain from Fz3 mutant mice, Fenstermaker et al. (2010) found that serotonergic ascending axons were misprojected in response to Wnt5a, whereas dopaminergic axons showed a decreased repulsive response to Wnt5a and a decreased attractive response to Wnt7b (Fenstermaker et al., 2010). Therefore, Wnt5a-stimulated attraction of serotonergic neurons and repulsion of dopaminergic neurons, as well as Wnt7b-stimulated attraction of dopaminergic

Table 1 | Activities of Shh and Wnts in vertebrate axon guidance.

Ligand(s)	Receptor(s)	Model	Animal	Activity	References
Shh	Boc/Smo	Pre-crossing commissural axons	Rodents	Attraction	Charron et al., 2003; Okada et al., 2006
Shh	Smo + ?	mDN axons	Mouse	Attraction	Hammond et al., 2009
Shh (low)	Ptc/Smo	RGC axons	Chick	Outgrowth	Kolpak et al., 2005; Fantetti and Fekete, 2012
		Statoacoustic ganglion axons			
Shh	Hhip + ?	Post-crossing commissural axons	Chick	Repulsion	Bourikas et al., 2005
Shh	Boc	Ipsilateral RGC axons	Rodents	Repulsion	Fabre et al., 2010
Shh (high)	Ptc/Smo	RGC axons	Chick, *Xenopus*	Repulsion	Kolpak et al., 2005; Gordon et al., 2010; Fantetti and Fekete, 2012
		Statoacoustic ganglion axons	Chick		
Shh	Ptc/Smo	Descending RST axons	Mouse	Repulsion	Song et al., 2012
Wnt4	Fz3	Post-crossing commissural axons	Rodents	Attraction	Lyuksyutova et al., 2003; Domanitskaya et al., 2010
Wnt5a/7a	?		Chick		
Wnt1, Wnt5a	Ryk	CST axons	Mouse	Repulsion	Liu et al., 2005
Wnt3	Ryk	RGC axons	Chicken	Repulsion	Schmitt et al., 2006
	Fz			Attraction	
Wnt5a	Ryk	Cortical axons	Mouse	Repulsion	Keeble et al., 2006
Wnt5a	Ryk/Fz	Cortical axons	Hamster	Repulsion	Li et al., 2009; Hutchins et al., 2011
Wnt5a	Ryk	Cortical axons	Hamster	Outgrowth	Li et al., 2009; Hutchins et al., 2011
Wnt5a	Fz3	mDN axons	Rodents	Repulsion	Blakely et al., 2011

Table 2 | Intracellular signaling pathways involved in axon guidance by Shh and Wnt.

Model	Ligand-receptor	Pathway components	References
Rodent pre-crossing commissural axons	Shh/Boc/Smo	SFKs	Yam et al., 2009
Rodent post-crossing commissural axons	Shh/Cell intrinsic	14-3-3 (β, γ), via PKA	Yam et al., 2012
Chick RGC axons	Shh/Smo	PKCα, ILK	Guo et al., 2012
Chick RGC axons	Shh/Smo	Rho GTPase, nonmuscle myosin II	Kolpak et al., 2009
Mouse post-crossing commissural axons	Wnt4/Fz3	PI3K-aPKC	Wolf et al., 2008
Cortical axons	Wnt5a/Fz/Ryk	Ca^{2+}, PLC, CaMKII	Li et al., 2009
5-HT and mDN neurons in mouse brainstem	Wnt5a/Wnt7b/Fz3	PCP (Fz3, Vangl2, Celsr3)	Fenstermaker et al., 2010
Mouse post-crossing commissural axons	Wnt5a/Fz3	PCP (Vangl2, Fz3, Celsr3, JNK)	Shafer et al., 2011
mDN neurons in the mouse brainstem	Wnt5a/Fz3	PCP (Fz3, Rac1)	Blakely et al., 2011

5-HT, 5-hydroxytryptamine (serotonergic); mDN, midbrain dopaminergic neuron; SFKs, Src family kinases; PKA, protein kinase A; PKC, protein kinase C; ILK, integrin-linked kinase; PI3K, phosphatidylinositol-3-kinase; PLC, phospholipase C; CaMKII, calcium/calmodulin-dependent protein kinase II; PCP, planar cell polarity pathway; JNK, c-Jun N-terminal kinase.

neurons is mediated by Fz3, which is known to act in the PCP pathway.

PCP signaling is also involved in A-P guidance of post-crossing commissural axons in the mouse. Celsr3, Fz3, and Vangl2 are all expressed in mouse commissural axons when they have to turn into the A-P axis (Torban et al., 2007; Shafer et al., 2011). Similarly, the phosphorylated version of JNK, which is a downstream component and represents a readout of PCP signaling activation (Boutros et al., 1998), is enriched in post-crossing commissural axons. Both the Lp mouse (which contains a destabilizing point mutation in the *Vangl2* gene) and the Celsr3 knockout mouse exhibit strong impairments of the directionality of post-crossing axons (Shafer et al., 2011), phenocopying the defects of the Fz3-deficient mouse (Lyuksyutova et al., 2003). Inhibition of JNK in open-book explant cultures also resulted in anteroposterior guidance defects. These results show that PCP signaling is responsible, at least in part, for the post-crossing commissural axon guidance *in vivo*. Shafer et al. (2011) also reported that in a heterologous system (HEK cells), co-expression of Dishevelled1 (Dvl1) with Fz3 reduced phospho-Jun levels after Wnt5a application compared to Fz3 overexpression alone, indicating that Dvl1 induced a feedback inhibition of the PCP pathway. This effect of Dvl1 was abolished by co-transfection of Vangl2, suggesting that Vangl2 antagonizes Dvl1's feedback inhibition. Moreover, in the same system Dvl1 induced phosphorylation of Fz3, which caused the accumulation of Fz3 in the plasma membrane and repression of the PCP pathway. This effect was antagonized by Vangl2 expression, which promoted the internalization of Fz3 and the consequent activation of the PCP pathway (Shafer et al., 2011). Since Vangl2 is highly enriched in the filopodial tips of commissural axon growth cones, the authors proposed that Wnt-stimulated growth cone turning might be mediated by the restricted antagonism of Fzd3 phosphorylation/internalization by Dvl1 and Vangl2 in filopodia (Shafer et al., 2011) (**Figure 8A**).

CONCLUDING REMARKS

Obviously, we are only at the beginning of understanding the signaling pathways downstream of Shh and Wnt in neural circuit formation. However, the central role of these morphogens in anteroposterior axon guidance has been clearly established in different species and in multiple neuronal populations (summarized in **Tables 1**, **2**). As occurs during tissue morphogenesis, the Wnt and Shh signaling pathways operate simultaneously during axon guidance to ensure the fidelity of axonal projections. Often, the signaling pathways appear to act in parallel, but still with opposite activities: one gradient to "push," the other gradient to "pull" axons in a particular direction. However, the pathways can also interact, as shown for the guidance of post-crossing commissural axons, where Shh acts directly as a repellent and indirectly by shaping an attractive Wnt activity gradient (**Figure 2D**; Domanitskaya et al., 2010).

Although recent studies have led to the identification of several novel, non-canonical signaling pathways by which these morphogens can rapidly elicit growth cone turning, a number of key issues remain unsolved. How are the early patterning gradients that are established during tissue morphogenesis maintained or modified during later stages of neural development? Do the transcriptional pathways act in navigating neurons to modulate axon guidance responses at intermediate targets? Both Wnt and Shh are bifunctional axon guidance molecules (attractive and repulsive), but many of the signaling components that elicit these distinct signaling outputs are unknown, especially for the Wnts. How are biphasic effects of Wnts and Shh possible, such that the same axon can respond differently in a concentration-dependent manner?

Future studies will reveal whether additional cross-talk between these pathways occurs at the level of cell surface receptors, signaling modulators or intracellular signaling components. Wnt and Shh signaling may also be influenced by post-translational modifications or the contributions of non-receptor binding molecules such as heparan sulfate proteoglycans. And finally, it is also unclear whether and how morphogens interact with more "classical" axon guidance cues, such as IgSF CAMs (immunoglobulin superfamily cell adhesion molecules), Semaphorins, Netrins, or Eph/Ephrins.

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LRRK2: an éminence grise of Wnt-mediated neurogenesis?

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The importance of leucine-rich repeat kinase 2 (LRRK2) to mature neurons is well-established, since mutations in *PARK8*, the gene encoding LRRK2, are the most common known cause of Parkinson's disease. Nonetheless, despite the LRRK2 knockout mouse having no overt neurodevelopmental defect, numerous lines of *in vitro* data point toward a central role for this protein in neurogenesis. Roles for LRRK2 have been described in many key processes, including neurite outgrowth and the regulation of microtubule dynamics. Moreover, LRRK2 has been implicated in cell cycle control, suggesting additional roles in neurogenesis that precede terminal differentiation. However, we contend that the suggested function of LRRK2 as a scaffolding protein at the heart of numerous Wnt signaling cascades provides the most tantalizing link to neurogenesis in the developing brain. Numerous lines of evidence show a critical requirement for multiple Wnt pathways in the development of certain brain regions, not least the dopaminergic neurons of the ventral mid-brain. In conclusion, these observations indicate a function of LRRK2 as a subtle yet critical mediator of the action of Wnt ligands on developing neurons. We suggest that LRRK2 loss- or gain-of-function are likely modifiers of developmental phenotypes seen in animal models of Wnt signaling deregulation, a hypothesis that can be tested by cross-breeding relevant genetically modified experimental strains.

Keywords: LRRK2, Wnt signaling, neurogenesis, Parkinson's disease, DVL, LRP6, GSK3

LEUCINE-RICH REPEAT KINASE 2

Leucine-rich repeat kinase 2 (LRRK2) is a protein that has been the subject of extensive research in recent years. This interest stems from the identification of LRRK2 as the product of the human *PARK8* gene, previously implicated as the cause of a familial form of Parkinson's disease in genetic linkage studies (Paisán-Ruíz et al., 2004; Zimprich et al., 2004). Furthering interest in LRRK2, *PARK8* has subsequently been associated with cancer, leprosy, and Crohn's disease (Hassin-Baer et al., 2009; Van Limbergen et al., 2009; Zhang et al., 2009). The importance of LRRK2 to these diseases will not be discussed further as the relevance to neuronal biology is limited. Nonetheless, the association of a single gene with four distinct medical conditions serves to highlight the complexity of LRRK2 function.

Parkinson's disease is a currently incurable late-onset neurodegenerative disorder with increasing public health implications in an aging population (Gasser, 2010). Therefore, uncovering the molecular events causing this condition with the ultimate aim of identifying therapeutic targets for disease modifying treatment has gained in importance. The *PARK* genes mutated in patients with familial Parkinson's disease represent an obvious starting point for this research. Although *PARK8* is just one of more than a dozen loci linked to Parkinson's disease, certain lines of evidence indicate that LRRK2 is of special relevance. Globally *PARK8* mutations are estimated to contribute to 1–5% of Parkinson's disease cases, which represents the greatest contribution from any known genetic or environmental cause (Kumari and Tan, 2009). In some populations, most notably North African Berbers, *PARK8* mutations are very common and account for as much as two-fifths of all Parkinson's disease cases (Lesage et al., 2005; Jasinska-Myga et al., 2010). Importantly, patients with *PARK8* mutations exhibit symptoms that are clinically indistinguishable from the more common idiopathic form of Parkinson's disease, while observed post-mortem brain pathologies are also largely identical (Zimprich et al., 2004). Thus, it seems likely that LRRK2 also plays a role in an as yet undetermined process that is deregulated very early in the pathogenesis of idiopathic Parkinson's disease (Kumari and Tan, 2009; Berwick and Harvey, 2011).

From a biochemical perspective, there are two immediate observations to be made about LRRK2. First, LRRK2 is a large (2527 amino acid) protein, containing multiple protein–protein interaction domains (**Figure 1**). Unsurprisingly, a vast number of interaction partners have been reported and LRRK2 has been suggested to function primarily as scaffolding protein (Berwick and Harvey, 2011; Lewis and Manzoni, 2012). Indeed, the breadth of reported interactors is so wide that LRRK2 probably functions in a number of distinct multi-protein complexes. Second, LRRK2 contains two separate enzymatic activities: serine–threonine phosphorylation (kinase activity) and guanine triphosphate hydrolysis (GTPase activity). Understandably, this has lead to the suggestion of alternative roles for LRRK2 as a "conventional" signaling protein, either functioning as a protein kinase or in an analogous manner to small GTPases such as Ras or Rac (Berwick and Harvey, 2011).

A detailed review of LRRK2 function is beyond the scope of this article, but what is most important to stress is that the function of LRRK2 remains unclear and in many cases is controversial. For example, 8 years of research have failed to find a reproducible kinase substrate other than LRRK2 itself, while there is still no agreement on whether the GTPase activity controls kinase activity

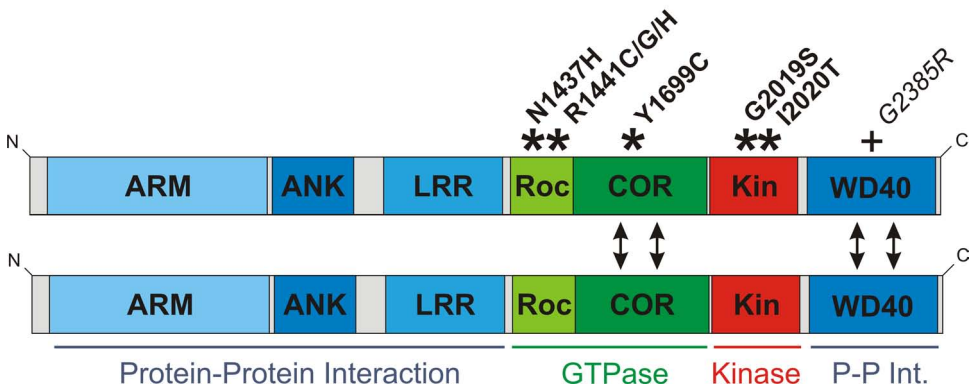

FIGURE 1 | Domain structure of LRRK2. Domains are color-coded according to function: those implicated in protein–protein interaction are depicted in blue; domains involved in GTPase function are green; and the kinase domain red. Here, LRRK2 is depicted as a dimer, although LRRK2 also exists as monomers and in higher molecular weight complexes (Greggio et al., 2008). Dimerization is likely to be mediated by the COR and/or WD40 domains (double-headed arrows). COR domains are established as dimerization devices in ROCO proteins (Gotthardt et al., 2008), whilst ablation of the WD40 domain has been reported to disrupt LRRK2 dimerization (Jorgensen et al., 2009). The location of pathological mutations proven to segregate with Parkinson's disease are shown with asterisks and bold font. Although only considered a risk factor, the G2385R mutation is also depicted with a plus sign, since this mutation is mentioned in the main text and is very frequent amongst Asian populations. ARM, armadillo repeat; ANK, ankyrin repeat; LRR, leucine-rich repeat; Roc, ras of complex proteins; COR, c-terminal of Roc; Kin, kinase.

or vice versa. It seems probable therefore that these functions are interdependent. In any case, the enzymatic activities of LRRK2 are certainly of some importance in the physiological and pathological function of this protein. To date all described *PARK8* mutations clearly segregating with Parkinson's disease cause changes in the GTPase or kinase domains, but not in any of the protein–protein interaction domains (**Figure 1**). Over-expression of LRRK2 in cultured cells and transgenic animals has been widely reported to cause cytotoxicity (Greggio et al., 2006; Smith et al., 2006; Iaccarino et al., 2007; West et al., 2007; Xiong et al., 2010; Stafa et al., 2012; Biosa et al., 2013). Whether this observation reflects an artifactual effect of over-expressing this large protein is unclear. Nonetheless, it is now generally accepted that this observed cytotoxicity is enhanced by *PARK8* mutations but ameliorated by loss of LRRK2 kinase or GTPase activity. In light of this, much work has been predicated on the idea that pathological effects of *PARK8* mutations require LRRK2 kinase activity, and in consequence a great deal of effort has gone into developing a pharmacological inhibitor of LRRK2 kinase activity (Ray and Liu, 2012). However, although the common G2019S mutation has been reproducibly shown to elicit increased LRRK2 kinase activity this mutation appears unique: all other mutations segregating with Parkinson's disease have no reproducible effect on kinase activity (Greggio and Cookson, 2009). Furthermore, the G2385R mutation, a risk factor within Asian populations, has been reported to have decreased kinase activity (Rudenko et al., 2012). Thus LRRK2 kinase activity appears to regulate the function of this protein, but whether increased kinase activity is responsible for pathogenesis in *PARK8* patients remains to be established. In conclusion, we favor a model where LRRK2 functions primarily as a scaffold that nucleates multiple protein complexes, but where protein function is nonetheless dependent on LRRK2 kinase and GTPase activities.

Despite considerable disagreement about LRRK2 function at the biochemical level, cell biological and transgenic animal studies have allowed advances to be made. In the following section we review aspects of LRRK2 biology where there are sufficient data to paint an overall picture that is beyond dispute, even if specific details are controversial or not yet known. We value the importance of work performed in lower organisms and will mention data obtained from these systems where pertinent, however, this review will focus on mammalian data. This distinction is justified since mammals express two LRRK proteins, LRRK1 and LRRK2, which despite strong similarities in sequence and structure appear to have contrasting functions. Lower organisms, in particular *Drosophila melanogaster* and *Caenorhabditis elegans*, encode a single LRRK protein, which is thus the ortholog of both LRRK1 and LRRK2. As we outline, there is sufficient evidence in existence to make roles for LRRK2 in three processes that underlie neurogenesis beyond debate. These are roles in synaptic and endosomal vesicle trafficking, macroautophagy, and regulation of microtubule dynamics are illustrated in **Figure 2**. Furthermore, an effect of LRRK2 on neurite outgrowth – a direct measure of the latter stages of neurogenesis – is extremely well supported. Coupled with growing evidence of roles in adult neurogenesis and in proliferation we contend that these data make a strong case for a central role for LRRK2 in multiple stages of neurogenesis.

ROLES FOR LRRK2 IN FUNCTIONS UNDERLYING NEUROGENESIS

Regulated membrane trafficking events underlie many key processes involved in neurogenesis. These include requirements for endocytosis for the proper function of neurogenic signaling pathways, such as Notch and Wnt cascades, and membrane

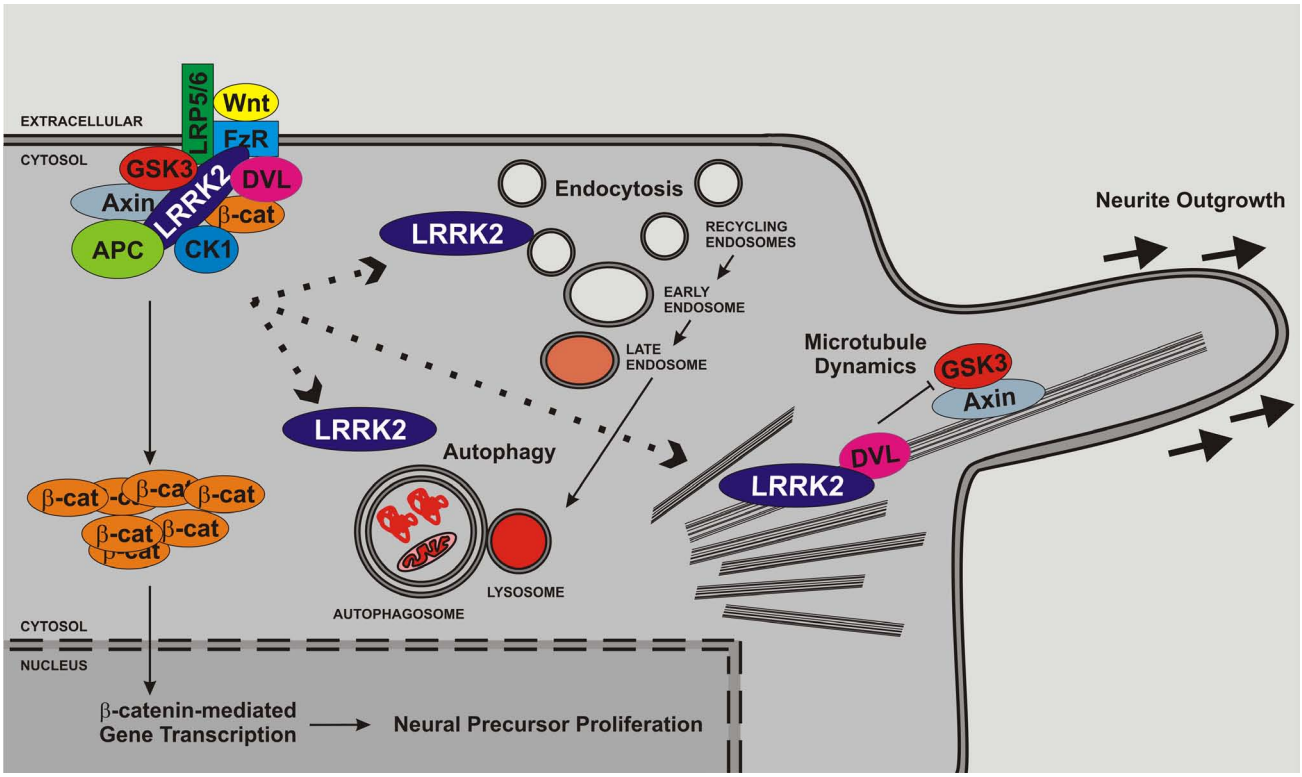

FIGURE 2 | LRRK2 regulates cell biological functions important for neurogenesis. Several lines of evidence support roles for LRRK2 in microtubule function, endocytosis/vesicle trafficking and autophagy. LRRK2 is likely to impact upon neurite outgrowth and the latter stages of neurogenesis through direct association with membrane structures and microtubules and/or regulation of signaling pathways. In addition, LRRK2 has been implicated in proliferation and may therefore also govern early stages of neurogenesis. The interaction between microtubule function, vesicle trafficking, autophagy and proliferation and the canonical Wnt pathway is depicted but other pathways are likely to play additional roles.

receptors involved in axonal outgrowth (Blitzer and Nusse, 2006; Le Borgne, 2006; Winckler and Yap, 2011). Importantly, roles for LRRK2 in membrane trafficking events are supported by data extending from early reports placing LRRK2 protein on cellular membranes (Biskup et al., 2006; Hatano et al., 2007) to evidence of vacuolation in LRRK2 knockout mouse kidney cells (Tong et al., 2012). Intriguingly, the kinase activity of LRRK2 appears to be enhanced at membranes (Berger et al., 2010), while the distribution of LRRK2 between membrane and cytosolic fractions can be regulated by extracellular stimuli (Berwick and Harvey, 2012a). These observations suggest that LRRK2 plays an active role in membrane trafficking events, and is not simply present as a by-stander.

The precise membranous compartments and/or vesicles LRRK2 inhabits remains contentious since many have been suggested, however, membrane compartments involved in two cellular processes – pre-synaptic vesicle trafficking and macroautophagy – stand prominent. Evidence of a role for LRRK2 in the trafficking of pre-synaptic vesicles comes from multiple experimental techniques. These include localization of LRRK2 to vesicles in synaptic terminals by confocal and electron microscopy (Xiong et al., 2010; Piccoli et al., 2011), demonstrable electrophysiological defects following knock-down or over-expression of LRRK2 in cultured neurons (Shin et al., 2008; Piccoli et al., 2011), and biochemical interaction and co-localization of LRRK2 with the early endosomal marker Rab5b (Shin et al., 2008). The function of LRRK2 in pre-synaptic vesicular compartments remains to be determined, although a recent report that the LRRK2 homolog in *Drosophila* phosphorylates endophilin A proteins is promising (Matta et al., 2012).

A wealth of data implicate LRRK2 in macroautophagy, the process by which damaged organelles and protein aggregates are "consumed" by engulfment by membranous autophagosomes that subsequently fuse with lysosomes (Codogno et al., 2012). Macroautophagy is traditionally considered a mechanism of cellular homeostasis and regulated cell death. However, neural development is especially dependent on this process, most likely for mediating the extensive physical remodeling required (Cecconi et al., 2007; Cecconi and Levine, 2008). Studies performed using mouse models of LRRK2 dysfunction have been particularly revealing, since multiple observations show impaired macroautophagy in the kidney of *Lrrk2* null animals (Tong et al., 2010, 2012; Herzig et al., 2011). These data include increased numbers of lysosomes and related structures, and an accumulation of the macroautophagy substrates p62 protein and lipofuscin granules. Much of this kidney phenotype appears to be replicated in transgenic mice that over-express LRRK2 containing an artificial kinase-inactivating mutation (Herzig et al., 2011). In

agreement with these observations, over-expression of LRRK2 impacts upon the autophagic pathway in human embryonic kidney 293 (HEK293) cells (Alegre-Abarrategui et al., 2009; Gómez-Suaga et al., 2012). Importantly, published data also support a role for LRRK2 in macroautophagic processes in the central nervous system. Most notably, brains from aged transgenic mice that over-express human LRRK2 variants containing either the R1441C or G2019S *PARK8* mutations have an accumulation of autophagic vacuoles (Ramonet et al., 2011). This observation appears to be corroborated *in vitro*, where over-expression of G2019S has similar effects in cultured primary neurons or differentiated SH-SY5Y cells (MacLeod et al., 2006; Plowey et al., 2008).

Studies in *Drosophila* suggest another connection between LRRK2 and macroautophagy: via interaction with the small GTPase Rab7 (Dodson et al., 2012). Rab7 is well-established as a key regulator of the fusion step between macroautophagic organelles and lysosomes (Codogno et al., 2012). If this observation can be replicated in mammalian systems it would be a fascinating result, since Rab7 is also involved in the latter stages of endocytosis, mediating late endosomal maturation and fusion with the lysosome (Wang et al., 2011). As mentioned above, LRRK2 has also been reported to interact with Rab5b (Shin et al., 2008). Since Rab5 proteins link pre-synaptic vesicle trafficking with early stages of endocytosis and Rab7 links autophagy with late-stage endocytosis, these observations together connect LRRK2 with the entire endocytic pathway.

In neurons endocytic vesicles can be trafficked over huge distances, particularly in axons. The importance of microtubules for this process is well described (Perlson et al., 2010). It is therefore interesting that an association between LRRK2 and microtubules has been reported by a large number of groups (Biskup et al., 2006; Gloeckner et al., 2006; Gandhi et al., 2008; Gillardon, 2009a,b; Sancho et al., 2009; Dzamko et al., 2010; Kawakami et al., 2012; Kett et al., 2012; Sheng et al., 2012). This physical association appears highly relevant to neurogenesis as LRRK2 has been reported to co-localize with microtubules within growth cones (Sancho et al., 2009).

The nature of the LRRK2-microtubule interaction is still unresolved. For example, there are conflicting reports about whether inhibition of LRRK2 kinase activity promotes (Dzamko et al., 2010) or weakens (Kett et al., 2012) the association. However, a role for LRRK2 in microtubule dynamics seems beyond dispute. Certain clues suggest LRRK2 may affect the stability of microtubules. LRRK2 has been reported to enhance the polymerization of bovine tubulin in the presence of microtubule-associated proteins (MAPs) *in vitro* (Gillardon, 2009b). In addition, LRRK2 has been linked to canonical Wnt signaling (Sancho et al., 2009; Lin et al., 2010; Berwick and Harvey, 2012a), which is well described as a modulator of the microtubule cytoskeleton in neurons (Salinas, 2007). Perhaps most strikingly though, numerous lines of *in vivo* data implicate LRRK2 in modulating the function of the MAP tau, best known for its role in Alzheimer's disease (Taymans and Cookson, 2010). Post-mortem analysis of Parkinson's disease brains carrying Y1699C, G2019S, or I2020T *PARK8* mutations have been reported to display "tau pathology" in a number of cases (Zimprich et al., 2004; Khan et al., 2005; Rajput et al., 2006; Ujiie et al., 2012). Tau hyperphosphorylation has also been reported in brains from transgenic mice over-expressing LRRK2 with the G2019S or R1441G mutations (Li et al., 2009; Melrose et al., 2010), while *Lrrk2* knockout has been reported to decrease tau phosphorylation (Gillardon, 2009b) although others have been unable to replicate this observation (Hinkle et al., 2012). G2019S LRRK2 has also been reported to promote tau phosphorylation in *Drosophila* (Lin et al., 2010). Mechanistically, the details linking LRRK2 to tau phosphorylation are lacking but one might predict the interaction between LRRK2 and microtubules would bring LRRK2 and tau into proximity. Indeed, a recent report suggests this may be the case (Kawakami et al., 2012). Whether LRRK2 phosphorylates tau directly remains unclear with one study supporting direct phosphorylation (Kawakami et al., 2012), and another suggesting that phosphorylation is performed by glycogen synthase kinase 3 (GSK3; Lin et al., 2010), a reported LRRK2 interactor (Lin et al., 2010; Berwick and Harvey, 2012a). These reports are not necessarily in conflict, since the experimental systems used are different and tau contains over 80 reported phosphorylation sites (http://cnr.iop.kcl.ac.uk/hangerlab/tautable). However, the possibility that phosphorylation is via GSK3 is intriguing, since this kinase has been implicated in the control of multiple MAPs besides tau, such as *adenomatous polyposis coli* (APC; Zhou et al., 2004) and collapsin response mediator protein 2 (Cole et al., 2004). Therefore, it is plausible that the control of microtubule dynamics by LRRK2 involves the modulation of a multitude of MAPs and takes place at a variety of microtubule sites, not just those regulated by tau in axons.

Parallel to a role for LRRK2 on microtubules, LRRK2 has also been connected to the actin cytoskeleton via the regulation of ERM (ezrin–radixin–moesin) protein phosphorylation. ERM proteins are three homologous proteins involved in anchoring actin filaments to the plasma membrane (Mangeat et al., 1999). Phosphorylation of ERM proteins is believed to induce a conformational change resulting in an open "active" shape (Mangeat et al., 1999). Two groups have found a positive correlation between LRRK2 levels and ERM protein phosphorylation (Jaleel et al., 2007; Parisiadou et al., 2009), that is most likely mediated by an indirect regulation (Nichols et al., 2009). Importantly, ERM proteins are essential for growth cone morphology and motility (Paglini et al., 1998), thus indicating that LRRK2 also impacts upon neurogenesis through ERM proteins. The connection between LRRK2 and the actin cytoskeleton is strengthened by a mass spectrometry study which found endogenous LRRK2 in HEK293 cells to associate with actin and a number of proteins known to modulate the actin cytoskeleton (Meixner et al., 2011). In light of these observations, it is interesting to speculate about a role for LRRK2 in coordinating neuronal microtubule as well as actin networks.

Thus, there are several lines of evidence for the importance of LRRK2 in a number of processes that underlie neurogenesis, but what evidence *directly* supports a requirement for LRRK2 in neurogenesis? Importantly, a comprehensive study in mouse embryos found a spatio-temporal LRRK2 mRNA expression pattern that is highly consistent with a key role for LRRK2 in neurogenesis (Zechel et al., 2010). Using *in situ* hybridization, LRRK2 mRNA expression was detected as early as day E10.5 in the developing central nervous system, with transcript detectable throughout the

cortex by day E12.5. Crucially, the authors describe embryonic expression of Lrrk2 as being most prominent in brain regions with "high proliferative and migratory activity, as well as sites of differentiation and cell death" (Zechel et al., 2010). These include the ventricular and subventricular zones of the telencephalon, in agreement with a previous report investigating older mice (Melrose et al., 2007). Lrrk2 was also found to be expressed in neural stem cells isolated from the dentate gyrus or striatal subventricular zone of E18.5 or adult mice. Thus LRRK2 expression patterns are consistent with a role in neurogenesis throughout life.

It is thus unsurprising that a growing body of experimental data show defects in neurogenesis caused by altered LRRK2 function. Experiments in cell and animal models utilized LRRK2 knockdown or knockout, over-expression of wild-type LRRK2, familial LRRK2 mutants and artificial kinase or GTPase dead mutants to measure predominantly neurite outgrowth and reported changes in length, number and branching of neurites *in vitro* and in brain slices. Most reports agree that the over-expression of familial LRRK2 mutants elicit decreased neurite length (MacLeod et al., 2006; Plowey et al., 2008; Parisiadou et al., 2009; Dächsel et al., 2010; Heo et al., 2010; Lin et al., 2010; Chan et al., 2011; Ramonet et al., 2011; Winner et al., 2011; Maekawa et al., 2012; Sheng et al., 2012; Stafa et al., 2012; Biosa et al., 2013; Cherra et al., 2013; Cho et al., 2013). Over-expression of wild-type LRRK2 was generally found to have no effect, or to cause mild neurite shortening, either reaching statistical significance or remaining as a trend. In contrast, loss of LRRK2 appears to have the opposite effect, allowing longer and more branched neurites to develop (MacLeod et al., 2006; Parisiadou et al., 2009; Dächsel et al., 2010; Heo et al., 2010; Stafa et al., 2012; Paus et al., 2013), although not all studies are in agreement (Gillardon, 2009b; Meixner et al., 2011). In general, these studies provide overwhelming evidence that the level of LRRK2 expression impacts upon neuritogenesis as predicted from the LRRK2 expression pattern throughout brain development.

Finally, we also note two publications investigating a role for LRRK2 in adult neurogenesis (Winner et al., 2011; Paus et al., 2013). These studies are of special relevance to Parkinson's disease, where impaired adult neurogenesis has been implicated in the development of non-motor symptoms (Marxreiter et al., 2013). A study in mice over-expressing the G2019S LRRK2 mutant reported decreased proliferation in the dentate gyrus and subventricular zones associated with decreased dendritic length and branching, and decreased cell survival (Winner et al., 2011). Another study by Paus et al. (2013) looked at adult neurogenesis in the dentate gyrus of *Lrrk2* knockout mice. As might be predicted, increased dendritic length and neurite arborization was observed. No defects in cell proliferation or survival were found however, although loss of Lrrk2 led to a greater number of doublecortin positive cells (Paus et al., 2013).

Thus a wealth of data implicates LRRK2 as a central player in the latter stages of neurogenesis – in particular neurite outgrowth and synaptogenesis – possibly via a combination of modulating vesicle trafficking and cytoskeleton dynamics. Nonetheless, emerging evidence also links LRRK2 to earlier stages of neuronal development, prior to cell cycle exit (Winner et al., 2011), supported by evidence of effects of LRRK2 on proliferation (Milosevic et al., 2009; Liu et al., 2012) and carcinogenesis (Hassin-Baer et al., 2009). Thus, LRRK2 might play a role from early mitotic neuronal precursors to terminal differentiation. As outlined above, we would suggest that LRRK2 is likely to function as a scaffolding protein in a number of distinct complexes, some of these important in neurogenesis. Nonetheless, two crucial questions remain: what is upstream of these complexes, and how are they regulated?

A ROLE FOR LRRK2 IN Wnt SIGNALING

Wnt (wingless/Int) signaling pathways constitute a family of highly conserved signal transduction cascades that have long been established as master regulators of animal development (Freese et al., 2010). The relevance of these pathways to neurogenesis is beyond doubt and will not be reviewed in detail in this article. Nonetheless the reader should be aware that a growing body of data also implicates Wnt signaling in the function of mature, post-mitotic neurons (Inestrosa and Arenas, 2010). Moreover, deregulated Wnt signaling pathways are suggested pathomechanisms for a number of neurological conditions, including Alzheimer's disease, Parkinson's disease, autism, and schizophrenia (De Ferrari and Moon, 2006; Inestrosa and Toledo, 2008; Berwick and Harvey, 2012b). Thus Wnt cascades can be considered essential for the central nervous system at all stages of life.

Wnt ligands themselves are secreted glycoproteins that bind to the extracellular domains of frizzled receptors on the plasma membrane of target cells. Signaling specificity is achieved in part through the large repertoire of Wnt ligands and frizzled receptors expressed in higher organisms, but also through the involvement of co-receptors. In the case of the canonical Wnt pathway, these receptors are low-density lipoprotein receptor-like proteins 5 and 6 (LRP5/6), which have also been reported to bind Wnt ligands at the cell surface. Upon binding of Wnt ligands to frizzled receptors and associated co-receptors the signal is relayed across the membrane, resulting in the activation of one or more intracellular cascades. Wnt signaling pathways relevant to neurogenesis and/or the function of mature neurons are depicted in **Figure 3**.

The best-described Wnt signaling cascade is the canonical Wnt pathway. This signaling mechanism ultimately results in the activation and nuclear recruitment of β-catenin protein, leading to the modulation of downstream target genes. In consequence, this pathway is sometimes referred to as the Wnt-β-catenin pathway. Canonical Wnt signaling is an unusual signaling mechanism as several events take place in the absence of a stimulus. In particular, β-catenin is sequestered into an inhibitory cytosolic complex known as the β-catenin destruction complex. Here, β-catenin is phosphorylated by GSK3 (the same protein implicated in tau phosphorylation). β-catenin phosphorylation results in the targeting of β-catenin for degradation by the proteasome. Therefore, in the absence of canonical Wnt pathway activators, β-catenin is continually degraded, and consequently unable to accumulate in the nucleus to regulate gene expression. Binding of Wnt ligand to frizzled receptors and LRP5/6 results in the recruitment of cytosolic dishevelled (DVL) proteins (key intermediates of most Wnt signaling branches) to the plasma membrane. Via interaction with key components of the β-catenin destruction complex such as Axin, DVL proteins cause the subsequent relocalization of the β-catenin destruction complex to the same juxtamembrane

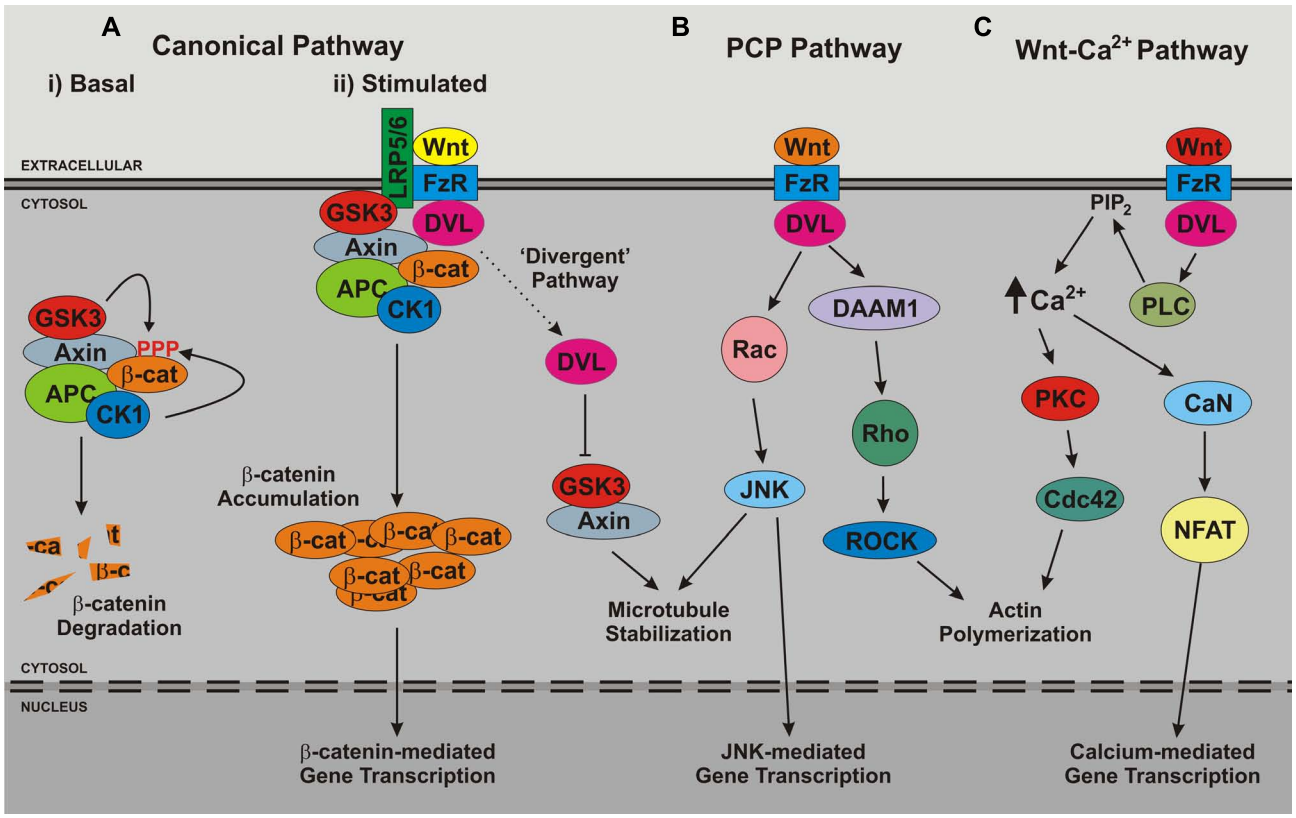

FIGURE 3 | Overview of Wnt signaling pathways. The three major branches of Wnt signaling – **(A)** the canonical, **(B)** planar cell polarity (PCP), **(C)** and Wnt-Ca^{2+} – pathways are illustrated. Note that in growing neurites a further branch has been reported, the so-called divergent canonical pathway, which impacts upon microtubule stability. APC, adenomatous polyposis coli; CaN, calcineurin; CK1, casein kinase 1; DAAM, dishevelled-associated activator of morphogenesis; DVL, dishevelled; FzR, frizzled receptor; GSK3, glycogen synthase kinase 3; JNK, c-Jun n-terminal kinase; LRP5/6, low-density lipoprotein receptor-related protein 5/6; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PLC, phospholipase C; ROCK, Rho-associated protein kinase.

site. This elicits the penultimate stage of canonical Wnt signaling, the inhibition of β-catenin phosphorylation, which allows β-catenin to become proteasome resistant and thus accumulate throughout the cell. However, the complexity of this mechanism is emerging. A requirement for the internalization of the cell membrane-associated protein complex containing Wnt ligand, frizzled receptor, LRP5/6, DVL proteins, and the β-catenin destruction complex into the endosomal system is now widely accepted. This internalized signaling complex passes through the endosomal system where it continues to signal from the cytosolic face of intracellular membranes (the so-called "signalosome" hypothesis). Finally, the signalosomes are sequestered from the cytosol into multi-vesicular bodies. Traditionally, by analogy to growth factor signaling pathways, this has been considered the termination step in Wnt signaling. However, recent data suggest that the sequestration of signalosomes into multi-vesicular bodies constitutes a final "signal activation" mechanism, since this leads to the removal of the canonical Wnt signaling pool of GSK3 from the cytosol (Dobrowolski and De Robertis, 2012).

Data from our laboratory has strongly implicated LRRK2 in multiple aspects of the canonical Wnt pathway. This work arose from a yeast two-hybrid screen identifying interactors of the Roc and/or COR domains of LRRK2 (Sancho et al., 2009). Since Roc and COR domains are expressed together throughout nature (Marín et al., 2008), it is reasonable to consider both domains part of a single functional unit conferring GTPase activity that was termed the RocCOR tandem domain. Using this LRRK2 RocCOR tandem domain as bait, the yeast two-hybrid screen returned cDNAs encoding the human DVL proteins DVL2 and DVL3 as potential interactors. Subsequent assays confirmed a direct interaction between the LRRK2 RocCOR domain and all three human DVL proteins, with the interaction site mapped to the DVL–Egl10–pleckstrin (DEP) domain of DVL1–3 (Sancho et al., 2009). Co-immunoprecipitation experiments confirmed that LRRK2 associates with DVL proteins in mammalian cells, while confocal microscopy revealed a striking recruitment of LRRK2 into polymeric DVL structures that are induced by over-expression of these proteins (Sancho et al., 2009).

Since DVL proteins are essential intermediates of all major branches of Wnt signaling (**Figure 3**), our work opened the possibility that LRRK2 may function in multiple Wnt cascades. However, follow-up studies focused on the canonical Wnt pathway, which can be assayed easily using TOPflash assays (Veeman et al., 2003). TOPflash assays are luciferase-based reporter assays

that quantitatively determine the level of β-catenin-mediated transcriptional activity in cells. Importantly, TOPflash assays revealed the LRRK2–DVL protein interaction to be functional as well as physical, since co-transfection of LRRK2 protein with any of the three human DVL proteins resulted in an enhancement of DVL-driven canonical Wnt activity (Berwick and Harvey, 2012a). This effect required the kinase and GTPase activities of LRRK2 and was increased further by targeting LRRK2 (and presumably, therefore, the LRRK2–DVL interaction) to membranes. Interestingly, Wnt3a treatment was found to increase the amount of endogenous LRRK2 present in membrane fractions of HEK293 cells (Berwick and Harvey, 2012a). Since the activation of canonical Wnt signaling takes place at intracellular membranes – consistent with the signalosome hypothesis – this lead us to investigate whether LRRK2 might physically interact with Wnt signaling receptors. Using a combination of confocal microscopy and co-immunoprecipitation LRRK2 was discovered to associate with LRP6, but not frizzled-1, frizzled-4, or frizzled-5. Yeast two-hybrid assays confirmed that the interaction was direct and, similar to interaction with DVL proteins, involved the LRRK2 RocCOR tandem domain (Berwick and Harvey, 2012a). These data are consistent with a role for LRRK2 in the activation of canonical Wnt signaling bringing DVL proteins to cellular membranes.

However, somewhat counter-intuitively, knock-down of LRRK2 was also found to potentiate DVL-driven TOPflash activity (Berwick and Harvey, 2012a). A similar effect was observed on basal and Wnt3a-driven β-catenin activity. In this regard knock-down of LRRK2 mimicked knock-down of AXIN1, an established component of the β-catenin destruction complex. Loss of AXIN1 is well-known to disrupt the β-catenin destruction complex thereby compromising β-catenin degradation and leading to an increase in basal canonical Wnt activity. We therefore wondered whether loss of LRRK2 might compromise an inhibitory role for LRRK2 in the β-catenin destruction complex. Consistent with this, co-immunoprecipitation of endogenous protein from mouse brain revealed Lrrk2 to exist in complex with multiple components of the β-catenin destruction complex, including GSK3 and β-catenin. Taken together, these results suggest a role for LRRK2 as a scaffold in canonical Wnt signaling. In the basal state, LRRK2 functions as part of the cytosolic β-catenin destruction complex and loss of LRRK2 compromises this role, leading to disruption of the complex and pathway activation. Following stimulation of cells with Wnt ligand, LRRK2 is recruited to cellular membranes. Here, via interaction with DVL proteins, the β-catenin destruction complex and LRP6, LRRK2 assists in the formation of Wnt signalosomes, enhancing the Wnt signal activity.

Our data are supported by work from other laboratories. Most notably, supporting the idea that LRRK2 associates with the β-catenin destruction complex, an interaction between LRRK2 and GSK3 has been reported in *Drosophila* (Lin et al., 2010). While this report does not investigate which cellular GSK3 pool associates with LRRK2 we note that the interaction modulated tau phosphorylation. Since Wnt signaling is well-known to regulate the phosphorylation of tau by GSK3 (Hooper et al., 2008), this would suggest that the GSK3 pool found to bind LRRK2 by Lin and colleagues could indeed represent the same Wnt-responsive fraction identified in our study (Lin et al., 2010; Berwick and Harvey, 2012a). Perhaps most interesting in this report, the LRRK2–GSK3 interaction was shown to be enhanced by the G2019S *PARK8* mutation (Lin et al., 2010). Curiously, the strength of the LRRK2 interaction with both DVL proteins and LRP6 was also affected by *PARK8* mutations (Sancho et al., 2009; Berwick and Harvey, 2012a). Unsurprisingly therefore, all investigated *PARK8* mutations decreased the capacity of LRRK2 to enhance the β-catenin activation elicited by DVL proteins (Berwick and Harvey, 2012a). This observation has obvious implications for the pathogenesis of Parkinson's disease, where perturbed Wnt signaling has already been suggested as a candidate pathomechanism (Berwick and Harvey, 2012b). However, as we outline below, decreased canonical Wnt signaling associated with familial *PARK8* mutations suggests that transgenic LRRK2 animal models of Parkinson's disease might present with discrete developmental phenotypes associated with Wnt dysfunction.

In addition to studies linking LRRK2 to Wnt signaling by protein–protein interaction strong circumstantial evidence from transcriptomics studies support this notion. In particular, an investigation into the effect of LRRK2 knock-down in human SH-SY5Y cells found mRNA species encoding a number of Wnt signaling proteins to be altered (Häbig et al., 2008). As it is well described that many Wnt signaling components are regulated at the transcriptional level by pathway activation, knock-down of LRRK2 would be expected to alter expression of other Wnt signaling proteins. Data from *C. elegans* also support this observation, with mRNA transcripts encoding Wnt signaling proteins being described as "coregulated with LRRK2" (Ferree et al., 2012).

Further support, albeit indirect, of a role for LRRK2 in Wnt signaling comes from studies investigating altered gene expression in animal models of Parkinson's disease. These investigations used a variety of different neurotoxins to elicit dopaminergic cell death resulting in parkinsonian-like motor phenotypes. L'Episcopo et al. (2011) reported increased *Wnt1* gene expression as well as deregulated *Fzd1* and β-catenin expression in the ventral mid-brain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. These experiments support the idea that canonical Wnt signal activation via increased *Wnt1* expression in astrocytes is neuroprotective (L'Episcopo et al., 2011). Another study used 6-hydroxydopamine (6-OHDA) to induce dopaminergic cell death in rats resulting in increased expression of the Wnt signal inhibitor Dickkopf-1 (Dkk1; Dun et al., 2012). Both models are in agreement with a neuroprotective role for Wnt signaling, since treatment with Dkk1 exacerbated toxic effects, whilst GSK3 inhibition was found to be protective (L'Episcopo et al., 2011; Dun et al., 2012). An unbiased genome-wide RNAseq approach in mice treated with a variety of pesticides showed altered expression of mRNAs encoding Wnt signaling components in ventral mid-brain and striatum (Gollamudi et al., 2012). Exposure to pesticides is a well-known environment risk factor for Parkinson's disease, further suggesting that dysregulated Wnt signaling might be a common mechanism underlying dopaminergic cell death in Parkinson's disease. Expression studies in human Parkinson's disease brains have not been as conclusive, although it is important to note that altered expression of Wnt pathway genes has been reported in women but not in men (Cantuti-Castelvetri et al.,

2007). These studies need to take into account that the brains analyzed are usually from individuals with symptomatic Parkinson's disease reflecting the loss of the majority of dopaminergic neurons in the substantia nigra. Therefore, gene expression changes are likely no longer reflective of the initial underlying etiology. However, in addition to work on LRRK2 outlined above, other clues from genetic causes of Parkinson's disease are consistent with altered Wnt signaling. Most notably Parkin, the product of the *PARK2* gene, has been reported to inhibit canonical Wnt signaling (Rawal et al., 2009), whilst the transcription factor Nurr1, which has been strongly linked to Parkinson's disease, is regulated by β-catenin (Jankovic et al., 2005; Kitagawa et al., 2007). Finally, it is not just Parkinson's disease-related genes that have been associated with Wnt signaling; Wnt signaling genes themselves have been linked to risk of Parkinson's disease. In particular, GSK3β has been suggested to modify disease risk in two studies (Kwok et al., 2005; Kalinderi et al., 2011) although a third failed to find any affect (Wider et al., 2011).

In summary, therefore, LRRK2 binds three central Wnt signaling components (Sancho et al., 2009; Lin et al., 2010; Berwick and Harvey, 2012a), while loss of LRRK2 and pathogenic *PARK8* mutations impact upon the activity of the canonical Wnt pathway (Berwick and Harvey, 2012a). In addition, connections between LRRK2 and Wnt cascades are strengthened by a number of studies supporting a role for dysregulated Wnt signaling in the early stage of Parkinson's disease. As outlined above, there is overwhelming evidence for a central function for LRRK2 in neurogenesis. Combining these ideas, we postulate a specific role for LRRK2 in Wnt-mediated neurogenesis. In the final section of this article, we will elaborate on this and suggest experimental approaches to test our hypothesis.

LRRK2 AS A MAJOR PLAYER IN Wnt-MEDIATED NEURONAL DIFFERENTIATION?

It is beyond dispute that Wnt ligands represent potent morphogens required for numerous aspects of neurogenesis, in particular the development of dopaminergic neurons of the ventral mid-brain (Brault et al., 2001; Castelo-Branco et al., 2010, 2004, 2003, 2010; Parish et al., 2008; Carpenter et al., 2010). Degeneration of these neurons underlies the typical motor symptoms associated with Parkinson's disease (Berwick and Harvey, 2012b). In this context, deregulated Wnt signaling caused by *PARK8* mutations might cause subtle defects in establishing neuronal circuitries, leaving these dopaminergic neurons more vulnerable to additional insults important for the pathogenesis of Parkinson's disease. In the remainder of this review, we describe roles for Wnt signaling pathways in modulating the same neurogenic events that were reported to be influenced by LRRK2. Combined with evidence of a function for LRRK2 as a Wnt signaling scaffold, this further supports the idea that LRRK2 is a central player in Wnt-mediated neurogenesis.

Evidence that Wnt ligands are major regulators of synaptic vesicle trafficking and synaptogenesis is accumulating (Farías et al., 2010; Inestrosa and Arenas, 2010). Published data support pre-synaptic and post-synaptic effects of multiple branches of Wnt signaling. Mammalian pre-synaptic development appears particularly dependent on Wnt-7a, an agonist of the canonical Wnt pathway (Farías et al., 2010). This Wnt ligand appears to be required for normal expression of the pre-synaptic vesicle protein, synapsin 1, in the developing mouse brain (Hall et al., 2000), with similar effects seen in mature neurons (Farías et al., 2007). Treatment of cultured neurons with Dkk1, an LRP5/6 antagonist, has confirmed this pre-synaptic effect of Wnt7a is through the canonical pathway (Davis et al., 2008). Curiously though, data from a number of laboratories suggest this effect is independent of transcription (Farías et al., 2010). This observation has led to the cascade by which Wnt7a modulates pre-synaptic and axonal (see below) function being described as a "divergent" canonical cascade (Ciani et al., 2004). Interestingly, LRRK2 is not just a key Wnt signaling protein interacting with LRP6 but was also found to interact with synapsin 1 and play a role in synaptic vesicle trafficking (Piccoli et al., 2011). The above evidence supports the idea of a Wnt7a-induced LRRK2-mediated canonical Wnt pathway with a direct transcriptionally independent effect on synapse formation and maintenance.

Both LRRK2 and Parkinson's disease pathogenesis have been linked to macroautophagy. Importantly, there is also evidence consistent with the idea that macroautophagy is modulated by Wnt ligands. Strikingly, knock-down of β-catenin alone appears sufficient to induce macroautophagy in carcinoma cells (Chang et al., 2013). Correspondingly, acute treatment of hippocampal neurons with the β-catenin agonist 2-amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine (Liu et al., 2005) was found to reduce oxygen–glucose deprivation-induced macroautophagy (Wang et al., 2012). Taken together, these observations suggest β-catenin to be a negative regulator of macroautophagy. In addition, GSK3 activity almost certainly impacts upon macroautophagy. GSK3 has recently been reported to phosphorylate TIP60, a histone acetyl transferase required for induction of macroautophagy. Mutation of the reported phosphorylation site to an alanine residue is sufficient to prevent growth factor deprivation-induced macroautophagy (Lin et al., 2012). In agreement with this, intranasal treatment with a GSK3 inhibitor peptide was reported to result in decreased autophagy and increased lysosomal acidification in brains from an Alzheimer's disease transgenic mouse model (Avrahami et al., 2013), with similar results obtained *in vitro*. These data predict a model where canonical Wnt pathway activation – resulting in GSK3 inhibition and β-catenin accumulation – would lead to decreased macroautophagy. However, the events do not appear straightforward, since GSK3 inhibition in a neuroblastoma cell line has been reported to induce increased lysosomal biogenesis, leading to increased macroautophagic flux (Parr et al., 2012). There are numerous reasons for this potential discrepancy, for example, cell lines and treatments used, however it is perhaps more relevant to observe that these studies are at very early stages. More pertinently still, none look at the regulation of macroautophagy during neural differentiation, where one would expect the requirements placed on the autophagic machinery of developing neurons to be more subtle than under conditions of stress. In conclusion, even though evidence supports the importance for LRRK2 and canonical Wnt signaling in macroautophagy, the specific signal transduction cascade, especially during neuronal differentiation, requires further investigation.

Wnt signaling is well-known to influence the dynamic instability of the microtubule cytoskeleton (Salinas, 2007). Multiple proteins involved in both the canonical and non-canonical Wnt pathway have been reported to affect microtubule stability, whilst GSK3 phosphorylates a variety of MAPs (Cole et al., 2004; Zhou et al., 2004; Salinas, 2007; Kim et al., 2011). Modulation of microtubule structures by Wnt pathways appear common to multiple cell types, for example Wnt ligands appear to be key regulators of mitotic spindles (Walston et al., 2004). In light of the above-described pre-synaptic function of LRRK2 and Wnt7a, it is important to emphasize that a large number of studies showing Wnt-mediated regulation of microtubules have used axon outgrowth as the model system. Much of this work was initiated by the observation that Wnt7a elicits axonal spreading and branching in cultured cerebellar granule cells (Lucas and Salinas, 1997), with corroborating data soon obtained *in vivo* (Hall et al., 2000). This effect is mimicked by GSK3 inhibitors and likely involves inhibition of phosphorylation of the microtubule-associated protein MAP1B (Lucas and Salinas, 1997; Lucas et al., 1998). Additional mechanisms involved in Wnt-mediated control of axonal microtubules include the identification of the β-catenin destruction complex members APC and AXIN1 as microtubule binding proteins in axons (Ciani et al., 2004; Purro et al., 2008). The precise details are still being elucidated, but it is fair to assume that, via interaction with microtubules, APC and AXIN1 create a spatially controlled signaling mechanism, specific to growing axons and growth cones. Importantly, LRRK2 also interacts with microtubules, induces hyperphosphorylation of the axonal MAP tau (Biskup et al., 2006; Gloeckner et al., 2006; Gandhi et al., 2008; Gillardon, 2009a,b; Sancho et al., 2009; Dzamko et al., 2010; Kawakami et al., 2012; Kett et al., 2012; Sheng et al., 2012), interacts with components of the β-catenin destruction complex (Lin et al., 2010; Berwick and Harvey, 2012a) and co-localizes with DVL1 to neurites in cell culture models at early stages of differentiation (Sancho et al., 2009). This further supports the idea of a LRRK2 mediated Wnt signaling pathway important during neuronal differentiation.

Above we have established good evidence for roles of LRRK2 and Wnt signaling in the regulation of pre-synaptic vesicle trafficking and microtubule dynamics, processes crucial for axonal outgrowth and synaptogenesis. While the evidence of a role for LRRK2 in macroautophagy is overwhelming, data supporting a role for Wnt signaling in modulating this process are more circumstantial. Nonetheless, the hypothesis that LRRK2 might function specifically in Wnt-mediated neuritogenesis is plausible, especially for the latter stages of neurogenesis. But what about the earlier stages? Here, the role for Wnt signaling is beyond doubt. For example, treatment with Wnt1, which activates the canonical Wnt pathway, causes expansion of ventral mid-brain precursors (Castelo-Branco et al., 2003). Conversely, loss of *Wnt1* in mice leads to a complete failure of mid- and hind-brain precursors to expand, leading to a near absence of these brain regions (Thomas and Capecchi, 1990). Similarly, Wnt3a (another canonical pathway agonist) secreted by hippocampal astrocytes has been shown to be essential for adult neurogenesis in the dentate gyrus (Lie et al., 2005). By contrast evidence of a function for LRRK2 in the proliferation of neural precursors and in adult neurogenesis are promising but at an early stage (Milosevic et al., 2009; Winner et al., 2011).

In conclusion, there is a remarkable degree of overlap between the effects of Wnt signaling and LRRK2 on neurogenesis. The importance of LRRK2 in canonical Wnt signaling further supports the notion of a specific function for LRRK2 in Wnt-mediated neurogenesis. This hypothesis can be investigated by crossing the relevant transgenic animals with known defects in Wnt-mediated neuronal differentiation with LRRK2 transgenics looking for an enhancement or rescue of phenotype. Of course, such experiments come with the usual important caveats associated with using animal models. For example, long non-coding RNAs are known to be poorly conserved between species making their study in model organisms of questionable relevance to humans (Pang et al., 2006). Human-specific transcriptional networks have also been reported in the brain (Konopka et al., 2012). However, at the level of protein function, conservation across species is usually very high, and thus, even though the data should be treated with caution, crossing of transgenic animal models is a justifiable approach. Indeed, this strategy has proven particularly useful for unveiling milder neurodevelopmental phenotypes. For example, crossing of *Wnt7a* and *Dvl1* knockout mice allowed a requirement for these genes in the development of cerebellar glomerular rosettes to be uncovered (Ahmad-Annuar et al., 2006). As *Lrrk2* knockout and familial *PARK8* mutant transgenic mice likely represent models of subtly increased and decreased canonical Wnt signaling, respectively, crossing of these lines with *Wnt7a* and/or *Dvl1* knockout animals would be of great interest.

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Sonic Hedgehog modulates EGFR dependent proliferation of neural stem cells during late mouse embryogenesis through EGFR transactivation

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Sonic Hedgehog (Shh/GLI) and EGFR signaling pathways modulate Neural Stem Cell (NSC) proliferation. How these signals cooperate is therefore critical for understanding normal brain development and function. Here we report a novel acute effect of Shh signaling on EGFR function. We show that during late neocortex development, Shh mediates the activation of the ERK1/2 signaling pathway in Radial Glial cells (RGC) through EGFR transactivation. This process is dependent on metalloprotease activity and accounts for almost 50% of the EGFR-dependent mitogenic response of late NSCs. Furthermore, in HeLa cancer cells, a well-known model for studying the EGFR receptor function, Shh also induces cell proliferation involving EGFR activation, as reflected by EGFR internalization and ERK1/2 phosphorylation. These findings may have important implications for understanding the mechanisms that regulate NSC proliferation during neurogenesis and may lead to novel approaches to the treatment of tumors.

Keywords: Shh, EGFR, Radial Glial Cells, transactivation, proliferation, cancer, neural progenitors

INTRODUCTION

The mammalian neocortex develops from neural stem/progenitor cells (NSCs) that generate neurons, astrocytes and oligodendrocytes under the control of a complex array of environmental factors (Caviness et al., 1995). The maintenance of these cells plays a crucial role in normal brain development and homeostasis, and its misbehavior has been related to the origin of tumors (McCarthy, 2012). Among the molecules that regulate NSC proliferation and maintenance, Sonic Hedgehog (Shh) has been addressed as an important mitogenic factor that regulates NSC proliferation, both in the embryonic and adult brain, acting together with the Epidermal Growth Factor (EGF) (Caric et al., 2001; Palma and Ruiz i Altaba, 2004; Palma et al., 2005). Shh and EGF synergic action is necessary to induce late NSC proliferation and cross talk between the Shh and EGF signaling pathways has been reported based on canonical Shh/Gli activity and modulation of EGFR expression (Palma and Ruiz i Altaba, 2004; Bigelow et al., 2005; Palma et al., 2005; Ruiz i Altaba et al., 2007). Importantly, recent evidence has also involved Shh/Gli and EGFR cooperative interaction in oncogenic transformation (Schnidar et al., 2009). Therefore, a detailed understanding of the downstream processes and molecular players involved in this cooperative growth factor interaction are important not only for brain development but also for the identification of novel drug targets and rational-based combination therapies (Mimeault and Batra, 2010; Mangelberger et al., 2012).

Canonical Hedgehog signaling involves the binding of Shh to its receptor, Patched1 (Ptch1), relieving a repression of Ptch1 upon the co-receptor Smoothened (Smo), which is a GPCR presumably coupled to an inhibitory G-protein (Gαi) (Ogden et al., 2008; Lappano and Maggiolini, 2011). Activation of Smo decreases the production of cAMP via Gαi, thus PKA is inhibited and no longer phosphorylates the Gli transcription factors, the only well-known mediators of Shh, ultimately leading to their stabilization and activation (Huangfu and Anderson, 2006).

The EGF receptor of the tyrosine-kinase ERbB family (ERbB1-4) is one of the most widely distributed control systems of cell proliferation and differentiation, not only by responding to its own ligands but also serving as a nodal element for a variety of other stimuli (Carpenter, 1999; Yarden and Sliwkowski, 2001). Upon ligand binding, the receptor dimerizes and its intracellular tyrosine kinase domain becomes activated leading to phosphorylation of the receptor itself and several intracellular proteins with signaling or vesicular trafficking functions (Schlessinger, 2000;

Abbreviations: NSC, neural stem cells; nsps, neurospheres; Shh, Sonic Hedgehog; CNS, Central Nervous System; Cyc, cyclopamine; Pur, purmorphamine.

Sorkin and Goh, 2008). Activated EGFR, as other tyrosine-kinase receptors, signals via the Ras/Raf/MEK/MAPK, STAT, PI3K/AKT, and PLC-γ pathways and changes its broadcasting location during endocytosis and recycling, remaining active during variable periods of intracellular trafficking before entering the lysosomal-degradation route that finally ends its signaling activity (Schlessinger, 2000; Piper and Luzio, 2007; Sorkin and Goh, 2008).

It is well-known that besides its own stimulus, the EGFR can also be indirectly transactivated by signals emerging from a variety of other receptors, most notably by GPCRs coupled to Gαi or Gαq (Daub et al., 1996; Carpenter, 1999; Gschwind et al., 2001; Buvinic et al., 2007). Transactivation usually involves metalloprotease (MMPs)-mediated release of soluble EGFR ligands by cleavage of transmembrane ligand precursors at the cell surface (Izumi et al., 1998; Prenzel et al., 1999). Transactivation of EGFR has not been explored during late central nervous system (CNS) embryogenesis. It remains unknown whether EGFR function in NSCs is controlled by Shh via transactivation as recently described in embryonic stem cells (Heo et al., 2007). Here we studied the acute effect of Shh signaling over EGFR function in NSCs during late brain development. These cells are biologically characterized by the *in vitro* double requirement for Shh and EGF for cell proliferation. We show for the first time that Shh is capable of modulating EGFR-dependent proliferation of late cortex NSCs through EGFR mediated transactivation and endocytosis. We identified a subpopulation of NSCs constituted by Radial Glial Cells (RGC) as the main target of Shh. Moreover, we extended our results providing evidence that Shh also induced EGFR to mitogenic signaling, and to become endocytosed but not degraded, in HeLa cells, a well-characterized cancerous cell model for the study of EGFR function (Salazar and González, 2002; Buvinic et al., 2007; Sigismund et al., 2008; Norambuena et al., 2009). Thus, Shh can modulate EGFR signaling in different cell contexts. Such kind of control likely contributes to regulate the function of stem and progenitor cells during brain development and also the pathogenic arising and progression of several cancers.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Cyclopamine (Infinity Pharmaceuticals, Inc.), recombinant octyl-modified Shh-N protein (R&D Systems), Purmorphamine (Calbiochem), EGF (human recombinant, Invitrogen), Tyrphostin (Calbiochem), Shh specific blocking antibody (5E-1), Shh-N plus the Gli inhibitor Gant61 (ALEXIS). Anti-phospho ERK, anti-total ERK, anti-β-actin and anti-β-tubulin, rabbit anti-GFAP, PD98059 were all from Sigma. Sheep anti-EGFR (Upstate), guinea pig polyclonal anti-GLAST, rabbit anti-Sox2, Ilomastat were from Chemicon, mouse anti-PKCλ (Transduction Labs), rabbit anti-caspase3 (Cell Signaling), polyclonal antibody EGFR984 (Biosonda Biotechnology), monoclonal antibody HB8506 (American Type Culture Collection), anti-phosphotyrosine 4G10 monoclonal antibody (gift kindly provided by Dr. Maria Rosa Bono, Universidad de Chile, Santiago, Chile). Fluorescent secondary antibodies used were anti-rabbit Alexa488 and anti-mouse Alexa555 (Invitrogen).

HeLa CELL CULTURE AND TREATMENTS

An in-house population of HeLa cells previously characterized for EGFR internalization and transmodulation (Salazar and González, 2002; Buvinic et al., 2007; Norambuena et al., 2009) were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), maintained at 37°C in a humidified atmosphere (95% air, 5% CO2). HeLa cells permanently expressing EGFR-GFP were obtained by transfection with pEGFP-N1-EGFR plasmid (kindly provided by Dr. Alexander Sorkin, University or Pittsburgh, USA) using the Lipofectamine 2000 method (Invitrogen). Selection was made in 1 mg/ml geneticin sulfate (G418) to obtain stable transfectants and the cells were then maintained in 0.8 mg/ml G418. Before the experiments, the cells were cultured to ∼80% confluence and serum-starved for 24 h in media supplemented with 0.3% fetal bovine serum (FBS), unless otherwise indicated. Treatments were performed at 3.3 μg/ml recombinant Shh, hedgehog inhibitor Cyclopamine (Cyc) at 10 μM, Shh specific blocking antibody (5E-1) at 5 mg/ml, Gli inhibitor Gant61 at 10 μM, Hedgehog agonist Purmorphamine (Pur) at 10 μM and EGF at 1 and 50 ng/ml.

HeLa CELL RT-PCR AND IMMUNOBLOT

HeLa RNA preparation and RT-PCR specific reaction conditions and sequences for the human *hprt*,*ptc1*, and *gli1* primer pairs were as described (Palma and Ruiz i Altaba, 2004). For HeLa cells immunoblot assays, 60 mg protein from total cell extracts prepared as described (Salazar and González, 2002) were resolved on 10% polyacrylamide SDS gels and transferred onto nitrocellulose (Schleicher and Schuell, Germany). When required, EGFR was immunoprecipitated with the monoclonal antibody HB8506 and resolved by SDS-PAGE and immunoblotted with anti-ubiquitin P4D1 antibody, as described (Salazar and González, 2002). For total EGFR detection, membranes were stripped and incubated with the polyclonal antibody EGFR984 (Salazar and González, 2002). Immunoblots were revealed with ECL (Amersham Biosciences) and the bands were digitalized in a VISTA-T630 UMax scanner driven by Adobe Photoshop CS (Adobe Systems, Mountain View, CA).

HeLa CELL IMMUNOFLUORESCENCE AND BrdU ASSAY

To analyze EGFR internalization, HeLa EGFR-GFP stable cell clones were grown on glass coverslips, treated and fixed for 30 min at room temperature with 4% paraformaldehyde in PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2 (PBS-CM). For BrdU incorporation assays, cells were grown in cover slips to ∼50% confluence and serum-starved for 24 h in media supplemented with 0.3% FBS. Cells were treated with 3 mM of BrdU and BrdU detection was performed as previously described (Palma and Ruiz i Altaba, 2004) and marker-positive cells were assessed as percentage of DAPI-positive cells (5 random areas per experiment). All digital fluorescence images were acquired at the time indicated on a Zeiss Axiophot microscope with a Plan-APOCHROMAT 63X/1.4 oil immersion objective and the 14-bit Zeiss Axiocam camera.

NEOCORTICAL EXPLANTS AND PRIMARY NEUROSPHERE CULTURE AND TREATMENTS

E18.5 cortical explants and Neurospheres (nsps) were obtained from outbred strains BalbC and C57/BL6 mice, respectively, and used for proliferation and differentiation and as described (Reynolds and Weiss, 1996; Dahmane et al., 2001), nsps for no more than three passages. Shh-N protein was used at 3.3 μg/ml. Other treatments included hedgehog inhibitor Cyclopamine (Cyc) at 10 μM, Hedgehog agonist Purmorphamine (Pur) at 10 μM and EGF 1 and 10 ng/ml. For acute stimulation of nsps Shh only was used. For proliferation assays nsps were plated on coated coverslips and cultured for 48 h in 10 ng/ml of EGF in the presence of Cyc or 1 ng/ml of EGF plus Shh (Palma and Ruiz i Altaba, 2004). To evaluate a possible role for the Shh pathway in RG cell maintenance nsps were deprived of EFG and cultured in the presence or absence of Shh alone to permit cell differentiation for 7 days, exchanging media every 3 days. Pharmacological inhibition with Cyc was performed similarly.

All animal procedures were in accordance with the Chilean legislation and were approved by Institutional Animal Care and Use Committees.

NEUROSPHERE BrdU INCORPORATION AND IMMUNOFLUORESCENCE

Incorporation of BrdU (3 mM, 2 h prior to culture fixation) and immunofluorescence detection on NSCs was performed as previously described (Dahmane et al., 2001) and marker-positive cells were assessed as percentage of DAPI-positive cells (5 random areas per experiment, from at least three independent experiments). Cells undergoing apoptosis were identified by caspase-3 immunodetection.

NEUROSPHERE IMMUNOBLOT AND IMMUNOPRECIPITATION

For immunoblot and immunoprecipitation, extracts of nsps or explants were prepared in lysis buffer (10mM Tris-Hcl, 5mM EDTA, 150 mM NaCl buffer and 1% Triton X-100) containing proteases and phosphatase inhibitors as described (Salazar and González, 2002). Proteins in immunoblots were visualized by ECL (Pierce) using horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research).

FLOW CYTOMETRY ON NEOCORTICAL EXPLANTS

Single cells dissociated from E18.5 neocortical explants prepared following protocol described in (Dahmane et al., 2001) were fixed in 2% paraformaldehyde for 15 min, permeabilized with 0.05% saponin and incubated for 30 min on ice in 1% PBS-BSA with anti-GFAP or anti-EGFR followed by secondary FITC-conjugated antibodies were analyzed in a flow cytometer (FACSort; BD Pharmingen) with CellQuest software. Statistical analysis.

Results were analyzed using Student's *t*-test and One-Way ANOVA. Values were expressed as mean ± the standard error of the mean (SEM). Significance was set as $p < 0.05$.

RESULTS

Shh and EGF synergic action has recently been involved in cell proliferation in embryonic stem cells (ES), and can occur via Shh induced EGFR transactivation (Heo et al., 2007), thus rising the question of how extensive this control system might be to other progenitor cell types or to other kind of cells, including cancerous cells. EGFR can be transactivated by a variety of stimuli. Depending on the kind and concentration of the ligand (e.g., EGF or TGF-a), the activated EGFR displays variations in signaling and cellular responses (Alwan et al., 2003; Sigismund et al., 2008; Madshus and Stang, 2009; Roepstorff et al., 2009). It is therefore important to analyze the contribution of Shh to the modulation of EGFR function according to cell type and context.

Shh MEDIATES NSC PROLIFERATION TROUGH EGFR MODULATION

During development, NSCs exhibit different requirements for growth factors. This is reflected in the growth of nsps, which require basic fibroblast growth factor (bFGF) at earlier embryonic stages and both Shh and EGF at late stages (Palma and Ruiz i Altaba, 2004). Considering that EGFR-mediated proliferation of cortical NSCs specifically requires EGF and Shh during late brain development, we first examined the effect of the specific inhibitors, Cyc (Hedgehog antagonist) and tyrphostin (EGFR inhibitor, AG1478) (Ward et al., 1994; Chen et al., 2002), on cell proliferation of E18.5 EGF-responsive nsps. Dissociated nsps were plated on substrate-coated dishes and grown in the presence of EGF (10 ng/ml) for 48 h. The cells were deprived of any growth factor for 2 h to cause cell growth arrest and maximal exposal of the EGFR to the cell surface, and were then stimulated with EGF for 2 h in the absence or presence of Cyc or AG1478, added 30 min before ligand addition. EGF-induced cell proliferation, measured by BrdU incorporation, was reduced by 70% with AG1478 (100 nM) and 50% with Cyc (10 μM) treatment, in agreement with previous reports confirming basal Shh activity (Dahmane et al., 2001; Palma and Ruiz i Altaba, 2004). Interestingly, simultaneous treatment with AG1478 and Cyc almost completely abrogated cell proliferation (about 96%) (**Figure 1A**), without significantly affecting apoptosis (**Figure 1B**). These results corroborate that the EGFR and Shh signaling pathways collaborate in the regulation of NSC proliferation during late embryogenesis. Interestingly, these observations also suggest that besides regulating the level of EGFR expression, Shh substantially contributes to the mitogenic response elicited by EGF signaling.

NSC PROLIFERATION INDUCED BY Shh REQUIRES EGFR TRANSACTIVATION

The collaborative action of EGF and Shh can be exerted by the activation of independent signaling pathways that might converge upon yet unknown downstream elements. Alternatively, the EGFR might constitute an upstream element shared by both EGF and Shh. An obvious possibility is that Shh acutely transactivates the EGFR and as a consequence activates the Ras/Raf/MEK/ERK1/2 pathway involved in cell proliferation (Traverse et al., 1994; Seger and Krebs, 1995). We first studied the effects of Shh treatment over EGFR tyrosine-phosphorylation. Strikingly, the EGFR immunoprecipitated from nsps treated with Shh (3 μg/ml) for 15 min showed an increased phosphotyrosine content in immunoblots, which was abrogated by AG1478 (**Figure 1C**), thus reflecting EGFR activation. This Shh-induced EGFR activation led to ERK1/2 activation, detectable within 15 min and reaching a maximum by 30 min (**Figure 1D**), which

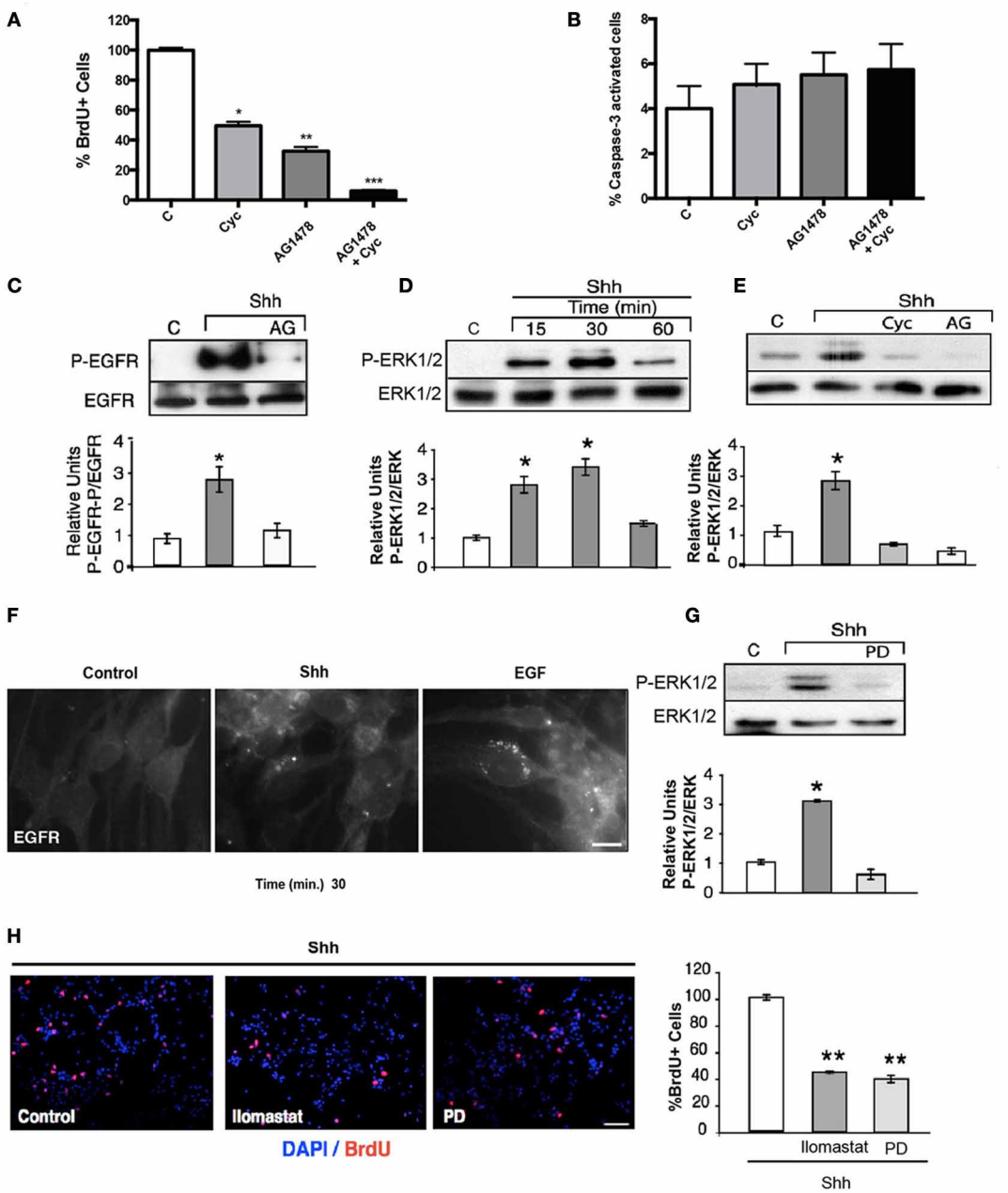

FIGURE 1 | Shh stimulation transactivates the EGFR leading to mitogenic signaling via the ERK1/2 pathway. (A) EGF-dependent NSC proliferation requires the Shh signaling pathway. Quantification of the percentage of BrdU+ cells. The mitogenic activity decreased significantly with either cyc (50%; $*p < 0.03$) or AG1478 (70%; $**p < 0.01$) and was almost completely abrogated with both inhibitors (90%; $***p < 0.001$). **(B)** Cells were treated with EGF (10 ng/ml), in the presence of either Cyc (10 μM), AG1478 (100 nM) or both drugs. Cells were fixed and cell death was evaluated by positive caspase-3 labeling. The histogram shows that treatment with inhibitors does not significantly change the percentage of caspase-3-activated cells. Data represent the results of three independent experiments. **(C)** Transactivation of the EGFR by Shh. Shh (3 μg/ml) induced an increment in the phospho-tyrosine activity of the EGFR, which was abolished by AG1478 (100 nM), added 30 min before. Tyrosine phosphorylation of EGFR was detected by immunoblot after immunoprecipitation. **(D,E)** Shh induces EGFR-dependent ERK1/2 activation. Nsps treated with Shh for the indicated time periods show increased phospho-ERK, detected by immunoblot, maximal at 30 min. Independent experiments show that such ERK1/2 activation measured after 15 min of Shh treatment is not only inhibited by Cyc (10 μM), but also by AG1478 (100 nM) ($*p < 0.03$). **(F)** Shh treatment in NSCs induces EGFR internalization. An adherent culture of nsps treated with 3.3 μg/ml Shh for 30 min shows EGFR internalization. EGF 50 ng/ml treatment was used as positive control. **(G)** MEK inhibitor PD98059 added 30 min before the stimulus inhibits Shh mediated ERK activation. ($*p < 0.03$). **(H)** Immunofluorescence for BrdU shows that ERK1/2 signaling and metalloprotease activity mediate Shh-dependent NSC proliferation. Quantification of the percentage of BrdU+ cells stimulated with Shh show a decreased proliferation of 60% by both PD98059 (100 μM) and the metalloprotease inhibitor Ilomastat (20 μM), added 30 min before stimulation. Data represent mean ± SEM of 3 independent experiments in triplicate ($**p < 0.01$). Bar = 10 μm **(F)** and Bar = 20 μm **(H)**.

was also abrogated by Cyc and AG1478 (**Figure 1E**). These results reveals for the first time that Shh transactivates the EGFR and its downstream Ras/Raf/MEK/ERK1/2 signaling pathway in cortical NSCs.

Because endocytosis plays a predominant role in EGFR function (Sorkin and Goh, 2008), and the intracellular trafficking and signaling consequences depend on the intensity of ligand-induced stimulation (Sigismund et al., 2008), we asked whether the transactivation elicited by Shh might led to receptor internalization. To answer this question we dissociated and plated nsps on substrate-coated dishes so as to obtain monolayers of cells that can be easily analyzed for EGFR endocytosis by indirect immunofluorescence. The cells were grown in the presence of EGF (10 ng/ml) for 48 h and depleted for 2 h before the experiments to induce maximal exposure of the EGFR to the cell surface. Treatment with Shh (3.3 μg/ml) induced a redistribution of EGFR from the cell surface to juxtanuclear endosomal compartments, although of less magnitude compared with EGF (50 ng/ml) used as positive control (**Figure 1F**). These results reveal that Shh transactivates the EGFR leading to its endocytosis.

In order to establish how much of the mitogenic effect of Shh is mediated by the EGFR-dependent ERK1/2 pathway we tested the effect of inhibiting its activating-kinase, MEK, with PD98059 (Alessi et al., 1995). PD98059 (100 μM) almost completely inhibited ERK1/2 activation (**Figure 1G**) and reduced the mitogenic activity of nsps grown in the presence of Shh by 60% (**Figure 1H**). Thus, the mitogenic effect of Shh upon NSCs seems to relay substantially on transactivation of EGFR and the subsequent engagement of the ERK1/2 pathway.

The mechanism of EGFR transactivation usually involves the proteolytic release of ligands from the cell surface through the activity of MMPs on ligand precursors (Izumi et al., 1998; Prenzel et al., 1999). Ilomastat/(GM6001), currently used as a general inhibitor of MMPs (Izumi et al., 1998; Prenzel et al., 1999), reduced Shh-dependent proliferation of nsps by 55% (**Figure 1H**), indicating that a similar MMP-dependent mechanism is triggered by Shh. These results indicate that the mitogenic signals conveyed by Shh acute stimulation requires transactivation of the EGFR through pathways involving metalloprotease mediated release of EGFR ligands from the cell surface.

RADIAL GLIAL CELLS (RG) ARE THE MAIN TARGETS OF Shh SIGNALING DURING LATE CORTICAL DEVELOPMENT

Shh has been implicated in the upregulation of EGFR expression observed in NSC during late development, determining increased responsiveness to EGF. RT-PCR analysis of neocortical tissue has revealed that cyc treatment decreases the EGFR expression *in vivo* (Palma and Ruiz i Altaba, 2004). However, it has not been demonstrated that this modulation of EGFR expression translates to effective changes at the protein level. To test this hypothesis we compared the effect of Shh, EGF and bFGF on the expression of EGFR. EGFR expression increased significantly in E18.5 neocortical explants treated for 48 h with Shh only in comparison to bFGF or control samples, reaching even slightly higher levels when compared to a positive control treatment with EGF (**Figure 2A**). We next address the population of cells targeted by Shh in our model system. One candidate are the RG cells that during late states of embryonic development start to express GFAP (reviewed in Imura et al., 2003; Ihrie and Alvarez-Buylla, 2008) and some of them are EGF-responsive stem cells (Doetsch et al., 1999). We analyzed the expression of GFAP by flow cytometry on neocortical explants and found a significant decrement after 48 h of Cyc treatment (**Figure 2B**), similar to the reduction in EGFR expression. This observation suggests that Shh might be acting on GFAP+ cells that express EGFR. In addition, immunostaining of GFAP on 1 week plated single cell suspensions of nsps, showed that GFAP expression is significantly affected, decreasing by Cyc treatment and increasing by Shh only (**Figure S1**). Of note, we were able to distinguish more cells with the characteristic RG cell morphology after Shh treatment in comparison to control growth factor deprived cultures. GFAP is indeed a marker of NSCs both in the cortical region of late embryogenesis and in the subventricular zone (SVZ) of the adult, but it is also expressed in many terminally differentiated astrocytes. Other criteria is then needed to confirm that the RG-NSCs are the target of Shh. RG cells are characterized by Sox2 expression, which marks multipotent NSCs during different stages of mouse ontogeny (Graham et al., 2003; Ellis et al., 2004). The glutamate transporter (GLAST) is also a well-established marker of RG cells. Therefore, we assessed the effect of Cyc on the expression of these two markers. Nsp cultures cells grown in the presence of EGF (10 ng/ml) plus Cyc for 48 showed reduced levels of Sox2 by immunoblot (**Figure 2D**). Nsps treated with Cyc also reduced the number of cells expressing GLAST as evaluated by immunoblot and the opposite was seen upon treatment with Shh (**Figure 2E**). All these results strongly suggest that Shh acts on a specific progenitor pool: the RG cells.

Taken together, our studies establish for the first time that the stimulatory actions of EGF on NSC proliferation are mediated partially by the activation of the Shh signal transduction pathway.

Shh ACUTE STIMULI MODULATE EGFR FUNCTION IN HeLa CANCER CELLS

To assess whether Shh modulation of EGFR function is a more general phenomena that might be extensive to cancerous cells, we next performed similar experiments in HeLa cells. These cells have been widely used to study EGFR function (Salazar and González, 2002; Buvinic et al., 2007; Sigismund et al., 2008; Norambuena et al., 2009) but to our knowledge have not been studied as a Hedgehog signaling model system. Once ensured that HeLa cells expressed the components of the Hedgehog pathway through reverse transcriptase PCR (**Figure 3A**), we examined the effect of Shh stimulation on HeLa cell proliferation. Cells were deprived of any growth factor for 24 h and then incubated in absence (control) or presence of recombinant Shh, Shh plus its specific blocking antibody (5E-1), Shh plus the Gli inhibitor Gant61, or EGF as positive control for another 24 h (**Figure 3B**). Cell cycling, measured by BrdU incorporation after application of a 1 h pulse, showed that Shh induced cell proliferation of HeLa cells, which on the other hand showed a significant reduction in the presence of the Shh blocking peptide, 5E1. Interestingly, Shh treatment showed only a slight decrease in proliferation with the canonical pathway inhibitor Gant61 (**Figure 3C**). These results suggest that Shh might promote HeLa cell proliferation

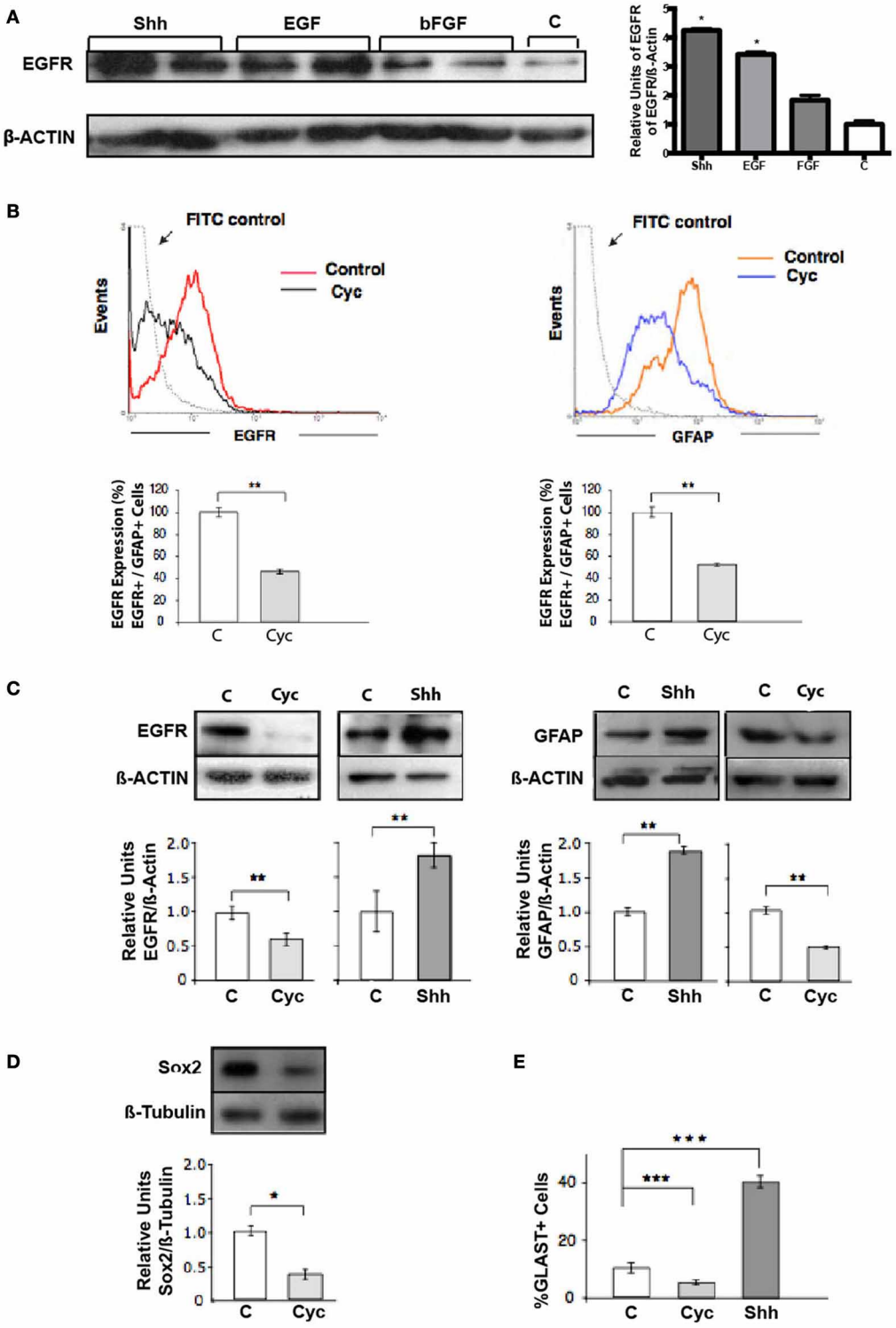

FIGURE 2 | Shh controls the pool of RG cells (GFAP+, Sox2+, GLAST+) that express EGFR. (A) Western blot and densitometry analysis of EGFR show higher levels of EGFR in Shh-treated explants in comparison to samples treated with bFGF and positive control EGF. **(B)** Flow cytometry of cells harvested from E18.5 cortical explants. Treatment for 48 h with Cyc (10 μM) provokes similar decreases in the pool of EGFR- and GFAP-expressing cells. Histograms are representative of three independent experiments expressed as the percentage of the mean ± and SEM (**$p < 0.01$). **(C)** Western blot and densitometry analysis of EGFR of E18.5 nsps cultures treated for 48 h as indicated. **(D)** Immunoblots of Sox2 in NSCs. Treatment with Cyc for 48 h showed a decreased in Sox2 mass respect to β-tubulin. **(E)** Histograms showing the percentage of GLAST+ NSCs after 24 h treatment as indicated. Data represent the results of three independent experiments expressed as the percentage of the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

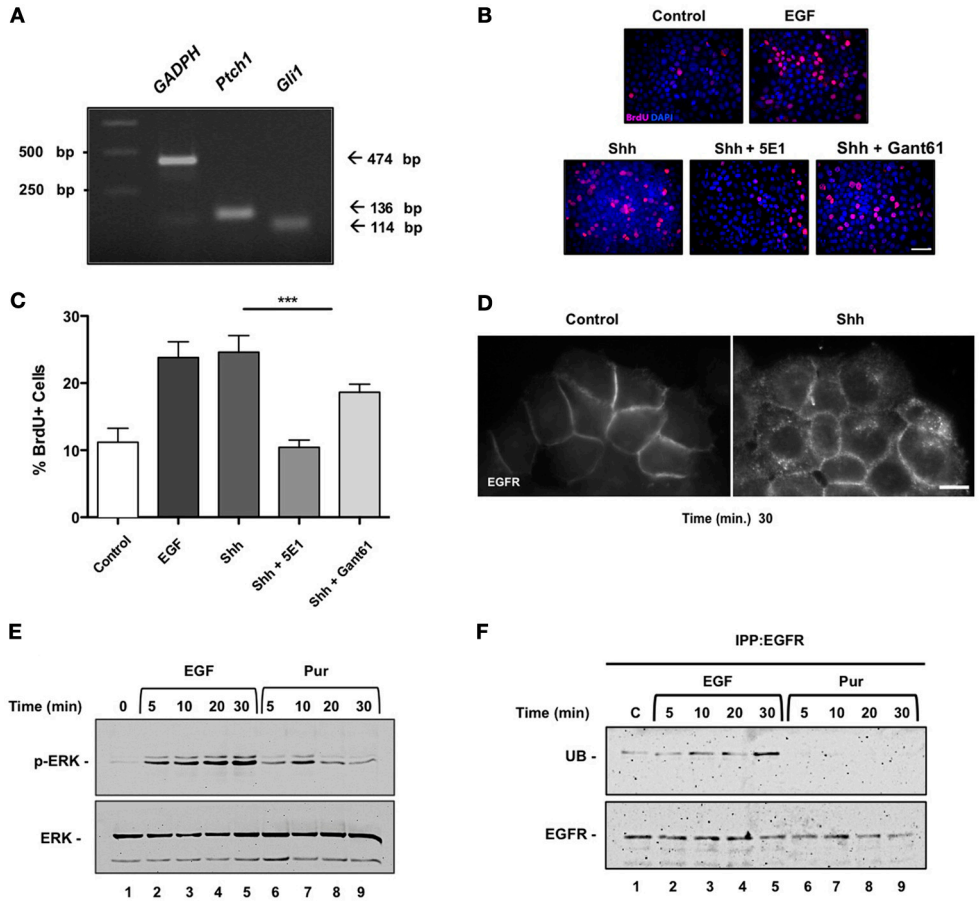

FIGURE 3 | Sonic Hedgehog induces internalization of EGFR, activation of ERK and proliferation of HeLa cells. (A) Expression of Hedgehog signaling pathway components in HeLa cells. mRNA expression levels of *Gli1*, *Ptch1*, and *GAPDH* were assessed by reverse transcription-polymerase chain reaction (RT-PCR) in HeLa cells. (*M* = molecular mass; bp = base pair). **(B)** Shh induces proliferation of HeLa cells in a non-canonical fashion. HeLa cells were untreated (control) or treated with recombinant Shh or EGF as positive control, for 30 min before stimulation with Shh, Shh blocking antibody 5E1 (5E1.5 mg/ml) or Gli inhibitor Gant61 (Gant 10 μM). Bar = 10 μm. **(C)** Quantification of the percentage of BrdU+ cells stimulated with EGF, Shh as indicated. A decrease in proliferation was observed in treatments with 5E1 and proliferation was not fully inhibited with Gant treatment. One-Way ANOVA $p = 0.0002$. **(D)** Shh treatment induces EGFR internalization. HeLa cells stably expressing EGFR-GFP were incubated in the absence (Control) or presence of Shh for 30 min and visualized under epifluorescence microscopy. Bar = 10 μm. **(E)** Both EGF (positive control) and Hedgehog agonist Pur acute treatments led to ERK1/2 signaling pathway activation in these cells. **(F)** Pur does not induce ubiquitination of EGFR. Cells were incubated in the absence (lane 1) or presence of either 50 ng/ml EGF (lanes 2–5) or 10 μM Pur (lanes 6–9) for the indicated time periods. EGFR was then immunoprecipitated with mAb-HB8506, and its ubiquitin (UB) content was assessed by immunoblot with anti-ubiquitin mAb P4D1. Total mass of EGFR detected after incubating blots with polyclonal antibody EGFR984. In contrast to EGF, Pur did not induce ubiquitination of EGFR.

from a non-canonical source, without direct mediation of the Gli transcription factors.

One possibility is that Shh might be promoting cell proliferation by EGFR transactivation. Although we could not detect an increment of tyrosine-phosphorylation in EGFR assessed by immunoblot we did observe internalization of the receptor. HeLa cells, stably expressing EGFR-GFP, were depleted and incubated with or without Shh and EGFR internalization was examined under fluorescence microscopy after 30 min. Shh (3.3 μg/ml) induced a redistribution of EGFR from the cell surface to intracellular compartments indicative of EGFR endocytosis (**Figure 3D**). Both Shh and Hedgehog agonist Pur acute treatments led to ERK1/2 signaling pathway activation in these cells (**Figures 2**, **3E**). Acute Pur treatment showed no evidence of EGFR degradation for up to 4 h (data not shown). Actually, as opposed to 50 ng/ml EGF treatment, we did not detect ubiquitination of the EGFR (**Figure 3F**). These results show that Shh drives EGFR internalization and promotes a transient mitogenic ERK1/2 signaling cascade activation. Shh seems to mimic the effects reported for low concentrations of EGF, which does not induce detectable ubiquitinylation and leads to EGFR recycling rather than degradation, thus contrasting with the effects of high ligand concentration (Sigismund et al., 2005, 2008, 2013). Actually, a threshold for EGFR ubiquitination has been recently reported in these cells (Sigismund et al., 2013). Overall, these results suggest that EGFR is also a downstream element in the Shh signaling pathway in HeLa cancer cells.

DISCUSSION

Acquisition and modulation of EGF responsiveness is critical in the regulation of several aspects of NSC development, such as self-renewal, differentiation and migration. Here we provide evidence that one of the main functions of Shh during late stages of brain embryogenesis is to promote and modulate EGF responsiveness of NSCs. We demonstrate that Shh has the capability to transactivate the EGFR and as a consequence strengthens the mitogenic intensity of the ERK1/2 signaling pathway triggered by EGF. This effect can explain the collaborative function of Shh and EGF exerted on the proliferation of late NSCs.

We identify RG (GFAP+, Sox2, and GLAST+) cells as the main target population of Shh, in which Shh induced transactivation, together with the reported EGFR expression (Dahmane et al., 2001) can all contribute to increase the EGF responsiveness. Our results are in line with a recent report by Dave et al., which showed *in vivo* that Shh pathway activation in the conditional $Ptc1^{Lox/Lox}$; $Nestin^{Cre}$ mutant cortex is mitogenic for cells that have a capacity to self-renew over an extended period of time. Authors identified RG cells- Nestin+/GLAST+ as the direct targets in the developing neocortex by mid neurogenesis (E14.5). Importantly, they showed reduction of Trb2 basal progenitor positive cells, suggesting that since RG cells can differentiate into basal progenitors, an indirect effect on this cell population could not be ruled out (Dave et al., 2011). In contrast Komada et al. reported that in *Shh*-CKO and *Smo*-conditional mutant embryos, the number of Tbr2-positive basal progenitor cells is significantly decreased in the SVZ/VZ of the developing neocortex (Komada et al., 2008). Furthermore, no significant differences could be observed in the Pax6 positive-RG cells among E15.5 wild-type and mutant mice. Clearly, this matter will require future investigation and we do not rule out that Shh signaling may be a mechanism for the regulation of both the number of RG cells and basal progenitors.

Our previous experiments in NSCs revealed synergism of the Shh and EGF signaling pathways reflected by increments of proliferative responses and induction of gene expression (Palma and Ruiz i Altaba, 2004). This up-regulation of EGFR expression levels induced by Shh indeed contributes to the collaborative action of Shh and EGFR on NSCs. EGFR expression has been associated with changes in progenitor cell behavior as a limiting factor for both proliferation and differentiation with response choice regulated in part by a concentration-dependent mechanism. Here we show that the mitogenic mechanism employed by Shh stimulus substantially depends on EGFR activity. Shh has been reported to provoke acute transactivation of EGFR, leading to cell proliferation in mouse embryonic stem cells (Heo et al., 2007). Our present results are the first to show that Shh transactivates the EGFR, leading to mitogenic ERK1/2 signaling in NSCs at late embryonic stages. Treatment of E18.5 cortical nsps with Shh for 15 min increases the tyrosine phosphorylation of the EGFR, a hallmark of its activation. Such Shh-induced EGFR phosphorylation was abrogated with an inhibitor of EGFR tyrosine kinase activity, and had functional consequences, as it resulted in activation of mitogenic ERK1/2 signaling. Recent observations suggest that the ability of cells to divide in response to EGFR activation requires the expression of a high level of the receptor in NSC (Lillien and Gulacsi, 2006). Because Shh can provoke an acute as well as a sustained status of EGFR activation (Bigelow et al., 2005; Heo et al., 2007), an initial event of EGFR transactivation can play a role in increasing the levels of the EGFR as well as in establishing regulatory loops acting on the proliferation machinery. Thus, Shh and EGF signaling pathways can functionally interact at different levels and may even establish a long-term regulatory loop depending on the cell context. How the synergistic signaling system of Shh and EGFR are integrated in NCSs and how does cooperativity between the pathways lead to selective activation of common response genes remains to be tested in future studies.

Endocytic trafficking is a crucial element in the regulation of EGFR function control (Goh and Sorkin, 2013; Pennock and Wang, 2003). The intensity, location and duration of receptor signaling can be modulated by the endocytic trafficking machinery, which is tightly intertwined with the mechanisms that control receptor signaling (Scita and Di Fiore, 2010; Goh and Sorkin, 2013). Activated EGFR undergoes transphosphorylation and ubiquitylation and these modifications define its endocytic trafficking/signaling behavior (Goh and Sorkin, 2013; Sigismund et al., 2013). Recent studies have shown that low ligand concentrations drive EGFR toward clathrin-dependent endocytosis followed by recycling, whereas high ligand concentrations promote EGFR internalization through a clathrin-independent route directed to lysosomal degradation (Sigismund et al., 2008). These alternatives have functional consequences in specifying the responses (Sigismund et al., 2008) and involve a threshold-controlled ubiquitylation system (Sigismund et al., 2013), which finely tunes the balance between EGFR signaling and trafficking. The ubiquitin-dependent down regulation route involves ESCRT-mediated sorting into intraluminal vesicles of multivesicular bodies that then fuse with lysosomes (Wegner et al., 2011). Thus, activated EGFR can remain signaling-competent for variable periods of time before degradation, determining different response outcomes (Sigismund et al., 2008; Rush et al., 2012). Our results, both in NSC and in HeLa cells show that Hedgehog pathway activation leads to EGFR internalization and to transient activation of the MAPK/ERK signaling cascade (**Figure 1D**, **Figure S2**), without detectable ubiquitination. This mimics the effect induced on EGFR by low EGF concentrations, which mainly leads to receptor recycling instead of degradation (Sigismund et al., 2008). In contrast, high EGF doses stimulation (50 ng/ml) induces EGFR ubiquitination and promotes its sorting to the lysosomal degradation pathway (**Figure 3F**). Besides mimicking EGFR stimulation by low EGF concentration, which by itself can constitute a strong mitogenic stimulus (Sigismund et al., 2008), another possibility of Shh-mediated regulation of EGFR function might occur through the reduction of PKA activity produced by activation of Smo via Gαi (Huangfu and Anderson, 2006). A decreased PKA activity has been shown to induce endocytosis and intracellular accumulation of empty/inactive EGFR, as well as an increment in the half-life of ligand-activated EGFR by delaying its sorting to the lysosomal degradation route (Salazar and González, 2002; Norambuena et al., 2010).

Our findings provide evidence that Shh, constitutes a mitogenic stimulating system, activating the EGFR and its MAPK/ERK signaling cascade. This potentiates the mitogenic effects of low

EGF concentrations. Taking into account the recently described Shh constitutive autocrine modulation in NSCs (Bigelow et al., 2005; Ruiz i Altaba et al., 2007; Martínez et al., 2013), our results suggest that downstram activation of EGFR might provide the necessary strength to the intracellular pathways to mount a mitogenic response in NSCs. This might eventually increase the expression of EGFR, as previously reported for other EGFR transactivating receptors (Buvinic et al., 2007).

We suggest that during late development, neural precursor cell proliferation would be regulated by an interplay of mechanisms involving different signaling pathways, which would control the size of the progenitor pool. Additional approaches addressing the relationship of Shh, EGFR and others signaling pathways will be required order to obtain a better understanding of how, when and where these pathways interact to control NSC behavior.

Although at the moment specific mechanisms of EGFR transactivation by Shh are not defined, we show the intermediary action of MMPs in Shh-induced proliferation in NSCs. Our results show that Shh elicits a mitogenic response through activation of GM6001-sensitive MMPs. Inhibition of MMP activity attenuates the Shh-mediated cell proliferation indicating that the release of soluble ligands by induction of GM6001-sensitive MMPs after Shh stimulation could be responsible for cell proliferation. Our findings complement those described recently in ES cells by Heo et al. (2007). However, in NSCs, the identity of MMPs and their mechanism still remain to be defined.

Transactivation of the EGFR by agonist-activated GPCRs typically requires growth factor cleavage mediated by MMPs. It is noteworthy that the transactivation of the EGFR, mediated by members of the ADAM (a disintegrin and metalloproteinase) family of zinc-dependent proteases, is relevant in the development and progression of diverse types of human tumors. Furthermore, there is evidence suggesting that brain tumors resemble stem cell niches (Oliver and Wechsler-Reya, 2004). Brain tumors contain cancer stem cells, which are essential for both development and recurrence of the tumors (Dahmane et al., 2001; Vescovi et al., 2006). Together, these studies raise the possibility that the factors that regulate normal cell lineages from neural precursors may serve similar functions in the development of brain cancers from stem-like cancer cells (Ruiz i Altaba et al., 2007). Integration of the Shh and EGFR pathways could be a critical step in cancer initiation and/or tumor growth. Our data obtained in HeLa cells indeed suggest a conserved essential role of Shh on the regulation of EGFR function, which have therapeutic interest. The recognized importance of the EGFR in tumorigenesis suggests the possibility that abnormally increased Shh signaling contributes to carcinogenesis, thus providing additional rational to Shh as a target for antitumor therapies.

In conclusion, the present work gives further evidence of the importance of Shh in the regulation of the homeostasis of the EGF signaling pathways and proliferation, emphasizing cross-talk processes. Our findings shed new insight into the complex signal transduction pathways that mediate the actions of growth factors in the developing brain.

AUTHOR CONTRIBUTION

Verónica Palma and Alfonso Gonzalez designed research; Gisela Reinchisi, Margarita Parada, Claudia Oyanadel, Ronan Shaughnessy, Verónica Palma, and Pablo Lois performed research; Gisela Reinchisi, Verónica Palma, and Pablo Lois analyzed data, Gisela Reinchisi, Margarita Parada, Alfonso Gonzalez, and Verónica Palma wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Cellular_Neuroscience/10.3389/fncel.2013.00166/abstract

Figure S1 | Shh maintains a specific GFAP+ -RG cell pool. Nsps were cultured for 48 h with EGF (10 ng/ml) and then EGF was removed and the cells were cultured in the presence of only Shh (3 μg/ml) or Cyc (10 μM) for 7 days. RG phenotype on treated nsps was assessed by immunofluorescence staining for GFAP, counterstaining the cell nuclei with DAPI Bar = 50 μm. The percentage of cells expressing GFAP is depicted in the corresponding histogram. Shh treatment increases 2-fold the number of GFAP+ cells, whereas Cyc treatment shows the opposite effect, decreasing by 50% the number of GFAP+ cells. Values are the mean ± SEM of three experiments per conditions. (**$p < 0.01$ vs. control).

Figure S2 | Shh treatment in HeLa cells induces activation of the mitogenic ERK1/2 transduction pathway. HeLa cells treated with 3.3 μg/ml Shh for the indicated time periods showed increased phospho-ERK, detected by immunoblot, maximal at 30 min.

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Wingless-type family member 3A triggers neuronal polarization via cross-activation of the insulin-like growth factor-1 receptor pathway

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Initial axonal elongation is essential for neuronal polarization and requires polarized activation of IGF-1 receptors (IGF-1r) and the phosphatidylinositol 3 kinase (PI3k) pathway. Wingless-type family growth factors (Wnts) have also been implied in the regulation of axonal development. It is not known, however, if Wnts have any participation in the regulation of initial axonal outgrowth and the establishment of neuronal polarity. We used cultured hippocampal neurons and growth cone particles (GCPs) isolated from fetal rat brain to show that stimulation with the wingless family factor 3A (Wnt3a) was sufficient to promote neuronal polarization in the absence of IGF-1 or high insulin. We also show that Wnt3a triggered a strong activation of IGF-1r, PI3k, and Akt in developmental Stage 2 neurons and that the presence of activatable IGF-1r and PI3k activation were necessary for Wnt3a polarizing effects. Surface plasmon resonance (SPR) experiments show that Wnt3a did not bind specifically to the IGF-1r. Using crosslinking and immuno-precipitation experiments, we show that stimulation with Wnt3a triggered the formation of a complex including IGF-1r-Wnt3a-Frizzled-7. We conclude that Wnt3a triggers polarization of neurons via cross-activation of the IGF-1r/PI3k pathway upon binding to Fz7.

Keywords: Wnt3a, IGF-1 receptor pathway, Frizzled-7, axonal outgrowth, neuronal polarity

INTRODUCTION

The establishment of neuronal polarity requires the action of two interrelated processes: axon specification and elongation. The initial signals and pathways that determine polarity are beginning to be understood. A particularly early event that occurs in neurons that have not yet exhibited a discernible axon (stage 2 of differentiation) is the segregation of activatable, membrane inserted IGF-1 receptors (IGF-1r) in a single neurite (Sosa et al., 2006). Subsequently, phosphatidylinositol-3 kinase (PI3k) and its product, phosphatidylinositol 3,4,5-trisphosphate (PIP3), accumulate in the distal region and growth cone of the neurite with the IGF-1r. These events are critical for the outgrowth of the future axon, together with activation of PI3k by IGF-1r (Shi et al., 2003; Sosa et al., 2006). Additionally, it has been shown that IGF-1 produces a robust and long lasting activation of the PI3k-Akt pathway through activation of the IGF-1r in hippocampal neurons (Zheng and Quirion, 2004). Activation of IGF-1r by their cognate ligand (IGF-1) and by insulin is also able to transactivate other receptor systems, such as epidermal growth factor receptors (EGFRs) (Roudabush et al., 2000).

Growth factors belonging to the Wnt family have been implicated in different aspects of axonal development (Rosso and Salinas, 2007). There is increasing published evidence indicating that Wnts are key mediators of axonal outgrowth and guidance (Lyuksyutova et al., 2003; Arevalo and Chao, 2005) and regulate axonal remodeling through inhibition of GSK3β (Krylova et al., 2000; Ciani et al., 2004). Interestingly one study showed that Wnt5a could accelerate initial axonal outgrowth by activating aPKC and stimulating the interaction between Dv1 and the polarity complex PAR3/PAR6/aPKC (Zhang et al., 2007). However, the above mentioned experiments were made in the presence of high insulin concentration, enough to fully activate the IGF-1r and trigger hippocampal neuron polarization in the absence of any other growth factor (Dotti et al., 1988; Sosa et al., 2006). Another member of the Wnt family, Wnt3a, has been shown to be essential for hippocampal development (Lee

et al., 2000) and regulates neurite outgrowth in neuroblastoma cells (Greer and Rubin, 2011). The experiments in the present paper were designed to investigate the involvement of Wnt family members (specifically Wnt3a) in the regulation of initial axonal outgrowth and the establishment of neuronal polarity. Moreover, we studied a possible relationship between Wnt3a and the IGF-1r pathway.

Using cultured hippocampal pyramidal neurons and growth cone particles (GCPs) prepared from fetal rat brain (GCPs), our results show that Wnt3a strongly stimulated the IGF-1r and the PI3k pathway through the formation of an IGF-1r (active)-Wnt3a-Frizzled-7 (Fz7) complex and, therefore, stimulated initial axonal outgrowth and the establishment of neuronal polarity.

MATERIALS AND METHODS

PRIMARY ANTIBODIES

The following primary antibodies were used: affinity-purified rabbit polyclonal antibody against βgc (Quiroga et al., 1995), which was diluted 1:200 for immunofluorescence and 1:500 for western blotting; mouse monoclonal antibody against IGF-1r clone αIR3 (Calbiochem), which was diluted 1 μg/ml for immunoprecipitation; rabbit monoclonal antibody against Frizzled-7 receptor (Sigma-Aldrich), which was diluted 1:500 for western blotting; rabbit polyclonal antibody to the phosphorylated (tyr 458) binding motif of p85 PI3k (Cell Signaling), which was diluted 1:200 for immunofluorescence and 1:1000 for western blotting; anti PI3k p85 rabbit monoclonal antiserum (Cell Signaling), which was diluted 1:1000 for western blotting; mouse monoclonal antibody against TrkB (Santa Cruz Biotechnology), which was diluted 1:100 for western blotting; rabbit polyclonal antibody to phosphorylated IGF-1r (Tyr 980) (Cell Signaling), which was diluted 1:100 for immunofluorescence and 1:1000 for western blotting; goat monoclonal antibody against phosphorylated TrkB receptor (Santa Cruz Biotechnology), which was diluted 1:200 for western blotting; rabbit polyclonal antibody against the Ror2 receptor (Cell Signaling), which was diluted 1:500 for western blotting; rabbit monoclonal antibody against Wnt3a (Cell Signaling), which was diluted 1:50 for immunoprecipitation and 1:500 for western blotting; mouse monoclonal antibody against GAP-43 (Sigma-Aldrich), which was diluted 1:500 for western blotting; mouse monoclonal antibody to the axonal marker Tau-1 (Calbiochem), which was diluted 1:800 for immunofluorescence; rabbit monoclonal antibody to β-III-tubulin (Sigma-Aldrich), which was diluted 1:6000 for immunofluorescence and 1:4000 for western blotting; mouse monoclonal antibody (clone 9F10) to c-myc (Roche Diagnostics, Germany) which was diluted 1:400 for immunofluorescence; rat monoclonal antibody to neural cell adhesion molecule L1 clone 324 (Millipore) 1 μg/ml and rabbit polyclonal antibody to phosphorylated Akt (Santa Cruz Biotechnology), which was diluted 1:500 for western blotting.

ANIMALS

All animal procedures were performed following approved protocols by the Board of Animal Welfare, School of Chemical Sciences, National University of Córdoba, Argentina.

CELL CULTURE

Dissociated hippocampal pyramidal cells were prepared from embryonic rat brain and cultured as previously described (Mascotti et al., 1997). To allow for neuronal survival, the medium contained a low level of insulin (5 nM), which was sufficient to stimulate insulin receptors but not fully activate IGF-1r [It has been reported that the affinity of IGF-1r for insulin is 100–500-fold lower than for its cognate ligand IGF-1 (Ballard et al., 1988); previous published experiments from our laboratory indicated that insulin significantly induced neuronal polarization at concentrations of 100 nM or higher (Grasso et al., 2013)]. Where indicated, 1.35 nM Wnt3a (Purro et al., 2008) or 20 nM IGF-1 [we have previously shown that IGF-1r was fully activated by IGF-1 at concentrations of 10 nM or higher (Quiroga et al., 1995)] was added to the culture medium. Neurons were considered to be at stage 3 when the length of the axon (positive to immunostaining with Tau-1) exceeded that of the average length of minor neurites by at least 20 μm (Craig and Banker, 1994).

CELL TRANSFECTION

cDNAs encoding shRNAs were inserted in a dicistronic vector pSuper.neo + GFP (pSuper RNAi System-OligoEngine) under the control of the H1 RNAIII polymerase promoter, and the transfection marker GFP was under the control of the PGK promoter. The target DNA sequence was GCCCATGTGTGAGAAGACC (Bohula et al., 2003; Sosa et al., 2006). A scrambled DNA target sequence (GAACGGTCGCAGTGTACCA) was created using the siRNA WizardTM, InvivoGen. The resulting plasmids were referred to as IGF-1r shRNA and scrambled sequence RNA (ssRNA). The Fz7 CRD was a generous gift from Dr. P. Salinas. The myc tagged, constitutively active p110 construct was a generous gift from Dr. L. Williams. The plasmids were mixed with Lipofectamine 2000 and added to the neurons 2 h after plating.

BLOCKING IGF-1r WITH ANTIBODIES

Neurons were grown in the presence of the mouse monoclonal antibody clone αIR3 (diluted to 1 μg/ml), which blocks the activation of IGF-1r. Clone αIR3 binds to an epitope within the α-subunit adjacent to the ligand binding site and blocks IGF-1 from binding to its receptor without affecting either insulin or IGF-II receptors (Kull et al., 1983; Rohlik et al., 1987). Clone αIR3 cross-reacts with the α-subunit of rat IGF-1r and has been extensively used in IGF-1r blocking experiments (Linseman et al., 2002; Sosa et al., 2006). Fresh antibody was added to the culture medium every 8 h.

PI3k INHIBITION

Neurons were grown in the presence (20 μM) or absence of the specific pharmacologic inhibitor of PI3k enzyme LY294002 (Sigma-Aldrich).

IMMUNOFLUORESCENCE

Cells were fixed and processed as previously described (Dupraz et al., 2009). Cells were observed using a Zeiss Pascal 5 confocal microscope. Images were captured and digitized using LSM Image software, all images were printed using Adobe Photoshop. The images were analyzed using ImageJ and/or StatSoft software.

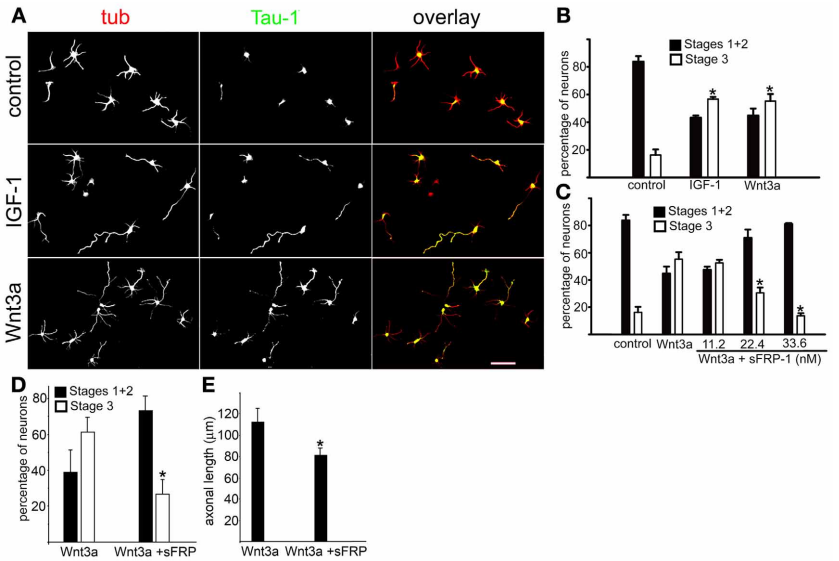

FIGURE 1 | Wnt3a triggered hippocampal neuron polarization. (A) Double immunofluorescence micrographs of hippocampal neurons after 20 h in culture showing the distribution of the neuronal marker β-III tubulin (tub) and the axonal marker Tau-1. Cells were cultured in control medium (5 nM insulin, top) or in medium containing either 20 nM IGF-1 (middle) or 1.35 nM Wnt3a (bottom). **(B)** Percentage (±sem) of neurons at different stages of differentiation grown for 20 h in control medium or in the presence of either 20 nM IGF-1 or 1.35 nM Wnt3a ($n = 3$ independent experiments). At least 100 cells were scored for each condition. $^*p < 0.005$ compared to control. **(C)** Percentage (±sem) of neurons at different stages of differentiation grown in control medium or in the presence of 1.35 nM Wnt3a or 1.35 nM Wnt3a plus different concentrations of sFRP-1. Note the significant dose-dependent inhibition of Wnt3a-mediated polarizing effects of sFRP-1 ($n = 3$ independent experiments). At least 100 neurons were scored for each condition. $^*p < 0.005$ compared to control. Scale bar 100 µm. **(D)** Percentage (±sem) of neurons at different stages of differentiation grown for 20 h in the presence of 1.35 nM Wnt3a and for further 24 h in the presence (or not) of 33.6 nM sFRP-1 ($n = 3$ independent experiments) At least 100 neurons were scored for each condition. $^*p < 0.02$ compared to Wnt3a. Neurons were considered to be at Stage 3 when the length of one of the processes positive to Tau-1 exceeded that of the average minor neurite by at least 20 µm. **(E)** Average length (±sem) of axons from neurons treated as in panel **(D)**. ($n = 3$ independent experiments) At least 100 neurons were scored for each condition. $^*p < 0.02$ compared to Wnt3a.

IMMUNOFLUORESCENCE OF ACTIVE IGF-1r AND ACTIVE PI3k

Cells were cultured as previously described. After 12 h in culture, cells were stimulated for 5 min with 20 nM IGF-1 or 1.35 nM Wnt3a, fixed, and processed for immunofluorescence using an antibody selective for the phosphorylated form of IGF-1r (Sosa et al., 2006) or a specific antibody against the phosphorylated form of the PI3k regulatory subunit p85 (Sosa et al., 2006).

ISOLATION OF GROWTH CONES

Axonal growth cones were isolated from developing brain as previously described (Pfenninger et al., 1983; Lohse et al., 1996). Briefly, brains of fetal rats at 18 days of gestation were homogenized. A low-speed supernatant (LSS) was prepared, loaded onto a discontinuous sucrose density gradient with steps of 0.83 M and 2.66 M sucrose, and spun to equilibrium at 242,000 g. The fraction at the load/0.83 M interface (designated "A") contained the GCPs.

IMMUNOPRECIPITATION EXPERIMENTS

Intact GCPs were incubated for 30 min in the presence or absence of Wnt3a (1.35 nM). To stabilize the ligand-receptor complexes, we used the membrane insoluble and cleavable crosslinker DTSSP, Pierce Protein Research Product, (1 mM) following the manufacturer protocols. GCPs were spun down for 6 min at 4000 g, and the pellet was lysed with 1X RIPA buffer containing 3 mM vanadate and a protease inhibitor cocktail. For immunoprecipitation, the supernatant was incubated with the indicated antibodies for 2 h at 4°C before adding protein A/G plus-coated beads. In some experiments (**Figure 7D**), total protein from hippocampal neurons in culture (in control conditions or stimulated with Wnt3a) was used as starting material.

GEL ELECTROPHORESIS AND WESTERN BLOT ANALYSIS

Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes and developed with ECL on x-ray films as previously described (Dupraz et al., 2009), or were analyzed using a Li-COR Odyssey Infrared Imaging System (LI-COR Biosciences) according to the manufacturer's instruction.

SURFACE PLASMON RESONANCE (SPR) ASSAY

The interaction of soluble recombinant human Insulin-like Growth Factor-1 (IGF-1) with its receptor (IGF-1r-recombinant human Met 1-Asn 932 extracellular moiety; R&D System, Minneapolis, USA) was measured by SPR analysis using a Biacore T100 instrument (Biacore Inc, Piscataway, NJ), that allows to determine the interactions between two molecules in real time (Karlsson et al., 1991). IGF-1r (ligand, 1.0 µg/ml) was dialyzed against 10 mM sodium acetate pH 4.5 and coupled to the carboxymethyl-dextran matrix of CM5 sensor chips (Biacore) using the Amine Coupling Kit as described (Johnsson et al., 1991).

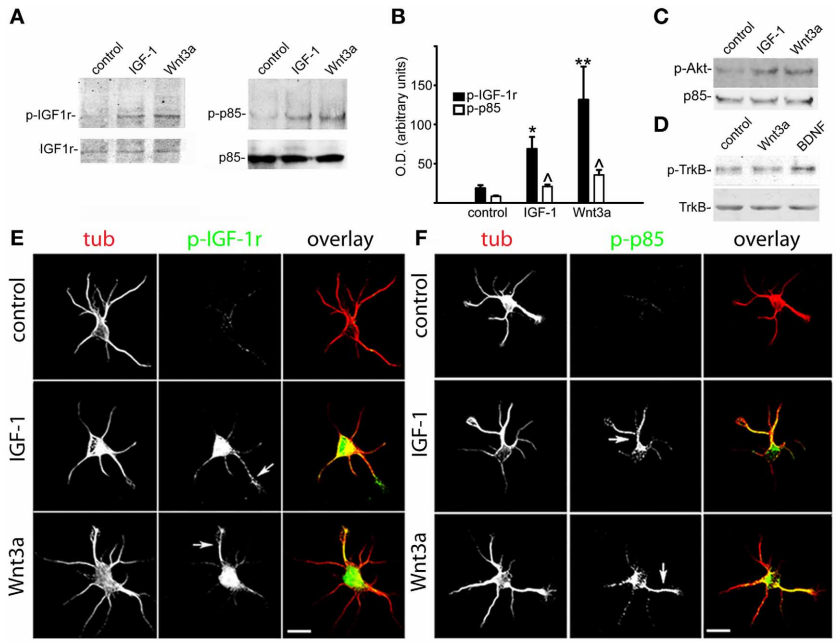

FIGURE 2 | Wnt3a elicited the polarized activation of IGF-1r and PI3k in hippocampal neurons. (A) Western blots of active (phosphorylated) IGF-1r (left) and phosphorylated p85 (right) in control GCPs and GCPs stimulated with either 20 nM IGF-1 or 1.35 nM Wnt3a. Growth cone particles were incubated for 30 min on ice with growth factors and incubated at 37°C for 5 min in the presence of 1 mM ATP. Note the noticeable increment in both the active form of IGF-1r (left) and phosphorylated p85 (right). IGF-1r (left) or total p85 (right) were included as loading controls. **(B)** Relative optical densities of active IGF-1r and phosphorylated p85 in the western blots [similar to those shown in panel **(A)**]. Note the significant increment of both active IGF-1r and phosphorylated p85 in the GCPs stimulated with IGF-1 ($*p < 0.01$; $\hat{}p < 0.05$) or Wnt3a ($**p < 0.001$; $\hat{}p < 0.05$) compared with control. Values are the average + sem of three independent experiments. **(C)** Western blots of active (phosphorylated) Akt in control GCPs or GCPs stimulated with either 20 nM IGF-1 or 1.35 nM Wnt3a. Growth cone particles were kept on ice for 30 min with the growth factors and incubated at 37°C for 5 min in the presence of 1 mM ATP, p85 was included as a loading control. **(D)** Western blots of active (phosphorylated) TrkB receptors in control GCPs or GCPs stimulated with 20 nM IGF-1, 1.35 nM Wnt3a, or 50 ng/ml BDNF. Growth cone particles were kept on ice for 30 min with the growth factors and incubated at 37°C for 5 min in the presence of 1 mM ATP. Total TrkB was included for comparison. **(E)** Double immunofluorescence micrographs of hippocampal neurons after 12 h in culture showing the distribution of the neuronal marker β-III tubulin (tub) and active IGF-1r (p-IGF-1r). Cells were cultured in control medium (control) and challenged for 5 min with either 20 nM IGF-1 (middle) or 1.35 nM Wnt3a (bottom). Note that active IGF-1r was polarized to one neurite in the cells challenged with either IGF-1 (middle arrowhead) or Wnt3a (bottom arrowhead) in stage 2 neurons that had not exhibited a discernible axon. **(F)** Double immunofluorescence micrographs of hippocampal neurons after 12 h in culture showing the distribution of the neuronal marker β-III tubulin (tub) and phosphorylated p85 (p-p85). Cells were cultured in control medium (control) and challenged for 5 min with 20 nM IGF-1 (middle) or 1.35 nM Wnt3a (bottom). Note that phosphorylated p85 was polarized to one neurite in the cells challenged with either IGF-1 (middle arrowhead) or Wnt3a (bottom arrowhead) in stage 2 neurons that had not exhibited a discernible axon. Scale bars: 20 μm.

The activation and immobilization periods were set between 3 and 7 min to couple the desired amount of proteins yielding between 1200 and 2000 resonance units (RU). Recombinant human IGF-1 (analyte) was dialyzed against phosphate-buffered saline (PBS) containing 150 mM NaCl and twofold dilutions were made in the same buffer.

In order to characterize the direct interaction between IGF-1r and mouse Wnt3a or Fz7 [Recombinant Human FZD7/frizzled-7 Protein, made in HEK293 (aa 45–169-Speed Biosystems, MD, USA) preincubated with Wnt3a] IGF-1r was immobilized on a CM5 sensor chip surface, as described above. Recombinant Wnt3a was reconstituted in PBS-0.55% of bovine serum albumin (BSA), and twofold dilutions were made in the same buffer.

All binding experiments were performed at 25°C. Dissociation was carried out in PBS. Pulses of PBS were used to regenerate the surface. SPR data were analyzed using Biacore T100-evaluation 2.0.1 software (Biacore). All the experiments were repeated at least three times and the standard deviations were typically less than 10%. Dissociation constants (K_D) and association and dissociation rates (*kon* and *koff*, respectively), were determined under kinetic conditions after correction for non-specific binding, in which the proteins were passed over blocked, no immobilized surfaces, as previously described (Fernandez et al., 2007, 2011).

RESULTS

Wnt3a TRIGGERED POLARIZATION OF HIPPOCAMPAL NEURONS IN CULTURE AS WELL AS POLARIZED ACTIVATION OF IGF-1r AND PI3k

IGF-1r (activated by IGF-1 and/or insulin) controls initial axonal elongation in hippocampal neurons by activating the PI3k/Akt/Cdc42 pathway (Sosa et al., 2006). To study the possible involvement of the Wnt growth factor family Wnt3a on this process, we cultured pyramidal hippocampal neurons in "control" conditions (in the presence of 5 nM insulin), and in the

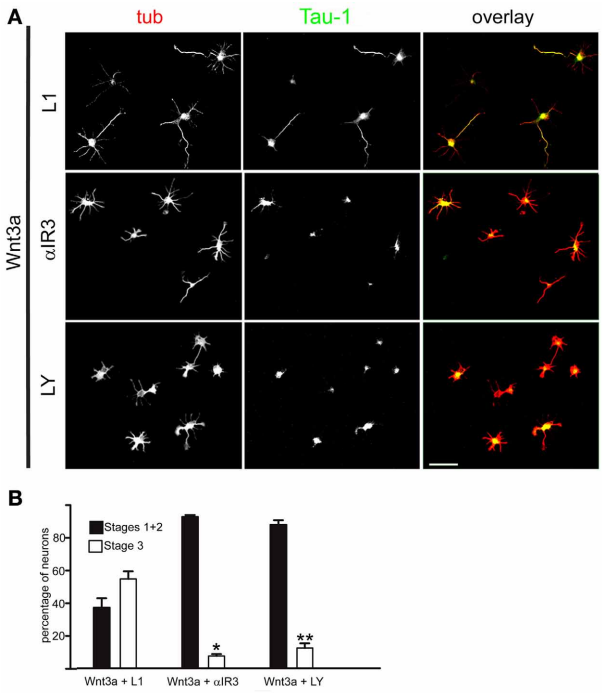

FIGURE 3 | An IGF-1r blocking antibody or the PI3k inhibitor LY294002 hindered the polarizing effects of Wnt3a. (A) Double immunofluorescence micrographs of hippocampal neurons after 20 h in culture showing the distribution of the neuronal marker β-III tubulin (tub) and the axonal marker Tau-1, Cells were cultured in medium containing 1.35 nM Wnt3a plus an antibody to L1 (top), 1.35 nM Wnt3a plus the IGF-1r blocking antibody αIR3 (middle), or 1.35 nM Wnt3a plus the PI3k inhibitor LY294002 (bottom). **(B)** Percentage (±sem) of neurons at different stages of differentiation grown for 20 h in medium containing 1.35 nM Wnt3a plus the anti-L1 antibody, 1.35 nM Wnt3a plus the IGF-1r blocking antibody αIR3, or 1.35 nM Wnt3a plus the PI3k inhibitor LY294002 ($n = 3$ independent experiments). At least 100 cells were scored for each condition. $*p < 0.001$ compared with Wnt3a + L1; $**p < 0.001$ compared with Wnt3a (**Figure 1B**, right). Scale bar: 100 μm.

presence of either 20 nM IGF-1 or 1.35 nM Wnt3a. **Figure 1A** shows that most neurons cultured in control conditions for 20 h failed to form axons; only short, minor neurites were present (**Figure 1A**, top). In contrast, most cells cultured in the presence of IGF-1 (**Figure 1A**, middle) or Wnt3a (**Figure 1A**, bottom) generated long, axon-like processes that were enriched in Tau-1 protein. To analyze this observation quantitatively, we scored the differentiation stages of neurons cultured for 20 h in control conditions or in the presence of IGF-1 or Wnt3a. We found that over 80% of the cells cultured in control conditions remained in stages 1 or 2 of differentiation, and less than 20% had formed a discernible axon (stage 3). In contrast, over 55% of the cells cultured in either the presence of IGF-1 or Wnt3a showed an identifiable Tau-1 containing axon (**Figure 1B**). To determine the specificity of Wnt3a axogenic effects, we used the Wnt pathway antagonist secreted frizzled-related protein 1 (sFRP-1), which binds to Wnt proteins (Rattner et al., 1997). Our results showed that sFRP-1 dose-dependently inhibited Wnt3a polarizing effects (**Figure 1C**). To investigate the possible role of Wnt3a in axon maintenance, we scored the differentiation stages in neurons cultured for 20 h in the presence of 1.35 nM Wnt3a (at this time over 50% of the neurons were in stage 3-see **Figure 1B**). Then, 33.6 nM sFRP1 was added to half of the cultures and the neurons were grown for further 24 h. We found that over 60% of the neurons cultured in the presence of Wnt3a for 44 h were in stage 3 of differentiation, in contrast less that 30% of the cells were in this stage when sFRP-1 was added to the cultures (**Figure 1D**). We also measured axonal length in neurons cultured as in **Figure 1D**. Our results indicated a significant reduction of axonal outgrowth in the neurons treated with sFRP-1 (**Figure 1E**).

Next, we investigated the possible relationship between Wnt3a axogenic effects and the activation of the IGF-1r/PI3k pathway. We initially analyzed the activation of IGF-1r in GCPs challenged with 20 nM IGF-1 or 1.35 nM Wnt3a using a specific antibody for the active (phosphorylated) form of the IGF-1r. We found that Wnt3a significantly activated IGF-1r to a higher degree than the cognate ligand IGF-1 (**Figure 2A**, left). In addition, Wnt3a also significantly activated PI3k (**Figure 2A**, right). Quantification of these results (by measuring the optical density of the different bands in the western blots) is shown in **Figure 2B**. Our results also showed a noticeable activation of Akt, a kinase downstream of PI3k (**Figure 1C**). We also analyzed the possible Wnt3a-induced activation of another tyrosine-kinase receptor, TrkB (the BDNF receptor), which is enriched at the growth cone of neurons (Pfenninger et al., 2003). Our results show that Wnt3a did not cause any noticeable activation of TrkB (**Figure 2D**). In the present study, we found that challenging the neurons with 1.35 nM Wnt3a resulted in a distribution of active IGF-1r that was essentially identical to that achieved upon challenge with 20 nM IGF-1 (**Figure 2E**-middle and bottom). We also found that challenging with either 20 nM IGF-1 or 1.35 nM Wnt3a resulted in a polarized distribution of phosphorylated p85, the PI3k regulatory subunit, to one neurite of cells in stage 2 (**Figure 2F**).

TRIGGERING OF INITIAL AXONAL OUTGROWTH AND THE ESTABLISHMENT OF NEURONAL POLARITY BY Wnt3a REQUIRED THE PRESENCE OF ACTIVATABLE IGF-1r AND ACTIVATION OF PI3k

To further investigate the relation between Wnt3a and the IGF-1r/PI3k pathway on the establishment of neuronal polarity, we designed experiments to study the possible mechanism(s) by which Wnt3a stimulates IGF-1r and PI3k. Our results indicate that the addition of an IGF-1r blocking antibody prevented polarization of Wnt3a-challenged neurons (**Figure 3A**, middle), compared with cells cultured in the presence of a monoclonal antibody to the neural adhesion molecule L1 added at the same protein concentration (**Figure 3A**, top); therefore, most of these neurons remained at stage 2 after 20 h in culture (**Figure 3B**). Moreover, in loss of function experiments using a shRNA derived from the IGF-1r sequence (which uniformly silenced the expression of βgc-containing IGF-1r, shown in **Figures 4A,B**) (Sosa et al., 2006) we observed that the silenced cells challenged with Wnt3a failed to form axons and generated only short, minor processes (**Figure 4A**). To analyze this observation quantitatively,

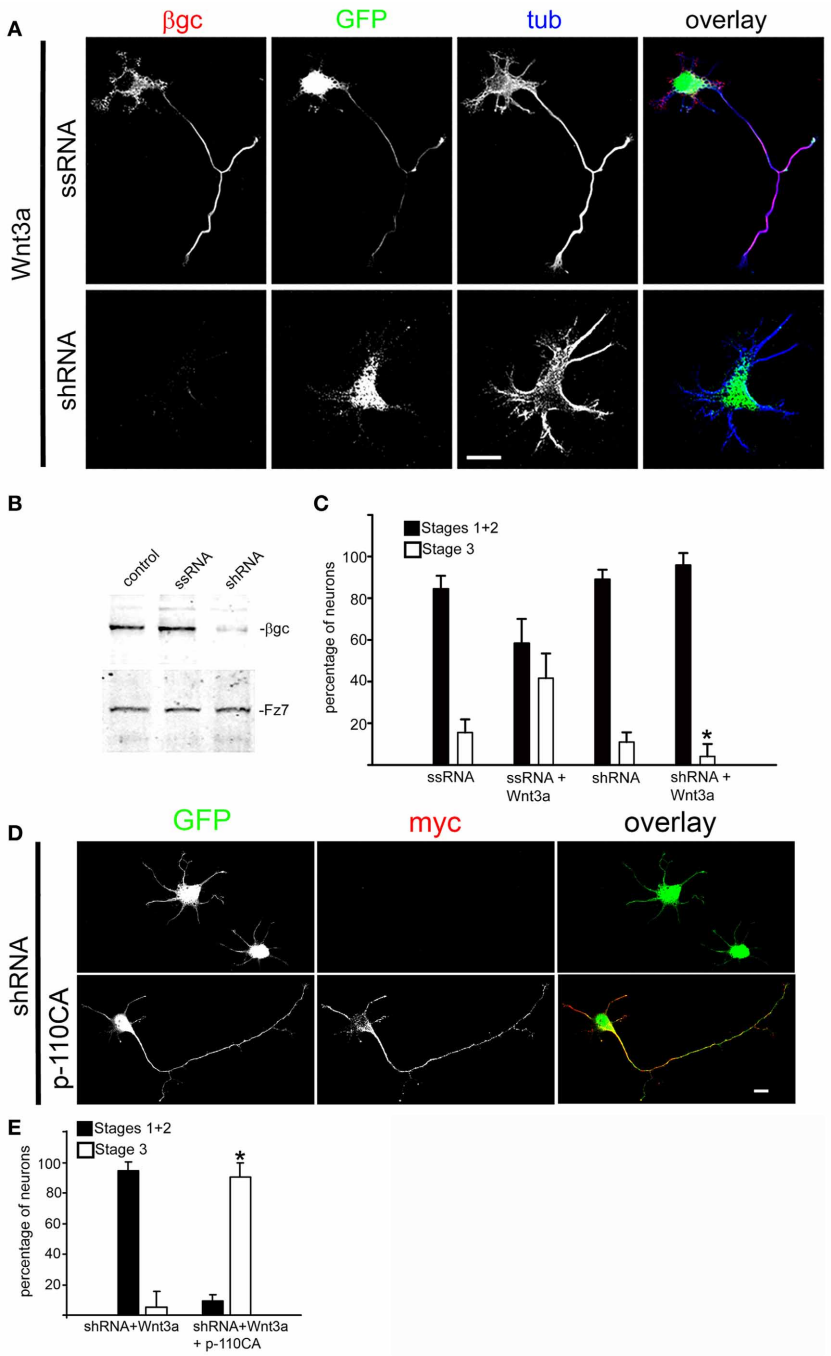

FIGURE 4 | Wnt3a polarizing effects required normal expression of IGF-1r. (A) Triple immunofluorescence micrographs of hippocampal neurons after 24 h in culture showing the distribution of the IGF-1r βgc subunit, a scrambled sequence RNA (GFP, top) or IGF-1r targeted shRNA (GFP, bottom), and the neuronal marker β-III tubulin (tub). Cells were cultured in medium containing 1.35 nM Wnt3a. The ssRNA transfected cells (top) showed a normal morphology. In contrast, the shRNA transfected cells (bottom) did not develop an axon. **(B)** Western blots showing protein levels of βgc, the Wnt receptor Fz7 (to control specificity, also serves as loading control) and cells transfected with a scrambled RNA sequence (ssRNA), and cells transfected with IGF-1r shRNA (shRNA). We observed a substantial decrease in βgc expression in the shRNA treated cells. **(C)** Percentage (±sem) of neurons at different specific stages of differentiation grown for 20 h in culture in control medium or in the presence of 1.35 nM Wnt3a and transfected with a scrambled sequence RNA (ssRNA) or IGF-1r targeted shRNA (shRNA) ($n = 3$ independent experiments). At least 100 transfected cells (GFP positive) were scored for each condition. $^*p < 0.005$ compared to ssRNA + Wnt3a. Scale bar: 20 μm. **(D)** Immunofluorescence of hippocampal neurons after 24 h of DIV showing the distribution of myc (marker of transfection with cDNA encoding for a constitutively active form of the PI3k catalytic subunit p110). GFP is a marker of transfection efficiency with the shRNA directed to IGF-1r.Calibration bar = 20 μm. **(E)** Percentage (±sem) of neurons at different specific stages of differentiation grown for 20 h in culture in the presence of 1.35 nM Wnt3a and transfected with IGF-1r shRNA (shRNA) or co-transfected with shRNA and a constitutively active form of the PI3k catalytic subunit p110 (p110CA). $n = 3$ independent experiments. Thirty or more transfected or co-transfected cells were scored in each experiment. $^*p < 0.02$ compared to shRNA.

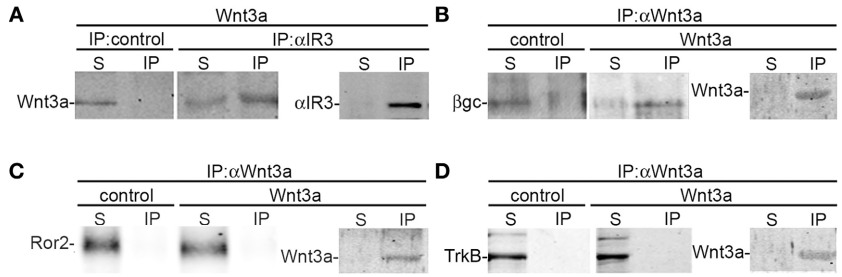

FIGURE 5 | Wnt3a interaction with IGF-1r. (A) Growth cone particles were stimulated with Wnt3a and immunoprecipitated with a non-relevant primary antibody (IP control) or the αIR3 antibody to pull down IGF-1r. Blots were probed with an anti-Wnt3a antibody. Immunoprecipitation of αIR3 is shown to confirm successful IP and as a loading control. **(B–D)** Growth cone particles were incubated in control buffer or in the presence of 1.35 nM Wnt3a, immunoprecipitated with anti-Wnt3a and probed with **(B)** βgc antibody to reveal IGF-1r; **(C)** anti-Ror2; and **(D)** anti-TrkB (BDNF receptor prominent at the growth cone of developing neurons) Immunoprecipitation of Wnt3a is shown to confirm successful IP and as a loading control. IP, immunoprecipitate; S, soluble fraction after immunoprecipitation. All blots are representative of at least three independent experiments.

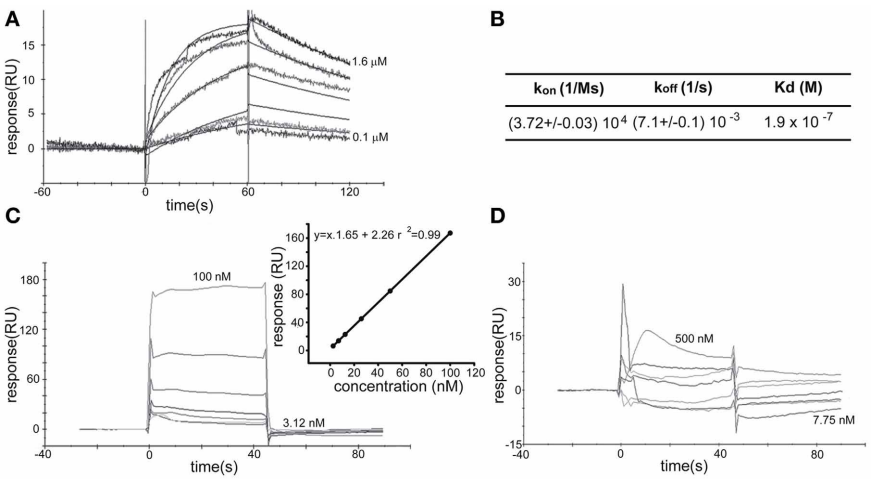

FIGURE 6 | Surface Plasmon Resonance Analysis. (A) SPR sensogram of the interaction between hIGF-1 (1600–100 nM) and IGF-1r (1600 RU immobilized) after correction for non-specific binding. **(B)** The apparent KD for the binding of hIGF-1 to immobilized IGF-1r was 1.3×10^{-7} M by kinetic analysis. kon and koff from where KD was calculated are also shown. **(C)** SPR sensogram of the interaction between soluble Wnt3a (3125–100 nM) and IGF-1r (1600 RU). (Inset) The linear dependence of the response in function of the concentration of soluble Wnt3a injected over immobilized IGF-1r support the idea of non-specific direct binding. **(D)** Soluble IGF-1r (7.75–500 nM) showed no specific biding to immobilized Wnt3a.

we scored the differentiation stages of neurons transfected with a scrambled sequence RNA (ssRNA) or with IGF-1r targeted shRNA after 24 h in culture. Quantification showed that only about 6% of shRNA-transfected neurons formed a discernible axon when challenged with Wnt3a, whereas 45% of controls containing ssRNA formed axons (**Figure 4C**). Since PI3k operates downstream of the IGF-1r and to eliminate possible artifacts due to toxic or deleterious effects of transfection with the shRNA, we cotransfected cells with the IGF-1r shRNA and with a cDNA encoding a myc-tagged constitutively active form of PI3k, p110. The results showed that co-transfection completely rescued the phenotype (**Figure 4D**), with over 90% of co-transfected cells exhibiting long, axon-like processes compared to less than 10% of cells transfected with IGF-1r shRNA alone (**Figure 4E**).

Most cells challenged with Wnt3a in the presence of the PI3k inhibitor LY294002 also failed to form axons (**Figure 3A**, bottom). As shown in **Figure 3B**, less that 10% of the neurons were in stage 3 after 24 h in culture, which was in contrast to Wnt3a-challenged neurons cultured in the absence of the PI3k inhibitor.

Wnt3a ACTIVATED THE IGF-1r-PI3k PATHWAY THROUGH THE FORMATION OF AN ACTIVE IGF-1r-Fz7-Wnt3a COMPLEX

To further study the association between Wnt3a and the IGF-1r, we performed immunoprecipitation experiments using GCPs challenged with Wnt3a and crosslinked with 3,3′-dithio*bis* (sulfosuccinimidylpropionate). The results revealed that an antibody to IGF-1r (clone αIR3) co-immunoprecipitated Wnt3a (**Figure 5A**). In addition, an antibody to Wnt3a co-immunoprecipitated IGF-1r in GCPs challenged with Wnt3a prior to crosslinking and immunoprecipitation (**Figure 5B**). Ror2 is a tyrosine kinase Wnt3a co-receptor found in non-neuronal cells (Li et al., 2008), and shown to be highly enriched at the leading edge

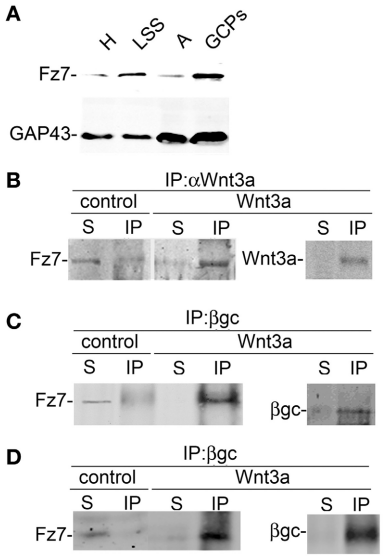

FIGURE 7 | Activation with Wnt3a triggers the formation of a IFG-1r/Wnt3a/Fz7 complex. (A) Fz7 is enriched in GCPs. Western blot analysis of the distribution of the Wnt receptor Fz7 in different subcellular fractions of fetal brain: homogenate (H), low-speed supernatant (LSS), A-fraction (A), and growth cone particles (GCPs). Equal amounts of protein were loaded in all lines. Note the enrichment of Fz7 in GCPs around fourfold compared to (H). A blot showing the distribution of GAP-43, a protein highly enriched in GCPs, was included for comparison. **(B**,**C)** Wnt3a triggered the formation of a complex containing IGF-1r-Wnt3a-Fz7. Growth cone particles were incubated in control buffer or in the presence of 1.35 nM Wnt3a and immunoprecipitated with **(B)** Anti Wnt3a and probed with an antibody to the Wnt receptor Fz7 and **(C)** immunoprecipitated with βgc antibody to pull down IGF-1r and probed with anti Fz7. Immunoprecipitation of Wnt3a and βgc are shown to confirm successful IP and as a loading control. IP, immunoprecipitate; S, soluble fraction after immunoprecipitation. **(D)** Total protein from hippocampal neurons cultured for 20 h in the presence of 1.35 nm Wnt3a, deprived of any growth factor for 4 h, and stimulated (or not) with 1.35 nM Wnt3a for 5 min immunoprecipitated with anti βgc antibody to pull down the IGF-1r and probed with anti Fz7. Immunoprecipitation of βgc is shown to confirm successful IP and as a loading control. IP, immunoprecipitate; S, soluble fraction after immunoprecipitation. All blots are representative of at least three independent experiments.

of hippocampal pyramidal neurons in culture (Paganoni and Ferreira, 2005). After crosslinking of GCPs challenged with 1.35 nM Wnt3a, we did not observe co-immunoprecipitation of Ror2 with the Wnt3a antibody (**Figure 5C**). Another prominent tyrosine kinase receptor of the growth cone in developing neurons is TrkB, the BDNF receptor, which also failed to co-immunoprecipitate with the Wnt3a antibody (**Figure 5D**). Taken together, these results indicate that only IGF-1r co-immunoprecipitated with Wnt3a, whereas related tyrosine kinase receptors, such as Ror2 and TrkB, did not. These experiments, together with those indicating that the IGF-1r blocking antibody prevents Wnt3a axogenic effects, suggested a possible direct interaction between Wnt3a and the IGF-1r. To assess this issue we performed surface plasmon resonance (SPR) experiments. To validate the method employed, the affinity between IGF-1 and its cognate receptor, the IGF-1r, was determined. Twofold dilutions of IGF-1 (1.6–0.1 μM) were passed on immobilized IGF-1r for 60 s and response units (RU) were recorded. Dissociation was carried out using PBS. The sensogram showed specific interaction (**Figure 6A**) and the kinetic parameters were calculated with the Biacore T100 Evaluation software fitting to a 1:1 binding model (**Figure 6B**). These results are in line with those found in the literature (Surinya et al., 2008). In contrast, when different concentrations of soluble Wnt3a were passed on IGF-1r, no specific binding could be detected (**Figure 6C**). This could also be appreciated by the linear response in the graphic of the RU in function of the concentration (**Figure 6C**, inset). To preclude any possibility of inactivation of the Wnt3a binding site by immobilization of IGF-1r, an interaction analysis in the reverse orientation was made. Thus, Wnt3a was immobilized (3500 RU) and different concentration of IGF-1r were assayed as analyte. **Figure 6D** shows no binding of these molecules corroborating the previous result that there is no direct interaction between these proteins in an isolated system. These results raised the possibility that binding of Wnt3a to IGF-1r and its consequent activation could be achieved through the formation of a complex via binding of Wnt3a to a cognate receptor protein. We found that the Wnt family receptor Fz7 is highly enriched at the growth cone of differentiating neurons (**Figure 7A**). As expected, the Wnt3a antibody co-immunoprecipitated Fz7 (**Figure 7B**). Moreover, an antibody to the βgc subunit of IGF-1r co-immunoprecipitated Fz7 only when the GCPs were preincubated with Wnt3a (**Figure 7C**). In addition, we obtained similar results by immunoprecipitating proteins from cultures of hippocampal neurons deprived of growth factors for 4 h and kept in control conditions (no co-immunoprecipitation was observed) or stimulated for 5 min with Wnt3a (**Figure 7D**). It follows that binding of Wnt3a to Fz7 promoted the formation of an IGF-1r (active)-Wnt3a-Fz7 complex at the growth cone of hippocampal pyramidal neurons in culture. To get more information about the characteristics of this complex we performed SPR experiments passing the extracellular moiety of Fz7 (aa 45–169) preincubated (or not) 1:1 with Wnt3a on immobilized IGF-1r (extracellular moiety). No specific interaction between the two receptors was detected in this experiment (not shown). Finally, we performed experiments in order to obtain direct evidence about the participation of the Fz7 receptor in Wnt3a induced neuronal polarization. For that purpose, we transfected hippocampal pyramidal neurons with the myc-tagged dominant negative Fz7 CRD which uncouples the binding of Wnts to Fz7 from the receptor activation (Wei et al., 2011). The results of these experiments are shown in **Figure 8**, a quantification of the transfected neurons indicated that less than 5% of the cells transfected with Fz7 CRD reached stage 3 of differentiation, exhibiting a Tau-1 positive axon, compared to over 60% of the control cells transfected with the plasmid expressing only myc (**Figure 8B**).

DISCUSSION

Secreted Wnts interact with their membrane receptors and activate three different intracellular cascades: (i) The canonical or Wnt/β-catenin pathway, which involves cytosolic stabilization of β-catenin, its translocation to the nucleus, and

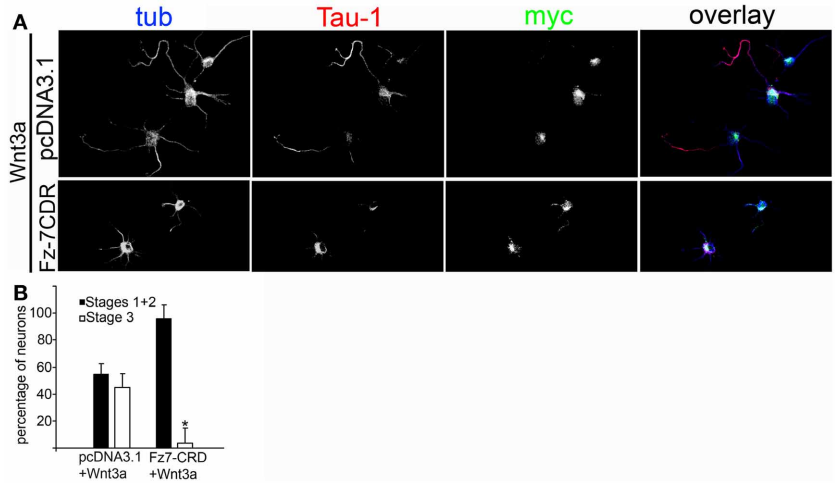

FIGURE 8 | (A) Wnt3a polarizing effect depends on the activation of Fz7. Double immunofluorescence micrographs of hippocampal neurons incubated for 24 h in the presence of 1.35 nM Wnt3a showing the distribution of the axonal marker Tau-1 and the neuronal marker β-III-tubulin. myc is a marker of transfection with the negative dominant form of Fz7-CRD. Scale bar: 20 mm. **(B)** Percentage (±sem) of neurons at different stages of differentiation grown for 20 h in medium containing 1.35 nM Wnt3a and transfected with pcDNA3.1-myc or Fz7-CRD ($n = 3$ independent experiments). At least 20 transfected cells were scored for each condition. $*p < 0.01$ compared with pcDNA3.1-myc.

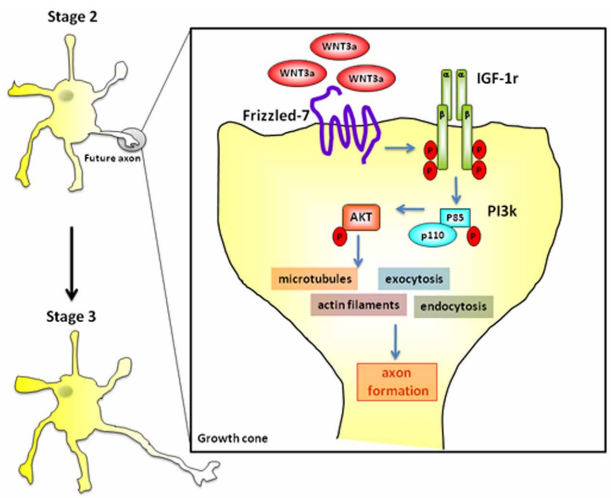

FIGURE 9 | Wnt3a binds to Fz7 and cross-activate the IGF-1-PI3k-Akt pathway inducing initial axonal outgrowth and neuronal polarization. Challenging with 1.35 nM Wnt3a resulted in a polarized distribution of active (phosphorylated) IGF-1r and phosphorylated p85, the PI3k regulatory subunit, to one neurite of cells in stage 2 (see **Figure 2F**).

the transcriptional activation of specific genes; (ii) The planar cell polarity pathway, which involves the activation of small Rho-GTPases and kinases that modulate cell orientation and organization; and (iii) The Wnt/calcium pathway, which regulates intracellular levels of calcium and the activation of Ca^{+2}-sensitive kinases (Ciani and Salinas, 2005). To activate intracellular pathways, Wnts interact with different families of receptors, such as seven-span transmembrane receptor molecules known as Frizzled, the co-receptors from the family of low-density lipoprotein receptor-related proteins (LRP 5/6), and two atypical tyrosine-kinase receptors Ror1 or 2 and Ryk (Angers and Moon, 2009). Wnts signal through the novel Ryk receptor via multiple mechanisms, including nuclear translocation of their intracellular domains and activation of pathways employing Src family kinases and members of the canonical Wnt pathway (Fradkin et al., 2010). It has also been shown that one member of the Wnt family involved in hippocampal formation, Wnt3a, induces Akt activation via direct activation of PI3k in fibroblasts (Kim et al., 2007). In addition, Wnt3a has been shown to activate Akt in PC12 cells (Fukumoto et al., 2001). The receptor system(s) involved in PI3k activation by Wnt3a, however, have not been identified. We have previously shown that activation of IGF-1r (and activation of PI3k) is essential for polarization of cultured hippocampal pyramidal neurons (Sosa et al., 2006). The results shown here demonstrate that Wnt3a can promote initial axonal outgrowth and neuronal polarization in the absence of IGF-1 and also suggest that Wnt3a is important for axon maintenance and further axonal outgrowth after polarization. These results and previously published data prompted us to investigate the possible activation of PI3k by Wnt3a in cultured hippocampal neurons to identify the receptor(s) involved in PI3k activation, and determine Wnt3a consequences on initial axonal outgrowth and neuronal polarization. Surprisingly, we found that Wnt3a could trigger a robust and significant activation of IGF-1r, PI3k, and Akt in GCPs (in the absence of IGF-1 or high insulin; **Figures 2A–C**). Activation of IGF-1r and PI3k was polarized to one neurite of cells in stage 2 of differentiation that had not exhibited a noticeable axon (**Figure 2C**). Moreover, challenge with Wnt3a in defined medium that did not contain any growth factors except insulin (5 nM) was sufficient

to trigger the establishment of neuronal polarity (**Figure 1**). Wnt3a polarizing activity required the presence of activatable, membrane inserted IGF-1r (**Figures 3**, **4**). Crosslinking and immunoprecipitation experiments of GCPs challenged with Wnt3a suggested interaction of Wnt3a with IGF-1r. However, by SPR experiments no direct interaction between IGF-1r and Wnt3a was observed neither when IGF-1r was immobilized nor when Wnt3a was attached to the chip surface (**Figures 6C,D**). Thus, SPR results suggested no direct, specific interaction between these proteins in an isolated system. Finally, by immunoprecipitation experiments we observed that stimulation with Wnt3a triggered the formation of a complex containing IGF-1r-Wnt3a-Fz7. Moreover, we demonstrated that Fz7 is essential for the Wnt3a observed effects in hippocampal neurons. It follows that axogenic effects of Wnt3a are due to a polarized cross-activation of the IGF-1r/PI3k pathway. Interestingly, there is a significant temporal overlap between high Wnt3a expression (Lee et al., 2000) and the initial outgrowth of axons in the hippocampus "*in situ*" (Fletcher and Banker, 1989). Since the expression of IGF-1 gene and its transcript is high in the developing brain but decreases significantly in the adult (Rotwein et al., 1988; Leroith et al., 1993), the activation of the IGF-1r by other growths factor, such as Wnts, could significantly expand the time window for the activation of this receptor. A cross-talk between the insulin receptor (homologous to the IGF-1r) and Wnt have been recently shown in non-neuronal cells involving co-receptor low density lipoprotein receptor-related protein-5 (Palsgaard et al., 2012). Besides its central role in the establishment of neuronal polarity, the PI3k-Akt pathways is involved in the regulation of many different phenomena in the central nervous system, including cell division (during neuronal proliferation), large scale cellular remodeling (during differentiation-including dendrite and synapses development), and synaptic specific changes during plasticity-(Knafo and Esteban, 2012). Many neurobehavioral disorders arise as a consequence of subtle developmental abnormalities and aberrant PI3k signaling has been indicated by many studies to be a contributing factor to the pathophysiology of disorders such as schizophrenia and autism (Waite and Eickholt, 2010). Moreover, PI3k signaling strongly enhances the resistance of several neuron types against aggressions such us oxidative damage and hypoxia. It follows that activation of PI3k by Wnt3a, in neurons, can have a significant physiological and/or pathophysiological impact.

As discussed above, numerous receptors and co-receptors have been identified for the members of the Wnt family growth factors, and Frizzled-3 (Fz3) has been proposed to have a role in neurite outgrowth and nervous differentiation of glioblastoma cells (Rampazzo et al., 2013). Also, a mouse Fz3 knock out exhibited defects in axonal outgrowth and guidance (Wang et al., 2006). In our experimental system, however, we did not find detectable expression of Fz3 using two different antibodies (not shown) at least inside the times of culture used in this study. In contrast, we found high levels of Fz7 in neurons dissociated from hippocampi at embryonic day 18 (E18) cultured for 20 h and GCPs prepared from fetal rat brain (E18). A high level of expression of Fz7 was also found in rat hippocampi (E18) (Varela-Nallar et al., 2012).

As mentioned above, we did not find evidences indicating a direct interaction of the IGF-1r with Fz7 bound to Wnt3a using SPR. Several reasons can explain this observation, including: (i) That the presence of transmembrane and/or intracellular regions of one or both receptors are necessary for the interaction, since we used the extracellular moiety of the receptors in our SPR experiments; (ii) That the interaction between the receptors is not direct. There are published results that can support this possibility. For example, cross activation of the LRP6 receptor by Wnts depends on the formation of a complex containing Frizzled, LRP6, Disheveled-1, and the axin Gsk3 complex (Zeng et al., 2008). Hence, more investigation will be needed to obtain a complete picture of the components of the IGF-1r-Wnt3a-Fz7 complex and the characteristics of the binding of these different components. Interestingly, it has been recently published that IGF-1 signaling interacts with canonical Wnt signaling to promote neuronal proliferation (Hu et al., 2012).

Taken together, our results indicate that cross activation of the IGF-1r/PI3k pathway by Wnt3a is sufficient for the establishment of neuronal polarity by regulating initial axonal elongation (a cartoon showing the proposed activation pathway is shown in **Figure 9**). Indeed, the present results suggest that Wnt3a, together with IGF-1, is one of the growth factors initiating axonal outgrowth in hippocampal neurons.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: María E. Bernis, Mariana Oksdath, Sebastián Dupraz, Alvaro Nieto Guil, Marisa M. Fernández, Emilio L. Malchiodi, Silvana B. Rosso, and Santiago Quiroga. Performed the experiments: María E. Bernis, Mariana Oksdath, Sebastián Dupraz, Alvaro Nieto Guil, and Marisa M. Fernández. Analyzed the data: María E. Bernis, Mariana Oksdath, Sebastián Dupraz, Alvaro Nieto Guil, Marisa M. Fernández, Emilio L. Malchiodi, Silvana B. Rosso, and Santiago Quiroga. Contributed reagents/materials/analysis tools: Sebastián Dupraz, Silvana B. Rosso, Marisa M. Fernández, and Emilio L. Malchiodi. Wrote the paper: María E. Bernis, Sebastián Dupraz, Marisa M. Fernández, Emilio L. Malchiodi, Silvana B. Rosso, and Santiago Quiroga.

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Chemokines induce axon outgrowth downstream of Hepatocyte Growth Factor and TCF/β-catenin signaling

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Axon morphogenesis is a complex process regulated by a variety of secreted molecules, including morphogens and growth factors, resulting in the establishment of the neuronal circuitry. Our previous work demonstrated that growth factors [Neurotrophins (NT) and Hepatocyte Growth Factor (HGF)] signal through β-catenin during axon morphogenesis. HGF signaling promotes axon outgrowth and branching by inducing β-catenin phosphorylation at Y142 and transcriptional regulation of T-Cell Factor (TCF) target genes. Here, we asked which genes are regulated by HGF signaling during axon morphogenesis. An array screening indicated that HGF signaling elevates the expression of chemokines of the CC and CXC families. In line with this, CCL7, CCL20, and CXCL2 significantly increase axon outgrowth in hippocampal neurons. Experiments using blocking antibodies and chemokine receptor antagonists demonstrate that chemokines act downstream of HGF signaling during axon morphogenesis. In addition, qPCR data demonstrates that CXCL2 and CCL5 expression is stimulated by HGF through Met/b-catenin/TCF pathway. These results identify CC family members and CXCL2 chemokines as novel regulators of axon morphogenesis downstream of HGF signaling.

Keywords: beta-catenin, axon, neurite outgrowth, chemokine, hippocampal neurons, hepatocyte growth factor

INTRODUCTION

The establishment of the neuronal morphogenesis is a complex process by which neurons extend and branch out an axon and dendrites, resulting in the proper assembly of the neuronal circuitry. A range of secreted molecules, including growth factors and morphogens, promote axonal, and dendrite outgrowth. Among them, the Neurotrophins (NT), Hepatocyte Growth Factor (HGF), and Wnts regulate neuronal survival, neurite outgrowth, synaptogenesis, and synaptic plasticity (Maina and Klein, 1999; Korhonen et al., 2000; Chao, 2003; Yu and Malenka, 2003; Ciani and Salinas, 2005; Nakano et al., 2007; Park and Shen, 2012). HGF signaling through its tyrosine kinase receptor Met provides neurotrophic signals to hippocampal neurons (Korhonen et al., 2000) and promotes axon outgrowth (David et al., 2008). How neurons interpret this variety of signals to develop unique axon arbor morphologies is just beginning to be understood.

β-catenin, a component of the cell–cell adhesion complex and an effector of canonical Wnt signaling, plays a key role in axon outgrowth, dendritogenesis, and synapse formation (Murase et al., 2002; Bamji et al., 2003, 2006; Yu and Malenka, 2003; Lu et al., 2004). Briefly, canonical Wnt signaling results in the cytosolic stabilization of β-catenin, which in the absence of Wnt is degraded through the ubiquitin-proteasome system (Li et al., 2012). Stabilized β-catenin translocates to the nucleus and together with Lymphoid Enhancer Factor-1 (LEF-1)/T-cell factor (TCF) transcription factors regulates the expression of Wnt target genes (Behrens et al., 1996; Molenaar et al., 1996). On the other hand, β-catenin binding to the adhesion complex components cadherin and α-catenin is altered by β-catenin tyrosine phosphorylation, resulting in the downregulation of cell adhesion and the promotion of migration (Nelson and Nusse, 2004; Heuberger and Birchmeier, 2010). We previously showed a requirement for β-catenin phosphorylation at Y654 and Y142 in the axon outgrowth promoted by the NT and HGF signaling, respectively. HGF signaling induces the phosphorylation of β-catenin at Y142 (PY142), which translocates to the nucleus and promotes axon morphogenesis through TCF4/β-catenin-dependent transcription of target genes (David et al., 2008). These findings highlight the relevance of β-catenin forms producing transcriptional regulation independent of Wnt signaling (Monga et al., 2002; Zeng et al., 2006; Heuberger and Birchmeier, 2010; Xi et al., 2012).

Chemotactic cytokines ("chemokines") are small proteins classified into four subgroups referred to as CXC/α, CC/β, CX_3C/δ, or C/δ families (Zlotnik and Yoshie, 2000; Tran and Miller, 2003) according to the position and spacing of cysteine residues important for their tri-dimensional structure. Chemokines are best known for their role in leukocyte migration in host immune surveillance and inflammatory responses. However, chemokines and their G-protein-coupled receptors are also expressed by neurons and glia in the nervous system. Interestingly, chemokines of the CXC and CC families have been implicated in proliferation, neurogenesis, and neuronal differentiation of neural precursors

(Tran and Miller, 2003; Edman et al., 2008a,b; Wu et al., 2009). Meningeal CXCl12/Sdf-1 signaling through its receptor CXCR4 regulates the migration of cerebellar progenitors (Zou et al., 1998; Reiss et al., 2002) and Cajal-Retzius cells (Borrell and Marin, 2006). CXCL12 also controls interneuron migration during cortical development (Stumm et al., 2003; Lopez-Bendito et al., 2008; Lysko et al., 2011; Sanchez-Alcaniz et al., 2011; Wang et al., 2011). Furthermore, CXCL12 reduces axon outgrowth and branching in hippocampal neurons (Pujol et al., 2005). Importantly, neuronal migration and morphogenesis are coordinated processes that appear inversely regulated by CXCL12 signaling. CXCL12 increases the rate of interneuron migration while reducing neurite branching, whereas blocking CXCL12 signaling enhances neurite branching, thus explaining how interneurons switch from migratory streams to invade the cortical plate and branch out extensively (Lysko et al., 2011). Moreover, astrocyte-secreted CCL5/Rantes induces the outgrowth of cortical neuron neurites (Chou et al., 2008). CCL5 secretion is suppressed in astrocytes from a Huntington mouse model (Chou et al., 2008), indicating that chemokine signaling is involved in neuronal physiology and pathology.

Here we asked which are the genes regulated by HGF/β-catenin signaling during axon morphogenesis. We observe that expression of CC and CXC chemokines is upregulated by HGF signaling in hippocampal neurons. We find that chemokines promote axon outgrowth in hippocampal neurons, the most remarkable one being CXCL2 that also stimulates axon branching. Experiments using chemokine blocking antibodies and pharmacological inhibitors of chemokine receptors demonstrate that chemokines act downstream of HGF signaling. We also show that chemokine expression is reduced upon Met and TCF inhibition. These results identify the chemokines as novel regulators of axon morphogenesis downstream of HGF and β-catenin/TCF signaling.

MATERIALS AND METHODS

MATERIALS

HGF was purchased from Peprotech, Wnt-3a from Millipore, Hoescht-33258, SU11274, and FH535 were from Sigma, and SB225502 and SB324837 from Tocris. Antibodies were purchased from the following companies: βIII-tubulin from Covance, β-actin from Sigma, β-catenin from Becton-Dickinson and anti-rat CCL20 and CXCL2 antibodies from R&D. Rat CCL5, CCL7, CCL20 were from R&D Systems and CXCL2 from Peprotech.

HIPPOCAMPAL CULTURES

Rat primary hippocampal neurons were isolated from 18–19 day embryos and cultured in DMEM medium supplemented with N2 and B27. Neurons were plated on poly-D-lysine coated (500 μg/ml) glass coverslips for immunostaining (40 cells/mm^2) or on plastic (1000–1500 cells/mm^2) for RNA isolation.

IMMUNOFLUORESCENCE AND AXON MEASUREMENTS

Neurons plated on coverslips were treated at the first day in vitro (1DIV) and fixed at 2DIV. Treatments were as follows: 10, 300, or 1000 ng/ml for chemokines; HGF 50 ng/ml; Wnt-3a 100 ng/ml; SU11274 2 μM; FH535 10 μM; SB225502 1.25 nM; SB324837 20 nM; blocking antibodies against rat CCL20 and CXCL2 (40 μg/ml) or ovalbumin at the same concentration. Neurons were fixed with 4% paraformaldehyde (PFA) for 20 min at RT. Cells were then washed with phosphate buffer saline (PBS) and blocked and permeabilized in PBS containing 5% Foetal calf serum, 5% Horse serum, 0.2% glycine, and 0.1% Triton X100, before incubation with βIII-tubulin antibody. Secondary antibodies were Alexa Fluor488 or Fluor594 (Molecular Probes). Coverslips were mounted on Mowiol. Micrographs were obtained using an inverted Olympus IX70 microscope (10×, 0.3 NA, or 20×, 0.4 NA) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti). Images were acquired using DPM Manager Software and processed using MacBiophotonics ImageJ software (www.macbiophotonics.ca). Axon length was measured using Adobe Photoshop software and the axon was identified as the longest neurite at this stage (2 DIV) of the hippocampal cell development. Images were inverted using Photoshop and are shown on a white background for a clearer visualization of their morphology. Branching was measured by counting Total Axonal Branch Tip Number (TABTN) (Yu and Malenka, 2003). Typically, 15–20 neurons were measured/condition in ≥ three independent experiments. Axon length and branching plots represent values compared to the corresponding untreated control, shown as average ± s.e.m. Significance was calculated by the Student's *t*-test. Asterisk (*) indicates statistical significance compared to the corresponding untreated control and hash (#) compared to stimulated controls (see legends for details).

LUCIFERASE ASSAY

To determine β-catenin transcriptional activation status, luciferase assay was performed following transfection of the TOP-Flash plasmid that carries a synthetic promoter containing three copies of the TCF-4 binding site upstream of a firefly luciferase reporter gene. Hek293T cells were plated at a density of 100 cells/mm^2 and transfected with Lipofectamine 2000 (Life Technologies) on the day next after plating. Treatments were given on the following day for 24 h (HGF 50 ng/ml; Wnt-3a 100 ng/ml; FH535 8 μM and SU11274 2 μM). After 48 h of transfection, cells were lysed in lysis buffer 25 mM glycylglycine, pH 7.8, 15 mM Mg_2SO4, 1% Triton X-100, 5 mM EGTA and rocket on ice for 15 min. Luciferase activity in the cell lysates was determined in Luciferase Buffer (25 mM glycylglycine, 15 mM $KHPO_4$, pH 7,8, 15 mM Mg_2SO_4, 1% Triton X-100, 5 mM EGTA, 1 mM dithiothreitol, 2 mM ATP, 100 mM acetyl-coenzymeA, and 100 mM luciferine) using a microplate luminometer. Luciferase activity was normalized for the total protein concentration in each condition.

shRNA EXPRESSION BY LENTIVIRAL INFECTION

shRNA vectors specific for rat β-catenin were transfected into Hek293T cells together with the plasmids psPAX2 and pMD2G, as previously described (David et al., 2008). The Hek293T medium was collected after 48 h of transfection and centrifuged at 50,000×g for 3 h. The viral pellet was re-suspended in sterile PBS plus 2% Bovine Serum Albumin. Hippocampal neurons were transduced 3–4 h after plating. According to GFP expression driven by the lentiviral vector ∼90% of neurons were transduced.

Neurons were treated with HGF alone or together with pharmacological inhibitors at 3 DIV and collected at 4 DIV for mRNA purification (RNA isolation kit; Macherey-Nagel). Efficiency of the silencing induced by shRNA was evaluated by Western-blotting of 4DIV hippocampal neuron cell lysates. Densitometric analysis of the bands was performed using Scion software and β-catenin levels were normalized to the intensity of β-actin band.

RNA ISOLATION

For RNA isolation, treatments were performed as for immunofluorescence studies. Pervanadate was applied for the last 2 h of HGF stimulation. RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel), including a DNase digestion step to remove contaminant DNA.

ARRAY PROCESSING AND ARRAY DATA ANALYSIS

RNA samples (800 ng) were amplified and labeled with Cy3-CTP using the One-Color Microarray-Based Gene Expression Analysis Protocol (Agilent Technologies, Palo Alto, CA, USA) and hybridized to Whole Rat Genome Microarray 4 × 44K (G4131F, Agilent Technologies).

Raw data files from the scanned arrays were extracted using Feature Extraction software version 9 (Agilent Technologies). Data files from Feature Extraction software were imported into GeneSpring® GX software version 9.0. (Agilent Technologies). Quantile normalization was performed (Bolstad et al., 2003) and expression values (log2 transformed) were obtained for each probe. Probes were also flagged (*Present*, *Marginal*, *Absent*) using GeneSpring® default settings. Probes with signal values above the lower percentile (20th) and flagged as *Present* or *Marginal* in 100% of replicates in at least one out of the two conditions under study, were selected for further analysis. Paired *t*-test was performed between conditions to be tested for differential expression analysis. Raw *p*-values were corrected for false discovery rate control using Benjamini–Hochsberg's method (Benjamini and Hochberg, 1995).

REVERSE-TRANSCRIPTASE PCR

mRNA was reverse-transcribed (RT) to cDNA (25°C for 10 min, 42°C for 60 min, and 95°C for 5 min) using random hexamers and Superscript II reverse transcriptase (Applied Biosystems). Negative control RT-minus reactions were carried out to confirm absence of DNA contamination in RNA.

SEMI-QUANTITATIVE PCR

To detect the relative expression of different chemokine genes in HGF-treated and untreated samples, semi-quantitative (sq) PCR was run. Equal volumes of cDNA were amplified by PCR using a couple of specific primers expanding at least two exons within the gene of interest. Sequences of the primers used were: CCL5 forward atatggctcggacaccactc, CCL5 reverse cccacttcttctctgggttg, CCL7 forward gggaccaattcatccacttg, CCL7 reverse cctcctcaacccacttctga, CCL20 forward gcttacctctgcagccagtc, CCL20 reverse cggatcttttcgacttcagg, CXCL2 forward agggtacaggggttgttgtg, CXCL2 reverse tttggacgatcctctgaacc. Ten microlitre aliquots taken from 25, 30, and 35 PCR cycles (CXCL2 and CCL7), 30, 34, and 38 PCR cycles (CCL5) or 24 and 28 PCR cycles (CCL20) were analyzed in 3% agarose gel. Densitometry of the DNA bands was performed using the Scion Image software (Scion Corporation) and comparing measurements from non-saturated PCR products. Loading was checked by amplification of the GAPDH transcript. Transcript analysis was performed from at least three independent simples.

REAL TIME PCR (qPCR)

cDNA processed from 1 μg RNA was used as the template. One microlitre aliquot of each cDNA was used per well. Samples were run in triplicate. Expression of the transcript levels were analysed using a FAM-labeled CXCL2 or CCL5 probes and compared to that of GAPDH, used as a loading control, in a ABI Prism 7000 HT sequence detection system (Applied Biosystems). Relative expression was calculated using the ΔΔC1 method.

RESULTS

CHEMOKINES OF THE CC AND CXC FAMILIES ARE UPREGULATED BY HGF TREATMENT

Previous work demonstrated that HGF signals through PY142 β-catenin and TCF4 to regulate the expression of target genes

A

	Array		sqPCR
Gene	Fold change	p value (n=4)	Fold change
Ccl5	4,086	0,01571	2,970 ± 0,67
Ccl7	3,700	0,00278	1,692 ± 0,34
Ccl13	3,358	0,00660	ND
Ccl7	3,298	0,00761	1,692 ± 0,34
Ccl20	2,730	0,01363	1,572 ± 0,31
Cxcl2	2,695	0,00580	2,484 ± 0,72
Ccl19	1,571	0,00266	1,487 ± 0,17

B

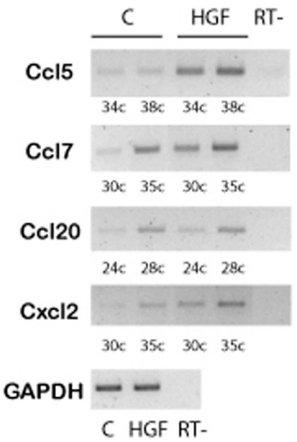

FIGURE 1 | Chemokine genes are upregulated by HGF signaling in 2DIV hippocampal neurons. (A) Summarized array data (left) indicating the chemokine genes that are upregulated in HGF-treated (50 ng/ml, 24 h) compared to untreated hippocampal neurons. (Right) Summary of the quantification of sqPCR experiments. Values indicate fold change of the chemokine expression in HGF-treated vs. untreated samples ± s.e.m. (≥3 experiments). **(B)** Representative sqPCR of samples taken at the indicated PCR cycle to compare the expression of chemokines in untreated and HGF-treated hippocampal neurons. GAPDH was used as a housekeeping gene (image corresponds to 30 PCR cycles). RT-indicates samples in which reaction was run without RT enzyme.

during hippocampal neuron development (David et al., 2008). We questioned which are the genes regulated upon HGF stimulation in axon morphogenesis. We performed array experiments using control and HGF-treated hippocampal neuron samples. Array results revealed an upregulation of several chemokine genes in HGF-treated neurons compared to untreated neurons (**Figure 1A**), which was confirmed by sqRT-PCR (**Figures 1A**,**B**). *In silico* analysis of the 2 kb region upstream of the ATG in the identified chemokine genes showed the presence of several copies of putative TCF-binding sites, as predicted for β-catenin/TCF-target genes (data not shown). These findings indicated that chemokines may be involved in the HGF-induced axon morphogenesis.

CHEMOKINE SIGNALING PROMOTES AXON MORPHOGENESIS

To address this possibility, we first tested whether chemokines induce axon outgrowth and branching. Hippocampal neurons were treated with CCL5, CCL7, CCL20, or CXCL2 at different concentrations (10–1000 ng/ml). CCL5 (10 ng/ml), CXCL2 (300 and 1000 ng/ml), and CCL20 (10 and 1000 ng/ml) significantly increased the total length of the axon compared to axon length values of untreated neurons (**Figure 2**). A cocktail of all the chemokines (10 ng/ml) also increased axon outgrowth (**Figure 2I**). The increases in axon length were in the range of that obtained by HGF stimulation (**Figure 2I**). In addition to increasing axon length, CXCL2 also produced axon branching (**Figure 2J**). Axon branching was not significant for the other studied chemokines at the tested concentrations (data not shown).

Having showed that exogenously added chemokines induce axon morphogenesis in hippocampal neurons, we studied whether blocking chemokine signaling would inhibit the effect of HGF on axon morphogenesis. To this end, we used blocking antibodies against the chemokines as well as the chemokine receptor antagonists SB2250002 and SB328437 (White et al., 1998, 2000). Neurons incubated with HGF together with antibodies against rat CXCL2 or CCL20 (40 μg/ml) displayed axon length and branching values similar to those of untreated neurons (**Figure 3**). However, the increase in axon length promoted by HGF was not affected by the presence of ovalbumin at the same concentration than the antibodies (40 μg/ml). Furthermore, treatment with HGF and the antagonist for the receptor of CXCL2 (CXCR2)

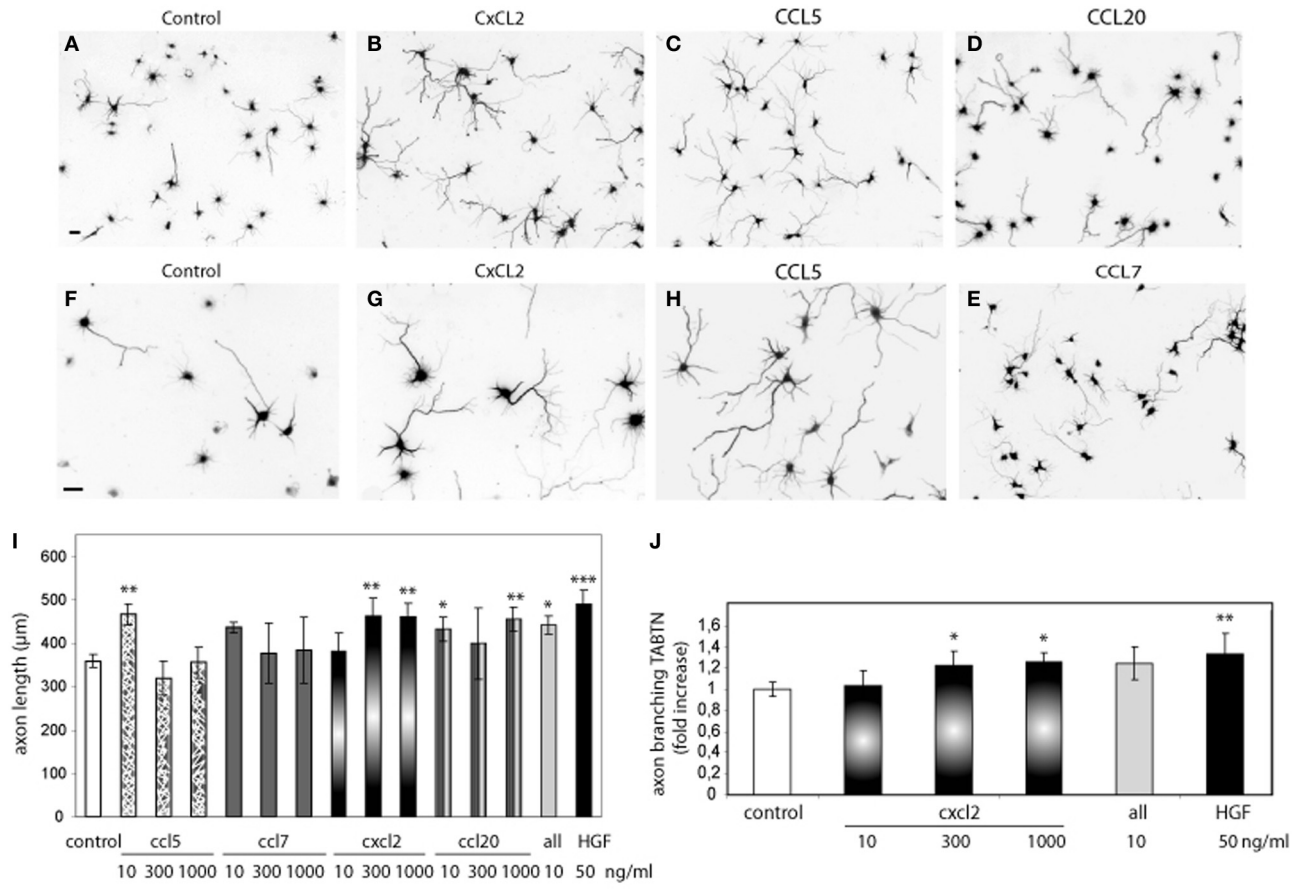

FIGURE 2 | Recombinant chemokines increase axon morphogenesis. **(A–H)** Hippocampal neurons (2 DIV) control or treated with CXCL2, CCL5, CCL20, and CCL7 (1000 ng/ml) and immunostained for βIII-tubulin to reveal the axon morphology. Images **(A–E)** were taken at 10× and **(F–H)** at 20×. Bars = 30 μm. Average axon length compared to control **(I)** and axon branching shown as an increase vs. control **(J)** for chemokine treatments at the indicated dose or HGF (50 ng/ml). *All* refers to a cocktail of the four chemokines (10 ng/ml). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

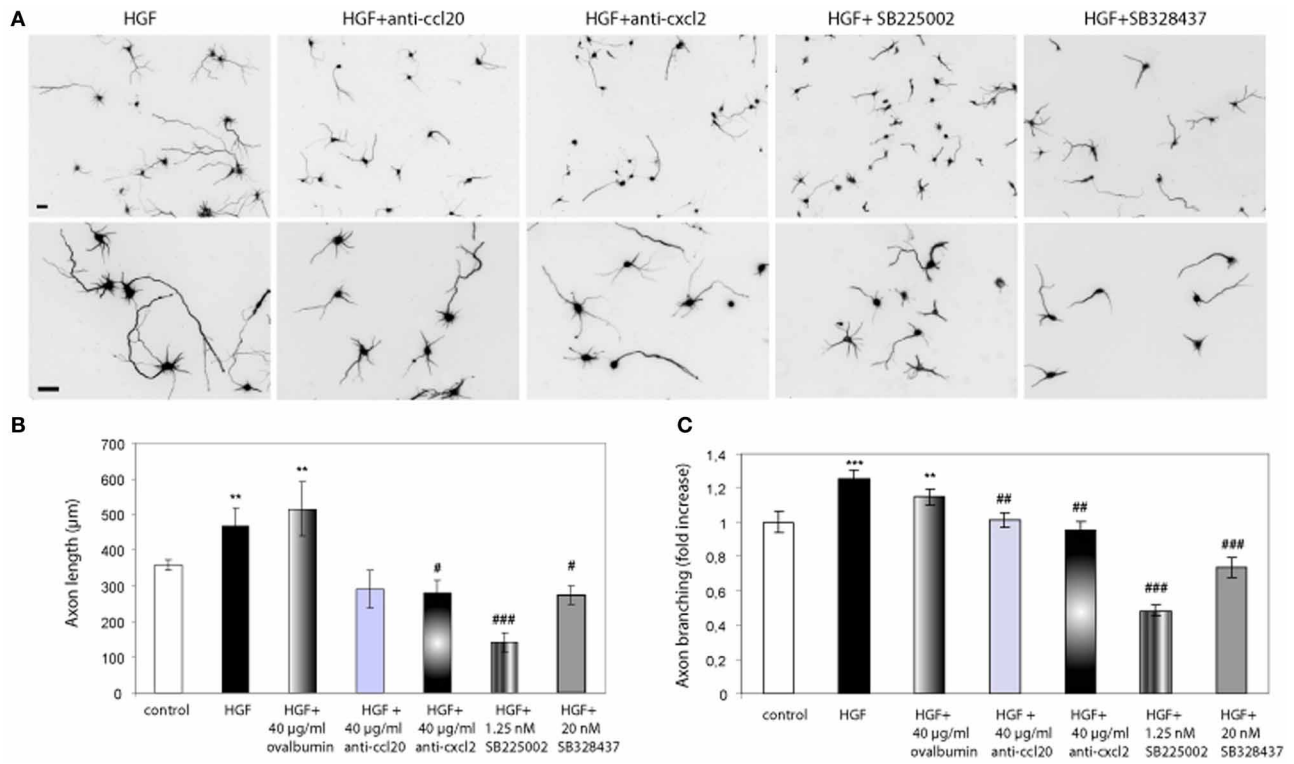

FIGURE 3 | Chemokine signaling is involved in the axon morphogenesis promoted by HGF. (A) Hippocampal neurons treated with HGF (50 ng/ml) together with anti-CCL20 (40 ug/ml), anti-CXCL2 (40 μg/ml), SB225502 (1.25 nM) or SB324837 (20 nM), and immunostained for βIII-tubulin. Top pictures were taken at 10× and bottom at 20×. Bars = 30 μm. **(B** and **C)** Average axon length measurements **(B)** and axon branching shown as an increase vs. control **(C)**. *All* refers to a cocktail of the four chemokines (10 ng/ml). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when comparing to control neurons. $\#p \leq 0.05$, $\#\#p \leq 0.01$, and $\#\#\#p \leq 0.001$ when comparing to HGF-treated neurons.

SB2250002, or with SB328437, an antagonist of CCR3 (that acts as the only receptor of CCL20 and one of the receptors of CCL5), potently inhibited axon outgrowth and branching to values below those of control neurons (**Figure 3**). These results suggest that CXCL2 and CCL20 are secreted upon HGF stimulation and that endogenous CXCL2 and CCL20 signaling plays a role in axon morphogenesis.

HGF REGULATES CXCL2 EXPRESSION THROUGH MET AND β-CATENIN/TCF

We sought to study the pathway regulating chemokine expression downstream of HGF signaling. We demonstrated that HGF stimulation induces PY142 β-catenin phoshorylation and transcriptional regulation by TCF/β-catenin (David et al., 2008). To check if chemokine expression is controlled by TCF and β-catenin downstream of HGF signaling, we followed both a pharmacological and a gene silencing approach. We used SU11274 that inhibits Met activity (Berthou et al., 2004) and FH535, which inhibits TCF/β-catenin by blocking the recruitment of β-catenin to the promoter of target genes (Handeli and Simon, 2008). First, we studied β-catenin transcriptional activation using the TOP reporter plasmid in Hek293 cells, in which HGF/Met signaling is active (Royal and Park, 1995). Although HGF did not stimulate luciferase reporter activity vs. control, treatment with SU11274 significantly reduced β-catenin transcriptional activation (**Figure 4B**). This result suggests that in Hek293 cells, HGF/Met signaling through β-catenin is already active in basal conditions, likely by an autocrine production of HGF. Furthermore, treatment with FH535 significantly reduced luciferase activity, demonstrating that TCF activation is involved in HGF/Met signaling (**Figure 4B**). We used Wnt-3a as a positive control, which produced a clear activation of luciferase activity and was also inhibited by FH535 (**Figure 4B**). These results confirmed FH535 as an effective TCF/β-catenin inhibitor and indicated that HGF/Met signal through TCF/β-catenin in Hek293 cells among other cell systems (Monga et al., 2002).

In hippocampal neurons, both SU11274 and FH535 inhibited the axon outgrowth induced by HGF signaling (**Figure 4A**). As a positive control we also treated neurons with Wnt-3a with or without FH535. As expected, TCF inhibitor blocked the axon outgrowth promoted by Wnt-3a. FH535 treatment also blocked the axon outgrowth promoted by HGF, rendering axon length values below those of control neurons, thus confirming that HGF signaling is dependent on TCF-driven transcription (David et al., 2008).

Next, we investigated whether chemokine expression is regulated through the HGF/Met/TCF/β-catenin pathway. Because

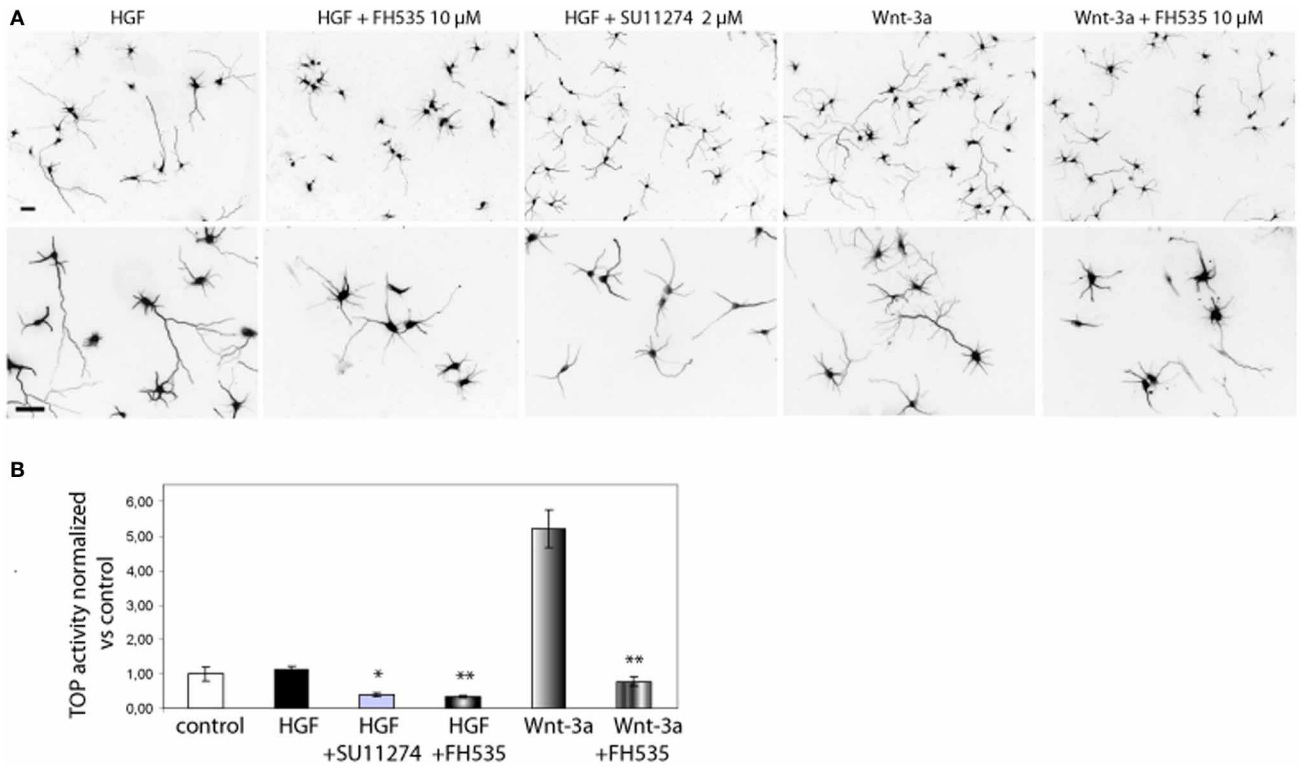

FIGURE 4 | Met and TCF inhibition reduce the axon outgrowth induced by HGF. (A) Hippocampal neurons treated with HGF (50 ng/ml) or Wnt-3a (100 ng/ml) alone or together with FH535 (10 μM) or SU11274 (2 μM) were immunostained for βIII-tubulin to reveal the axon morphology. Top pictures were taken at 10× and bottom at 20×. Bars = 30 μm. **(B)** β-catenin transcriptional activity determined using the TOP reported plasmid in transfected Hek293 cells. Relative luciferase activity was determined at 48 h after transfection. Treatments were applied for the last 24 h (see Materials and Methods for details). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

CXCL2 promoted both axon outgrowth and branching, we focused on this chemokine and on CCL5 as a member of the CC family. We analyzed the expression of these chemokines using real time qPCR from control neurons, neurons treated with HGF or treated with HGF together with SU11274 or FH535. Expression of CXCL2 increased by 1.6-fold in HGF-treated neurons compared to untreated neurons, but was reduced to values below those of untreated neurons following the treatment with HGF and SU11274 (**Figure 5A**). Pervanadate (a tyrosine phosphatase inhibitor) was previously used to stabilize the PY142-β-catenin form (David et al., 2008). However, HGF stimulation increased CXCL2 expression in pervanadate-treated neurons in a similar way than in the absence of pervanadate (**Figure 5B**). Furthermore, the increase in CXCL2 expression induced by HGF signaling was lost upon the co-treatment of HGF and FH535 (**Figure 5A**), indicating that it was mediated by TCF/β-catenin. In addition, we analyzed CXCL2 mRNA levels in hippocampal neurons in which β-catenin was silenced following lentiviral-driven shRNA expression. The silencing efficiency obtained with this β-catenin shRNA at four days was around 40–50% as similarly reported (David et al., 2008, 2010). Whereas HGF was able to increase the expression of CXCL2 in neurons expressing scrambled shRNA, this increase was lost in neurons expressing β-catenin shRNA (**Figure 5C**). We also analyzed the expression of CCL5, which increased nearly 2-fold upon HGF stimulation and was significantly reduced in the presence of HGF and FH535 (**Figure 5D**).

DISCUSSION

Aiming to identify the genes regulated by HGF signaling in developing hippocampal neurons during axon morphogenesis, we found the upregulation of several chemokines of the CC and CXC families. Following array experiments, we demonstrated that CCL5, CCL20, and CXCL2 significantly promote axon outgrowth and, in the case of CXCL2, also axon branching. PCR data confirmed that chemokines are upregulated by HGF signaling. By blocking chemokine signaling, we demonstrated that CCL20 and CXCL2 act downstream of HGF signaling in axon outgrowth and branching. During the establishment of the axon arbor morphology, HGF signaling induces β-catenin PY142 and TCF4-dependent transcriptional regulation of target genes (David et al., 2008). We inquired whether chemokines are regulated through TCF/β-catenin downstream of HGF signaling. TCF inhibition or β-catenin silencing reduced CXCL2 and CCL5 expression upon HGF stimulation. We conclude that chemokines are new molecules modulating axon outgrowth in hippocampal neurons, which expression is regulated by HGF through TCF/β-catenin signaling.

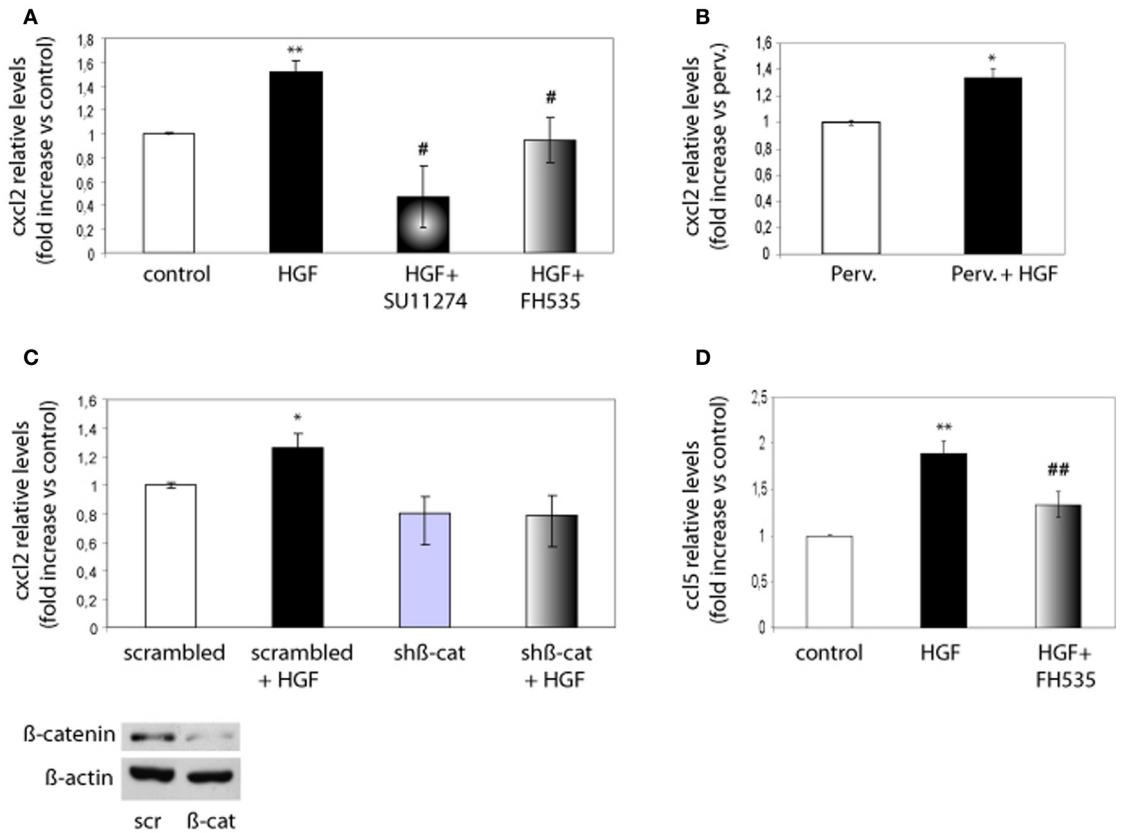

FIGURE 5 | CXCL2 and CCL5 expression is regulated by HGF signaling through TCF/β-catenin. (A) qPCR analysis indicates that CXCL2 relative levels in 2 DIV hippocampal neurons increase upon HGF stimulation (50 ng/ml, 24 h), decrease below control levels in neurons co-treated with SU11274 (2 μM) plus HGF and remain similar to control levels in neurons co-treated with FH535 (10 μM) plus HGF. **(B)** CXCL2 relative levels upon HGF stimulation in pervanadate (perv)-treated (last 2 h) neurons. **(C)** CXCL2 relative levels in 4 DIV hippocampal neurons transduced with lentiviral vectors driving the expression of scrambled or β-catenin shRNAs, untreated or treated with HGF stimulation, indicate that HGF does not increase CXCL2 levels in neurons expressing β-catenin shRNA. Lower panel: Western-blot for β-catenin shows a reduction in β-catenin levels in hippocampal neurons expressing scrambled (scr) or β-catenin (β-cat) shRNAs. β-actin was used as a loading control. **(D)** CCL5 relative levels (4 DIV) increase by HGF and significantly decrease when neurons were co-treated with HGF and FH535. $*p \leq 0.05$, $**p \leq 0.01$ when comparing to control neurons. $\#p \leq 0.05$, $\#\#p \leq 0.01$ when comparing to HGF-treated neurons.

CHEMOKINE SIGNALING IN NEURONAL MIGRATION AND AXON OUTGROWTH

Chemokines are well-established chemotactic molecules inducing the migration of leukocytes and hematopoietic progenitors (Rossi and Zlotnik, 2000). In the nervous system, chemokines and chemokine receptor expression are regulated under a variety of conditions, including brain repair (Babcock et al., 2003; Miller et al., 2008; Jaerve et al., 2012). Thus, chemokine signaling has been involved in neuroinflammation, the pathogenesis of chronic pain (White et al., 2005), myelination (Kury et al., 2002) and human immunodeficiency virus-1 (HIV-1)-associated neuropathology (Tran and Miller, 2003). Furthermore, a role for chemokines (in particular for CXCL12/Sdf-1) in regulating axon outgrowth and guidance has also been described during nervous system development (Tran and Miller, 2003). CXCL12-CXCR4 signaling is involved in the guidance of motoneuron's axon (Lieberam et al., 2005) and downstream of Sonic Hedgehog signaling in retinal ganglion cell axon pathfinding (Stacher Horndli and Chien, 2012). CXCL12 can either produce growth cone repulsion or attraction depending on the levels of cGMP (Xiang et al., 2002). Remarkably, CXCL12 signaling regulates the migration of different neuron and neuronal progenitor populations: gonadotropin-releasing hormone-1 neurons emerging from the nasal placode (Casoni et al., 2012), interneurons moving from the medial ganglionic eminence toward the cortical plate (Lopez-Bendito et al., 2008; Lysko et al., 2011), cerebellar progenitors (Zou et al., 1998; Vilz et al., 2005) and sensory neuron progenitors toward dorsal root ganglia (Belmadani et al., 2005). CCL2, CCL7, and their receptors are expressed during midbrain development, promoting the differentiation of dopaminergic neurons and also neuritogenesis (Edman et al., 2008a,b). To our knowledge, this is the first work reporting that CC chemokines induce axon morphogenesis in hippocampal neurons. In agreement with our data, *in situ* hybridization data freely available online reveals the expression of chemokines in the mouse hippocampus during embryonic and adult life (see Genepaint and Allen Brain Atlas webpages). CXCL12 was shown to promote the extension of perforant fibers from the entorhinal cortex to dentate gyrus neurons

and the migration of dentate granule cells during hippocampal development (Bagri et al., 2002; Lu et al., 2002; Ohshima et al., 2008). In addition, CXCL12 reduces axon elongation while promoting axon branching in dissociated hippocampal neurons (Pujol et al., 2005). We found that CXCL2 increases total axon length and axon branching in hippocampal neurons, suggesting that it plays a role *in vivo* during hippocampal development. In line with an effect of chemokines inducing axon development, mice lacking chemokine receptors showed impairments in hippocampal cognitive function and synaptic plasticity (Rogers et al., 2011; Belarbi et al., 2013).

HGF/β-CATENIN SIGNALING AND CHEMOKINES

Our findings establish a relationship between HGF and chemokine signaling in hippocampal neurons. HGF-treated neurons displayed increased expression of chemokines. Antibodies against CCL20 and CXCL2, as well as the CCR3 and CXCR2 antagonists SB22502 and SB328437, inhibited the axon outgrowth and branching promoted by HGF, implying that chemokines are downstream of HGF signaling. Moreover, treatment with SB22502 or SB328437 resulted in axon length and branching values clearly below those of untreated neurons, suggesting that endogenous chemokine production by hippocampal neurons impacts on axon development. Furthermore, affecting CCL20 signaling by anti-CCL20 antibodies or SB328437 treatment reduced axon branching in the presence of HGF. However, CCL20 at the tested concentrations (10–1000 ng/ml) did not promote significant axon branching. These findings suggest that promotion or inhibition of axon branching exhibit different EC50/IC50 values. Alternatively, CCL20 concentrations lower than tested may induce axon branching.

β-catenin is a classical effector of Wnt signaling and a transcriptional coactivator of LEF/TCF. We and others have described the interaction between Met and β-catenin (Monga et al., 2002; Zeng et al., 2006; David et al., 2008), which results in β-catenin phosphorylation at Y142 *in vitro* (David et al., 2008). In hippocampal neurons, HGF signaling increases PY142-β-catenin, which moves to the nucleus and regulates axonal morphogenesis through TCF4-transcriptional activation (David et al., 2008). Here, we confirm that HGF/Met signaling supports axon outgrowth through TCF/β-catenin transcriptional activity. In line with these findings, the phosphorylation of β-catenin and α-catenin downstream of tyrosine kinase receptor and/or src activation is emerging as a Wnt-independent pathway that promotes β-catenin transcriptional activation and migration of cancer cells (Ji et al., 2009; Xi et al., 2012). In this work chemokines were identified as transcriptional targets of HGF in developing in hippocampal neurons. CXCL2 and CCL5 expression analysis confirmed that these chemokines are regulated by HGF signaling. In addition, TCF inhibition and β-catenin silencing blocked the upregulation of CXCL2 by HGF. A previous paper (Halleskog et al., 2011) described the upregulation of cytokines and chemokines—including CXCL2—by Wnt-3a and β-catenin signaling in activated microglia. TCF inhibition also reduced the expression of CCL5 following HGF stimulation (by ∼30% compared to HGF-treated neurons), suggesting that CCL5 is at least in part regulated through TCF/β-catenin downstream of HGF signaling. It is possible that HGF signaling activates another pathway, i.e., NFkB pathway that affects CCL5 expression (Chou et al., 2008).

In summary these findings add different chemokines to the growing list of secreted molecules that modulate axon outgrowth, and highlights new developmental roles for signaling molecules known to regulate immune cell biology. As chemokines play a role in post-ischemic brain repair (Wang et al., 2012) and recruiting stem cells after spinal cord injury (Jaerve et al., 2012), it is tempting to speculate that chemokines at the injury site may serve to improve axon regeneration.

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SCO-spondin from embryonic cerebrospinal fluid is required for neurogenesis during early brain development

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The central nervous system (CNS) develops from the neural tube, a hollow structure filled with embryonic cerebrospinal fluid (eCSF) and surrounded by neuroepithelial cells. Several lines of evidence suggest that the eCSF contains diffusible factors regulating the survival, proliferation, and differentiation of the neuroepithelium, although these factors are only beginning to be uncovered. One possible candidate as eCSF morphogenetic molecule is SCO-spondin, a large glycoprotein whose secretion by the diencephalic roof plate starts at early developmental stages. *In vitro*, SCO-spondin promotes neuronal survival and differentiation, but its *in vivo* function still remains to be elucidated. Here we performed *in vivo* loss of function experiments for SCO-spondin during early brain development by injecting and electroporating a specific shRNA expression vector into the neural tube of chick embryos. We show that SCO-spondin knock down induces an increase in neuroepithelial cells proliferation concomitantly with a decrease in cellular differentiation toward neuronal lineages, leading to hyperplasia in both the diencephalon and the mesencephalon. In addition, SCO-spondin is required for the correct morphogenesis of the posterior commissure and pineal gland. Because SCO-spondin is secreted by the diencephalon, we sought to corroborate the long-range function of this protein *in vitro* by performing gain and loss of function experiments on mesencephalic explants. We find that culture medium enriched in SCO-spondin causes an increased neurodifferentiation of explanted mesencephalic region. Conversely, inhibitory antibodies against SCO-spondin cause a reduction in neurodifferentiation and an increase of mitosis when such explants are cultured in eCSF. Our results suggest that SCO-spondin is a crucial eCSF diffusible factor regulating the balance between proliferation and differentiation of the brain neuroepithelial cells.

Keywords: SCO-spondin, cerebrospinal fluid, neuroepithelium, neurogenesis, posterior commissure, mesencephalon, subcommissural organ

INTRODUCTION

After the closure of the anterior neuropore, the cranial region of the neural tube enlarges and generates the encephalic vesicles. During early development stages, these vesicles are delineated by the neuroepithelium, a pseudostratified epithelium that will eventually generate all the neurons and glial cells of the anterior adult central nervous system (CNS). The brain vesicles are filled with embryonic cerebrospinal fluid (eCSF), which plays important roles in encephalic development at both embryonic and fetal stages, regulating the survival, proliferation, and neural differentiation of the neuroepithelial progenitor cells (Gato et al., 2005; Salehi and Mashayekhi, 2006; Gato and Desmond, 2009; Zappaterra and Lehtinen, 2012). It has been proposed that the eCSF exerts its function by controlling neuroepithelial proliferation in response to internal liquid pressure (Desmond et al., 2005) and by facilitating the interaction of diffusible factors with the neuroepithelial cells apical surface (Gato and Desmond, 2009). The existence and importance of such diffusible factors have been demonstrated *in vitro* on mesencephalic and cortical explants, which develop normally in the presence of eCSF, whereas the absence of eCSF causes a decrease in neuroepithelial cells proliferation, differentiation, and survival (Gato et al., 2005; Lehtinen et al., 2011). The eCSF of avians (chick) and mammals (rodents and human) displays a dynamic expression pattern of hundred of proteins including essential growth and survival factor for the developing brain (Parada et al., 2005, 2006; Zappaterra and Lehtinen, 2012). Indeed, the main constituents of eCSF are proteins whose enrichment is 30-fold higher at embryonic stages than in the adult cerebrospinal fluid (Birge et al., 1974; Dziegielewska et al., 1980). Proteomic analysis of eCSF revealed the presence of several factors related to cell differentiation or proliferation such as fibroblast growth factors (Martin et al., 2006), insulin-like growth factors (Salehi et al., 2009), sonic hedgehog (Huang et al., 2010), Wnts (Lehtinen et al., 2011), lipoproteins (Parada et al., 2008), and nerve growth factor (Mashayekhi et al., 2009). While most of these proteins have a serous origin (Gato et al., 2004); some of them are directly secreted into the eCSF, such as Shh (Huang et al., 2009) or IGF-1 which are produced by the choroid plexus (Salehi et al., 2009).

Although the analysis of eCSF has gained recent attention as a promising avenue in the success of neuronal stem cell technology (Zappaterra and Lehtinen, 2012), the factors responsible

for its effects are only beginning to be uncovered. One possible candidate as an eCSF morphogenetic molecule is SCO-spondin. This high molecular mass glycoprotein is secreted to the eCSF by the subcommissural organ (SCO), a highly conserved brain gland present throughout the vertebrate phylum (Rodriguez et al., 1992, 1998; Meiniel and Meiniel, 2007). The SCO is one of the first structures to differentiate in the chick brain, expressing SCO-spondin as early as the third day of development (Didier et al., 2007; Caprile et al., 2009). This structure is located at the dorsal diencephalic-mesencephalic boundary, which, according to the prosomeric model, corresponds to the roof plate of prosomere 1, underneath the posterior commissure (PC). The SCO is composed of radial glial cells whose apical domains face the third ventricle and, hence, contact the cerebrospinal fluid, whereas their basal domains extend single processes that cross the nerve bundles of the PC and are attached to the pial membrane (Sterba et al., 1982; Rodriguez et al., 1992, 1998).

In spite of the fact that the sequence of SCO-spondin was reported more than 10 years ago (Didier et al., 2000), its precise function still remains to be elucidated. With respect to its biochemical structure, SCO-spondin is a giant glycoprotein of more than 5000 amino acids that display a multidomain organization, including the presence of several thrombospondin repeats (TSR), low-density lipoprotein receptor type A repeats (LDLrA), EGF-like domains, von Willebrand factor domains (vWF), one emilin (EMI) motif, and a C-terminal cystine knot (CTCK) (Didier et al., 2007). The presence of some of these domains has been reported in other proteins related with neurogenesis like thrombospondin 1 or reelin (Adams and Tucker, 2000; Panteri et al., 2006; Lu and Kipnis, 2010).

SCO-spondin is secreted apically, to the cerebrospinal fluid, as well as basally, toward the extracellular matrix contacting the axons of the PC (Caprile et al., 2009). The best characterized route of SCO-spondin secretion is toward the cerebrospinal fluid where it aggregates and forms the Reissner's fiber (RF); a thread-like dynamic structure that grows caudally from the SCO through the fourth ventricle and the central canal of the spinal cord, where it is finally degraded at the level of the *ampulle caudalis* (Molina et al., 2001). The RF has been proposed to regulate CSF production, composition, and circulation (Cifuentes et al., 1994; Rodriguez and Yulis, 2001; Caprile et al., 2003). However, the appearance of RF occurs several days after the onset of SCO-spondin secretion, indicating that, at least during this period, this protein remains soluble in the eCSF. The possible SCO-spondin neurogenic role during early development is suggested by *in vitro* experiments, where solubilized RF or peptides derived from the SCO-spondin sequence promote the survival (Monnerie et al., 1997) and differentiation (El Bitar et al., 2001) of neuronal cells.

Considering the early secretion of SCO-spondin, its biochemical structure, and the neurodifferentiation effect observed *in vitro*, we hypothesized that SCO-spondin affects the behavior of neuroepithelial cells during early brain development. To test this hypothesis, we used a loss of function approach in chick embryos by injecting and electroporating a SCO-spondin-specific shRNA expression vector into the neural tube. Our results show that SCO-spondin is crucial for PC formation and for proper brain development. The absence of this protein generates an increase in neuroepithelial cells division *in vivo*, showing ectopic cellular cluster in the diencephalon and mesencephalon, at the expense of cellular differentiation toward the neuronal lineage. The long-range mode of action of this protein is further supported by *in vitro* experiments, in which mesencephalic explants cultured in SCO-spondin depleted eCSF leads to a dramatical reduction of neurodifferentiation and an increase in mitosis of neuroepithelial cells.

MATERIALS AND METHODS

CHICK EMBRYOS

Fertilized chick eggs were incubated at 38°C in a humidified incubator for specific time intervals. Embryos were staged according to Hamburger and Hamilton (1992). Experiments were conducted following the guidelines outlined in the Biosafety and Bioethics Manual of the National Commission of Scientific and Technological Research (CONICYT, Chilean Government) and the Ethics Committee of the University of Concepción.

IMMUNOHISTOCHEMISTRY

Embryos were fixed for 24 h in Carnoy, dehydrated in ascending concentrations of alcohols and embedded in paraplast. Brains were oriented to obtain 5–7 μm thick sagittal sections of prosomere 1. Sections were immunostained with mouse monoclonal primary antibodies raised against vimentin and NCAM cytoplasmic domain (H5 and 4D, respectively, from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) as well as with a rabbit anti Reissner's fiber glycoproteins antibody (AFRU) that recognizes SCO-spondin (Caprile et al., 2009), a mouse anti-βIII tubulin antibody (clone Tuj1, Promega, Madison, WI, USA) and an anti-proliferating cell nuclear antigen (PCNA, PC10 ab29 Abcam). Antibodies were diluted in Tris-HCl buffer containing 1% bovine serum albumin (TRIS-BSA). Goat anti-mouse Alexa-546 and anti-rabbit Alexa-488 antibodies (Invitrogen, Carlsbad, CA) were diluted 1:100 in TRIS-BSA and incubated for 2 h at room temperature. Nuclei were visualized with TOPRO-3 (Invitrogen, Carlsbad, CA). For peroxidase staining, sections were incubated with a secondary goat anti-rabbit IgG coupled to peroxidase (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in the same buffer. Images were acquired with a laser confocal Nikon Eclipse TE2000-U microscope.

The immunohistochemistry of mesencephalic explants was made following the same protocol and using anti-BrdU (G3G4, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti cleaved caspase-3 (ASP175, Cell Signaling Technology), and anti-βIII tubulin (clone Tuj1, Promega, Madison, WI, USA) antibodies.

SCANNING ELECTRON MICROSCOPY

Stage HH18 Chick embryos were fixed for 2 h by immersion in 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate. After manually performing a sagittal cut through the midline of the brain, the tissue was dehydrated in ethanol until critical-point drying, ion covered with gold, and examined with an Etec microscope (Etec Corp., Hayward, CA) (del Brio et al., 2000).

eCSF EXTRACTION

eCSF from stages HH23-HH34 embryos was obtained as previously described (Gato et al., 2004) with small modifications. In order to avoid contamination with neuroepithelial cells, eCSF was gently sucked up under the dissecting microscope with a glass micro-needle, carefully introduced into the middle of the mesencephalic cavity. To minimize protein degradation, eCSF samples were kept at −15°C with a protease inhibitor cocktail (Sigma P2714), aliquoted, and frozen at −80°C until used.

WESTERN BLOT

For immunoblot analysis, 25 μg of total proteins were extracted from stage HH23–34 eCSF or from DMEM (Sigma) conditioned with stage HH36 subcommissural organ explants. Samples were fractionated by electrophoresis in 3%–15% linear gradient sodium dodecyl sulfate polyacrylamide gels and subsequently electrotransferred onto nitrocellulose membrane in a buffer containing 25 mM TRIS-HCl, pH 8.3, 192 mM glycine, 0.2% SDS and 20% methanol, at 25 mA, for 14 h (Towbin et al., 1979). Non-specific protein binding sites were blocked by incubating the nitrocellulose membranes with 5% non-fat milk in 0.1 M phosphate buffered saline buffer containing 0.1% Tween-20, for 2 h at room temperature. Membranes were probed with the AFRU antibody (1:15,000) overnight. Anti-IgG rabbit secondary antibodies (1:5000) (Jackson Immunoresearch) were incubated for 2 h at room temperature. Immunoreactive proteins were detected with an enhanced chemiluminescence system (SuperSignal, Pierce, Rockford, IL), as instructed by the manufacturer.

PLASMID CONSTRUCTION

The shRNA-SCO-spondin plasmid was constructed using the kit siSTRIKETM U6 Hairpin Cloning System- hMGFP (Promega, Madison, WI). The shRNA for SCO-spondin was designed using the programs www.promega.com/sirnadesigner and www.rnaiweb.com/RNAi/siRNADesign. Oligonucleotide sequences were as follows (5′ to 3′): shRNA-SCOspondin-Forward ACC GGA CAG AGC AGG TAA CAG ATT CAA GAG ATC TGT TAC CTG CTC TGT CCT TTT TC; shRNA-SCOspondin-Reverse TGC AGA AAA AGG ACA GAG CAG GTA ACA GAT CTC TTG AAT CTG TTA CCT GCT CTG TC; Scrambled-Forward ACC GGA AGA CCG AAA CGG TAA GTT CAA GAG ACT TAC CGT TTC GGT CTT CCT TTT TC; Scrambled-Reverse TGC AGA AAA AGG AAG ACC GAA ACG GTA AGT CTC TTG AAC TTA CCG TTT CGG TCT TC. These oligonucleotides were annealed and ligated into the siSTRIKE-hMGFP vector and the ligation was confirmed by PstI (NEB Inc.) digestion. Oneshot® Top10 (Invitrogen, Carlsbad, CA) cells were transformed with the resulting plasmids and grown in LB media (MO BIO Laboratories, Inc. Carlsbad, CA, USA) in the presence of ampiciline 1 mg/ml (USBiological, Swampscott, MA). Plasmid purification was made with HiSpeed Plasmid Maxi Kit (Quiagen GmbH, Hilden, Germany).

CULTURE AND TRANSFECTION OF SCO-CELLS

Cultured glial SCO cells were obtained from HH34 chick embryos. Briefly, the prosomere 1 roof plate was dissected and digested for 10 min with trypsin 0.12% (wt/vol) in phosphate buffer 0.1 M (pH 7.4, 320 mOsm) and further triturated to homogeneity with a fire-polished Pasteur pipette. 100,000 cells per well were plated onto glass coverslips in a 12-wells plate and incubated for 3 days with Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Transfection of shRNA-SCOspondin was carried using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The expression of SCO-spondin was analyzed by immunohistochemistry. The transfection of the plasmid was corroborated by the expression of GFP. Images were acquired with a laser confocal Nikon Eclipse TE2000-U microscope.

INJECTION AND ELECTROPORATION OF shRNA-SCO-SPONDIN *in ovo*

The injection and electroporation *in ovo* was performed as described in Krull (2004) with some modifications. Briefly, the neural tube of HH 9–11 embryos was injected with 1 mg/ml plasmid DNA containing 0.1% Fast Green (Sigma) for visual monitoring of the injection. Several drops of chick Ringer's solution were dropped onto the embryo after DNA injection. Electrodes were placed above (cathode) and below (anode) the diencephalon. Conditions used for electroporation were five Squarewave electrical pulses of 25 V, 50 ms pulse length, using the Ovodyne electroporator TSS20 (Intracel, Royston Herts, UK) and platinum electrodes. Following manipulation, the eggs were sealed with Parafilm (American National Can™, Greenwich, CT) and returned to the incubator. Twenty-four to thirty-six hours after electroporation, GFP expression was analyzed and embryos displaying expression at the level of the dorsal diencephalon were returned to the incubator until harvesting at HH29.

ORGANOTYPIC CULTURES OF MESENCEPHALIC NEUROECTODERM

Organotypic cultures of HH20 optic tecta were performed as described by Gato et al. (2005) and maintained at 37°C with 5% CO2 for 24 h in the presence of 0.01 mM 5-Bromo-2′-deoxyuridine (BrdU, Sigma) and one of the four following media: (1) DMEM (Sigma), (2) SCO-spondin positive conditioned medium obtained from the supernatant of HH36 SCO organ cultures maintained for 4 days in DMEM, (3) 80% DMEM with 20% stage HH25 eCSF, and (4) 80% DMEM with 20% stage HH25 eCSF and supplemented with a 1:300 dilution of the AFRU anti SCO-spondin antibody. After 24 h, the explants were fixed in paraformaldehide 4% and processed for immunohistochemistry to monitor proliferation (anti-BrdU antibody), apoptosis (anti-caspase 3 antibody), and neuronal differentiation (anti-βIII tubulin antibody). The positive areas of explants stained with each antibody as well as the total explant area were analyzed with the Image J program. Error bars represent s.e.m. and statistical analyses were performed using the Student's *t*-test. Differences were considered significant for $p < 0.05$.

RESULTS

SCO-SPONDIN IS PRESENT IN THE eCSF FROM EARLY STAGES OF DEVELOPMENT

To precisely describe the spatiotemporal expression pattern of SCO-spondin, we performed immunohistochemical staining on

chick embryo sections from early developmental stages. SCO spondin was first detected at stage HH17 in the diencephalic roof plate (**Figures 1A–D**) where it was restricted to the apical domain, suggesting its secretion to the eCSF (arrows in **Figure 1D**). At this stage, the diencephalic roof plate is similar to the rest of the neuroepithelium, consisting of a pseudostratified epithelium, whose basal and apical domains contact the eCSF and the external limiting membrane, respectively (**Figures 1E–F**).

The possible secretion of SCO-spondin to the eCSF was confirmed by western blot performed on eCSF from chick embryos at different stages of development (**Figure 2**). The results show that at HH23 (fourth day of development) the anti SCO-spondin recognizes four bands of 175, 140, 65, and 50 kDa; while at later stages additional bands of 350, 300, and 200 kDa appear, which is in agreement with previous reports (Hoyo-Becerra et al., 2006; Vio et al., 2008). Similar bands are found in the conditioned medium from HH36 SCO explants, with the exception of the smaller bands of 65 and 50 KDa (**Figure 2**, CM lane).

SCO-SPONDIN BINDS TO THE APICAL DOMAIN OF NEUROEPITHELIAL CELLS *in vivo*

The presence of SCO-spondin in the eCSF of early chick embryos led us to investigate if this glycoprotein interacts with the apical side of neuroepithelial cells. For this purpose, we realized immunohistochemistry with anti SCO-spondin on sectioned HH23 chick brains embryos (**Figure 3**). The results show that the immunoreaction is exclusively localized to the cells bodies present in the diencephalic roof plate (**Figures 3A,B,D,E**). At this stage the immunoreaction covers the entire cell, including the apical region in contact with the eCSF (arrows in **Figures 3B,E**) as well as the basal region in contact with the NCAM-positive axons of the PC (arrowheads in **Figure 3E**). The rest of the neuroepithelium is immunonegative for SCO-spondin, except for a thin line covering the apical region of mesencephalic apical membrane (arrows in **Figure 3C**) and a weak signal observed in the medial and basal part of the mesencephalic cells. In order to confirm the SCO-spondin binding to the neuroepithelial apical membrane, the SCO-spondin antibody was injected to the eCSF of live HH24 embryos. After 24 h, animals were sacrificed and the localization of the SCO-spondin antibody was assessed with anti-rabbit IgG. Our results confirm the binding of anti-SCO-spondin to the apical membrane of neuroepithelial cells *in vivo* (**Figures 4A–C**). The negative control, where unrelated antibodies were injected in the same way showed no immunoreaction (**Figures 4D–F**).

In ovo INHIBITION OF SCO-SPONDIN

In order to analyze the function of SCO-spondin *in ovo* during early CNS development, we designed a plasmid allowing the co-expression of GFP with a SCO-spondin specific shRNA or with a control scrambled shRNA. The high efficiency of the shRNA was first confirmed on primary culture of chick SCO-cells expressing SCO-spondin, showing that even though the number of transfected cells was low, all of them were immunonegative for SCO-spondin (**Figures 5A,B**). After the injection of the vector into the neural tube of HH11 embryos (**Figure 5C**), electroporation of the diencephalic roof plate was performed by placing the positive electrode at the dorsal diencephalic region and the negative electrode beneath the embryo (**Figure 5D**). One day after electroporation, the GFP expression was monitored in order to ensure that the expression of the plasmid occurred in the accurate region (**Figure 5E**), and the selected embryos were left to develop until stage HH29 before being examined. From the total of the

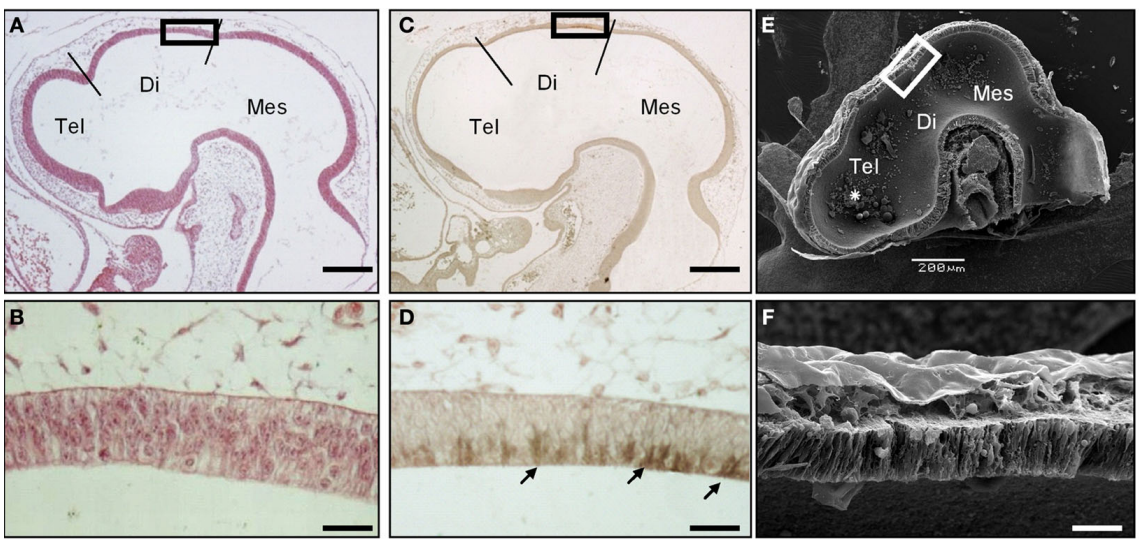

FIGURE 1 | SCO-spondin is expressed in the SCO at early developmental stages. Sagittal sections of stage HH17 chicken brain. **(A–B)** Hematoxilin-Eosin staining. **(C–D)** Immunohistochemistry with anti-SCO-spondin showing the presence of this protein in the apical region of caudal diencephalic roof plate (arrows in **D**). **(E–F)** Scanning electron microscopy showing the presence of aggregates within the brain cavities (asterisk in **E**). **(B,D,F)** Higher magnification of the diencephalic roof plate region boxed in **(A,C,E)**, respectively. At this early developmental stage, while the diencephalic roof plate does not present any marked morphological difference from the rest of the neuroepithelium, it strongly expresses SCO-spondin. Tel, Telencephalon; Di, Diencephalon; Mes, Mesencephalon; Scale bars represent 200 µm in **(A,C,E)**; 25 µm in **(B,D,F)**.

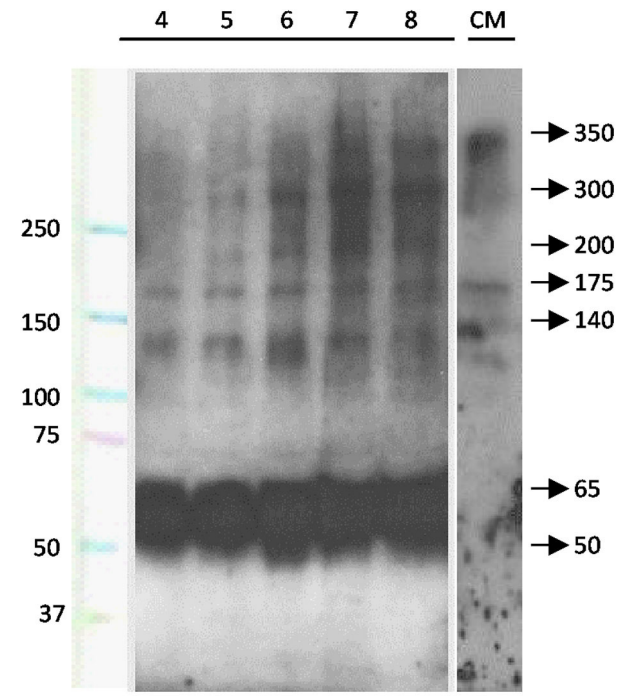

FIGURE 2 | SCO-spondin is present in the eCSF as early as the fourth day of development. Lanes 4–8: western blot analyses performed with anti-SCO-spondin antibody on eCFS extracted from chick embryos at stages comprised between the fourth and eighth day of development (HH23 to HH34). CM: western blot analyses performed with anti SCO-spondin antibody on conditioned medium obtained from HH36 SCO explants cultured in DMEM for four days. Lane 1 indicates molecular weight standards. From the fourth day of development onward, the eCSF contains four AFRU-immunoreactive bands of 175, 140, 65, and 50 kDa. Larger bands of 200, 300, and 350 kDa appear progressively during the subsequent days. Note the presence of the 140 and 175, 200, 300, and 350 kDa bands in the conditioned medium.

electroporated embryos, nine of them displayed GFP expression at the level of the diencephalic roof plate and survived until stage HH29. Immunohistochemical analysis with the anti-SCO-spondin antibody revealed that these animals can be separated into two different groups according to their phenotypes: (1) animals inhibited at the level of the cephalic region of the SCO. This group includes animals with complete inhibition and others expressing SCO-spondin exclusively at the level of the caudal region, and (2), animals in which SCO-spondin is specifically and exclusively inhibited at the level of the caudal or the central region of the SCO. Below, we will describe the consequences of these different types of inhibition on the development of the diencephalon (**Figure 6**) and the mesencephalon (**Figure 7**).

The diencephalon developed normally in animals that received the scrambled shRNA. Such control animals present a SCO-spondin positive region of 700 μm in length occupying an area of approximately 9800 μm^2, located precisely below the PC (**Figures 6A–C**). Under normal conditions, cells of the SCO extend basal prolongations that emerge from the cell body, traverse the PC, dividing this axonal tract in fascicles, and attach to the external limitant membrane (arrows in **Figure 6C**). The size of the PC fascicles grows progressively in rostro-caudal direction, being smaller at the rostral region. Hence, the length of the basal prolongations of the SCO cells is variable and closely correlates with the thickness of the PC fascicles. At this stage, the primordium of the pineal gland is present at the level of the dorsal diencephalon, rostraly to the SCO (**Figure 6A**). The diencephalon of animals in which SCO-spondin is inhibited caudally ($n = 3$) presents a diminution of 32 $\pm$ 9% in the PC area, which nevertheless displays a normal degree of axonal fasciculation, and a pineal gland primordium similar to the control (**Figures 6D–F**). We obtained one animal showing inhibition at the level of the central region, whose phenotype was similar to animals inhibited caudally with a small fasciculated PC, and presence of a normal pineal gland primordium (**Figures 6G–I**). In such animal, SCO cells have lost their radial morphology and present nuclei in the entire thickness of the SCO. Remarkably, while central inhibition abrogates SCO-spondin expression in the cell bodies, this protein is still strongly detected on the apical side of the SCO cells (asterisk in **Figure 6I**). Cephalic inhibition of SCO-spondin (**Figures 6J–L**) does not affect the PC area but causes a higher grade of axonal defasciculation (arrow in **Figure 6L**). Such animals ($n = 3$) lack the pineal gland primordium and show ectopic cellular clusters located in the dorsal diencephalon (asterisks in **Figure 6J**). Likewise, animals with complete inhibition ($n = 2$, see **Figures 6M–O**) lack a pineal gland and exhibit ectopic cellular cluster in the dorsal diencephalon (asterisks in **Figure 6M**). Additionally, complete inhibition causes a drastic diminution in the number of PC axons which are replaced by βIII tubulin positive cell bodies (inset in **Figure 6O**).

With respect to the mesencephalic region, the optic tectum of WT and control animals that received the scrambled shRNA display a normal and homogeneous thickness of 75$\pm$15μm which positively stains for βIII tubulin in its dorsal-most border (**Figures 7A–C**). The level of cell proliferation was revealed by the presence of a nuclear immunostaining of PCNA in 31 $\pm$ 4.3% of the cells (**Figures 7D–E**). Histological analysis reveals a discrete presence of mitotic spindles on the cells contacting the cerebrospinal fluid (arrows and inset in **Figure 7F**). The apical border of these cells consists of an homogeneous, uninterrupted, epithelium, and presents immunoreaction for anti SCO-spondin (inset in **Figure 7C**). Animals in which SCO-spondin was inhibited in the caudal region of the SCO (**Figures 7G–L**) present a normal optic tectum with a SCO-spondin immunoreactivity at the level of the apical surface (arrows in **Figure 7I**) and a level of βIII tubulin (**Figure 7H–I**), and PCNA (**Figures 7J–K**) immunoreaction similar to the control embryos. By contrast, the general mesencephalic morphology was severely affected in embryos with total inhibition, or in embryos displaying an inhibition localized to the cephalic region of the SCO (**Figures 7M–X**). Such embryos present a thicker neuroepithelial wall, including the presence of numerous undifferentiated cells (asterisk in **Figures 7M,S**). βIII tubulin immunoreactivity is highly reduced, particularly in animals with total inhibition (**Figure 7U**) and the PCNA immunoreactivity is dramatically increased and is present in both the cell nucleus and the cytoplasm. This localization of PCNA has been described in other proliferative cell types, during the M phase of the cell cycle (Iwao et al., 2005). In agreement with these results,

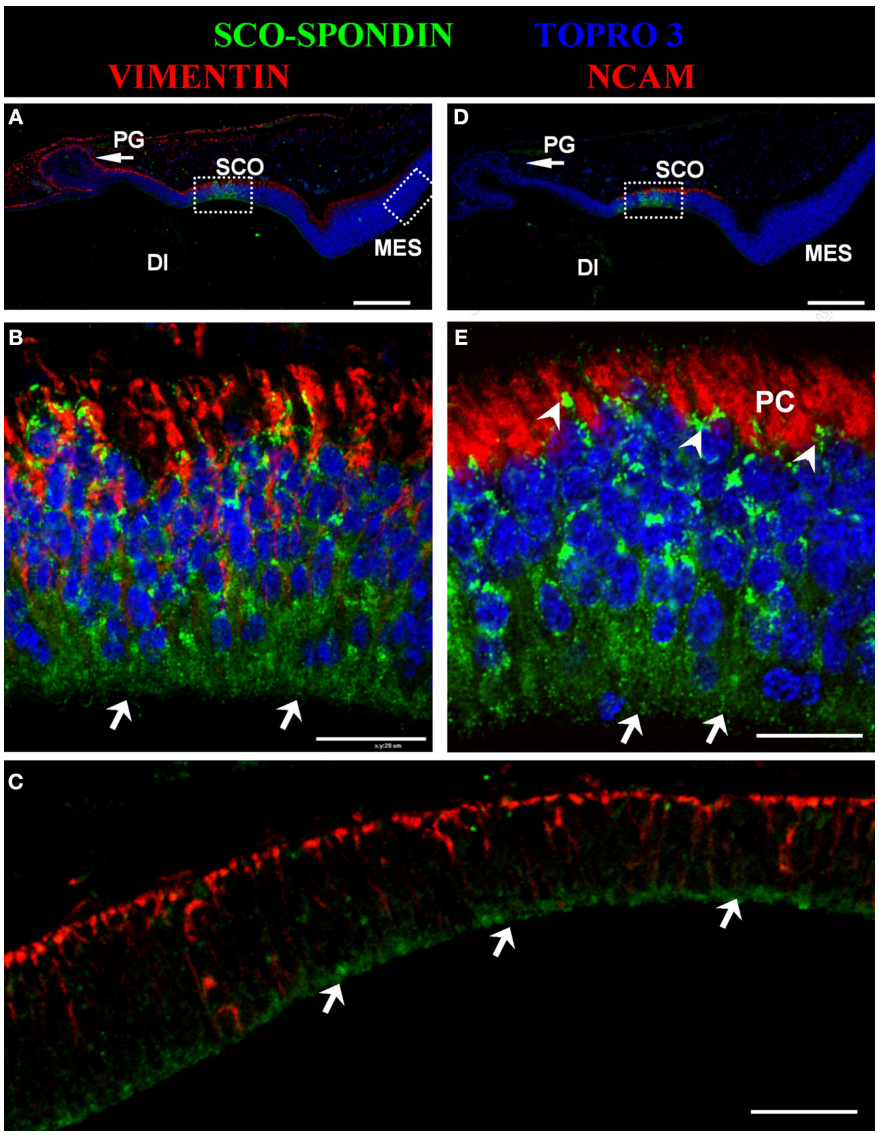

FIGURE 3 | Secreted SCO-spondin diffuses posteriorly and binds to the apical membrane of the mesencephalon. Sagittal sections of dorsal diencephalon and mesencephalon of HH23 chicken embryos. **(A–C)** Immunohistochemistry with antibodies against SCO-spondin and vimentin counterstained for nuclei with TOPRO-3. **(B)** Higher magnification of the diencephalic area boxed in **(A)**, showing the localization of SCO-spondin in the cells of diencephalic roof plate (SCO). **(C)** Higher magnification of the mesencephalic area boxed in **(A)**, showing the localization of SCO-spondin at the level of the apical membrane of the mesencephalic neuroepithelium (arrows in **C**). **(D–E)** Immunohistochemistry with antibodies against SCO-spondin and NCAM counterstained for nuclei with TOPRO-3. **(E)** Higher magnification of the area boxed in **(B)** showing the localization of SCO-spondin in contact with the axons of the posterior commissure (arrowheads in **E**) and in the apical region of the SCO cells in contact with the eCSF (arrows in **B** and **E**). Di, Diencephalon; Mes, Mesencephalon; PC, Posterior Commissure; PG, Pineal Gland; SCO, subcommissural organ. Scale bars represent 100 μm in **(A,C,D)**; 200 μm in **(B,E)**.

hematoxilin-eosin staining shows a dramatic increase in the number of mitotic cells of mesencephalic neuroepithelium (see arrows in **Figures 7R,X**, and inset in 7X). Furthermore, the apical border of mesencephalic cells is irregular, with the presence of detached cells (**Figures 7R,X**), and has lost its immunoreactivity for SCO-spondin (**Figures 7O,U**).

In summary, the animals with a SCO-spondin cephalic inhibition have a wider mesencephalon than controls (136 ± 11 μm vs. 75 ± 15μm, **Figure 7A′**), and also display a smaller area staining positively for βIII tubulin (9.5 ± 7% vs. 25.6 ± 1.7%, **Figure 7B′**). By contrast, animals with inhibition of SCO-spondin at the level of the caudal region show a mesencephalon similar to control animals concomitantly with a reduced PC area (67.7 ± 9% compared to control animals, **Figure 7C′**).

EFFECT OF SCO-SPONDIN ON MESENCEPHALIC EXPLANTS

The mesencephalic malformations found in animals in which SCO-spondin was inhibited led us to investigate the long-range

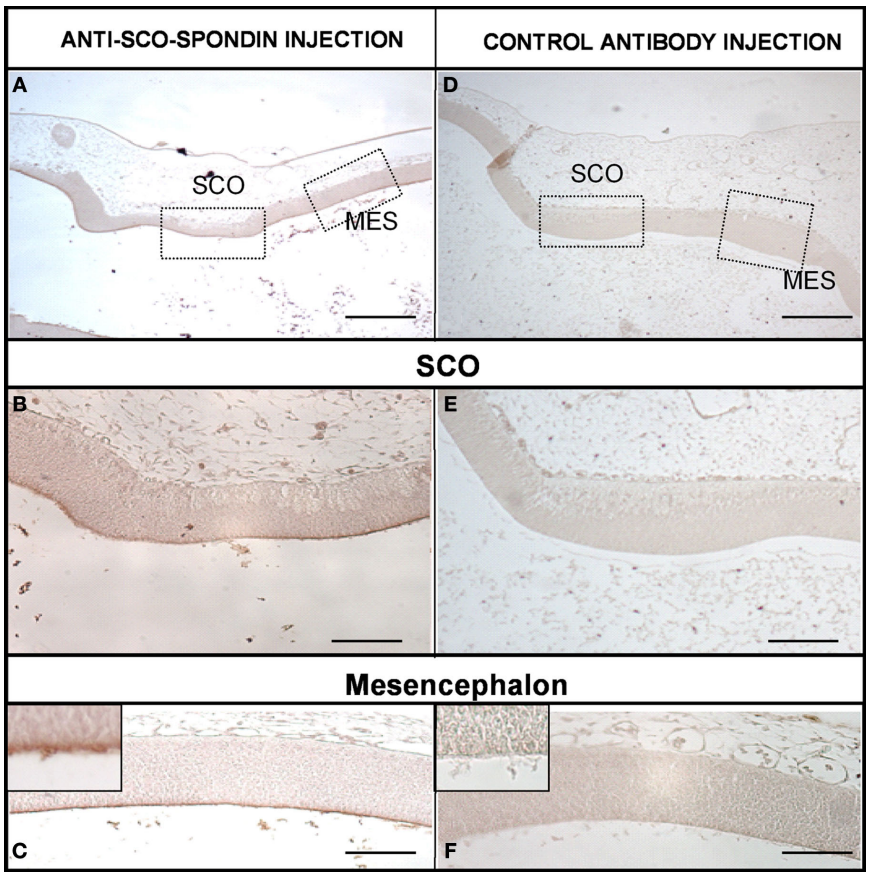

FIGURE 4 | SCO-spondin is bound to the neuroepithelium apical membranes *in vivo*. Sagittal sections of dorsal diencephalon and mesencephalon of HH27 chicken embryos. **(A–C)** Embryos were injected with anti–SCO-spondin antibody and left to develop for 24 h before being sacrified and immunostained using anti-rabbit IgG. Area boxed in **(C)** shows the presence of a thin immunoreactive line at the level of the neuroepithelial cells apical membrane. **(D–F)** Control experiment with an unrelated antibody. Scale bars represent 400 μm in **(A,D)**; 150 μm in **(B,E)**; 200 μm in **(C,F)**.

function of this protein *in vitro*, using optic tecta explanted from HH20 chick embryos (**Figure 8**). On the one hand, we analyzed the effect of SCO-spondin gain of function by comparing DMEM with conditioned DMEM medium that has been in contact with SCO explants that secrete SCO-spondin (**Figure 2** CM). On the other hand, we performed loss of function experiment by comparing normal eCSF with SCO-spondin-depleted eCSF. The gain of function experiment revealed that conditioned medium from SCO-explants produces a fivefold increase in neurodifferentiation (2.6 ± 1.2 vs. 12.15 ± 1.2; see **Figures 8E–F**) a threefold decrease in apoptosis (5.92 ± 1.3 vs. 1.6 ± 1.7; see **Figures 8A–B**) and a diminution in proliferation (6.04 ± 0.9 vs. 3.4 ± 2.6; see **Figures 8I–J**). Additionally, SCO-spondin inhibition generates a threefold increase in apoptosis (1.2 ± 0.6 vs. 4.8 ± 2.3; see **Figures 8C–D**) and proliferation (4.4 ± 1.8 vs. 11.4 ± 5.3; see **Figures 8K–L**), as well as a fourfold decrease in neurodifferentiation (23.4 ± 3.8 vs. 6.2 ± 3.09; see **Figures 8G–H**). Taken together, these *in vitro* results are similar to the *in vivo* situation, where the inhibition of SCO-spondin generates an increment in the mesencephalic proliferation at the expense of neurodifferentiation.

DISCUSSION

In this study, we performed, for the first time, an *in vivo* inhibition of SCO-spondin expression. Furthermore, by targeting the inhibition to different regions of the diencephalic roof plate, we showed that SCO-spondin is a pleiotropic protein, fulfilling different functions according to its secretion mode (**Figure 9**). When apically secreted, SCO-spondin remains soluble in the eCSF (**Figure 9A**) and binds to the apical membrane of neuroepithelial cells, thereby affecting their differentiation and proliferation, while its basal secretion at the level of the PC seems to contribute to the fasciculation and attraction of the PC axons (**Figures 9D,E**). These results are in agreement with previously reported *in vitro* experiments in which either SCO-spondin or peptides derived from its sequence promote fasciculation (Stanic et al., 2010), neurite outgrowth (Meiniel et al., 2003; Stanic et al., 2010), and differentiation (Monnerie et al., 1997; El Bitar et al., 2001).

BASALLY SECRETED SCO-SPONDIN REGULATES PC FORMATION

The following previous lines of evidence have led some authors to propose that SCO-spondin contributes to the PC development (Meiniel et al., 2008; Caprile et al., 2009; Hoyo-Becerra

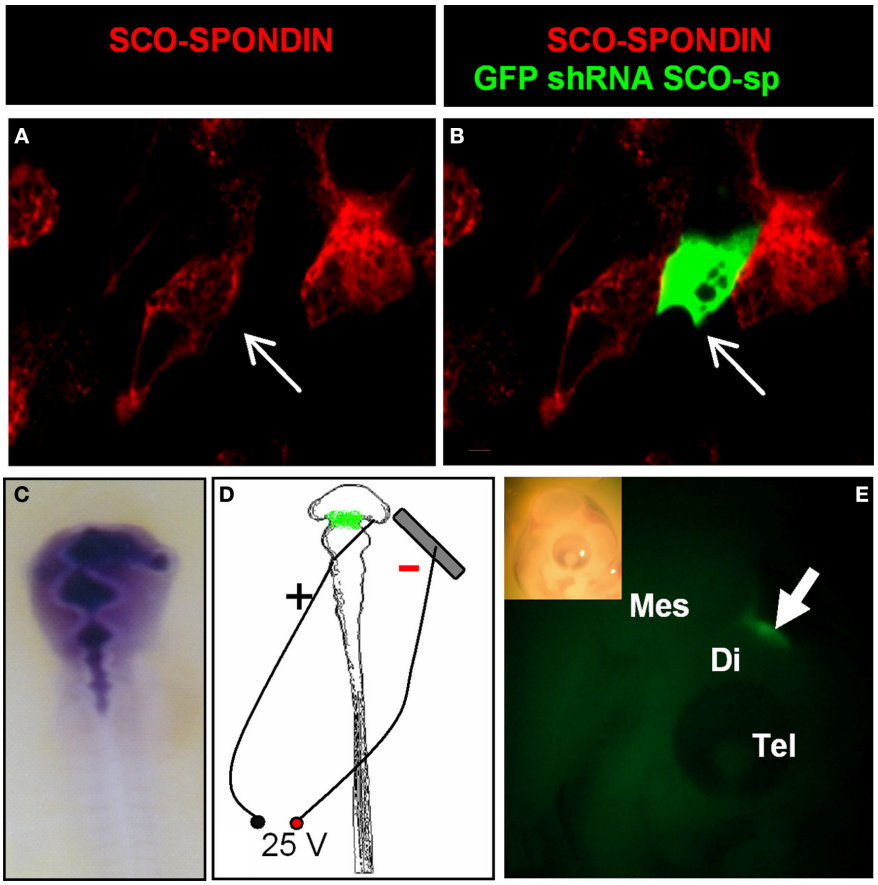

FIGURE 5 | Efficiency of the SCO-spondin shRNA expression plasmid and standardization of the electroporation procedure. (A–B) Primary culture of HH34 SCO cells were transfected with SCO-spondin shRNA plasmid and immunostained for SCO-spondin. Note that GFP-positive transfected cells are immunonegative for SCO-spondin (arrows in **A–B**). **(C)** Co-injection of the SCO-spondin shRNA expression plasmid with Fast Green into the neural tube at HH11. **(D)** Schematic drawing showing the respective position of the positive electrode on the dorsal diencephalic region and negative electrode below the embryo. **(E)** Lateral view of a chick embryo head 24 h post-electroporation (inset) showing GFP expression at the level of the dorsal diencephalic- mesencephalic boundary (arrow in **E**).

et al., 2010; Stanic et al., 2010; Grondona et al., 2012): (1) the concomitant formation of SCO and PC, (2) the similarity of SCO-spondin with other molecules involved in axonal guidance, (3) the early secretion of this protein toward the extracellular matrix surrounding the PC axons, and (4) *in vitro* experiments where the addition of SCO-spondin or peptides derived from its sequence increase neurite length and fasciculation. Here, we provide direct *in vivo* evidence that SCO-spondin is crucial for PC formation, as its loss of function either causes a marked decrease in the number of axons (animals with total inhibition), a moderate diminution in the number of axons (inhibition at the caudal region), or axonal defasciculation (animals with cephalic inhibition). The different roles observed for SCO-spondin when it is expressed in the cephalic region (fasciculation) and caudal region (incorporation of new axons) could be due to the steep SCO-spondin rostro-caudal expression gradient (Stanic et al., 2010). In this respect it is interesting to note that the presence of integrin β1 (the hypothetical SCO-spondin receptor) in the axonal membrane is negatively correlated to the concentration of its ligand (Condic and Letourneau, 1997). Hence, it is tempting to propose that, in the caudal region, the lower local concentration of SCO-spondin will promote the formation of integrin/SCO-spondin complexes, leading to axonal outgrowth and incorporation of new axons to the PC. According to this model, a higher availability of SCO-spondin in the cephalic region will induce the internalization of surface integrins, diminishing the interaction between the axons and their surrounding extracellular matrix, and, in turn, favoring the interaction between neighboring axons (i.e., fasciculation), a process mediated by axonal adhesion molecules, such as NCAM (Van Vactor, 1998).

APICALLY SECRETED SCO-SPONDIN REMAINS SOLUBLE IN THE eCSF AND BINDS NEUROEPITHELIAL CELLS

The apical secretion of SCO-spondin to the CSF and its polymerization to form the RF during late development and adulthood, are widely accepted. However, the presence of a functional and soluble form of SCO-spondin in the eCSF is a matter of recent studies (Hoyo-Becerra et al., 2006; Vio et al., 2008). Our work reveals that from the third day of chick development onward, SCO-spondin is secreted to the eCSF and that it remains soluble at

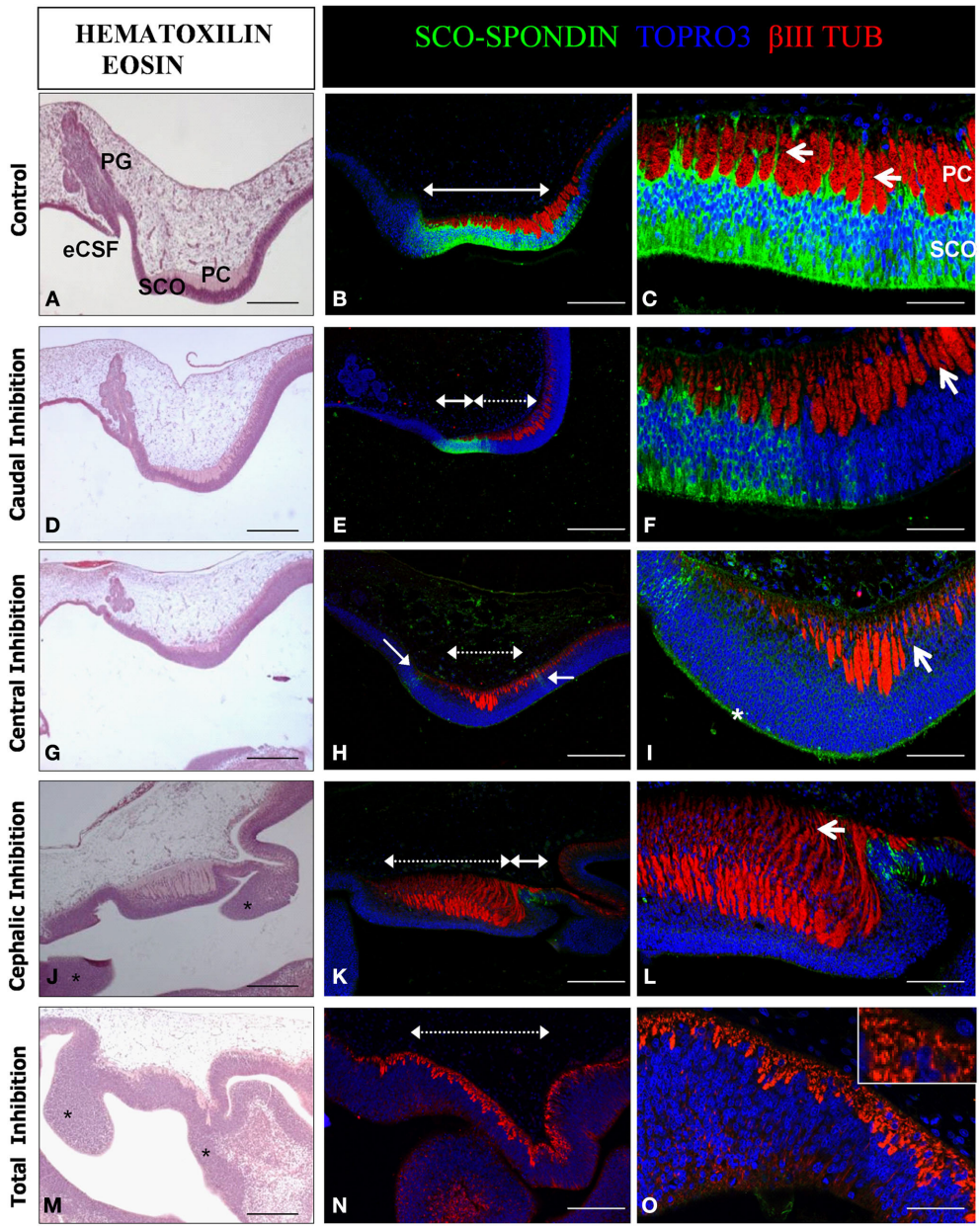

FIGURE 6 | Effect of the SCO-spondin loss of function on diencephalic development. Sagittal sections of dorsal diencephalon of HH29 chick embryos with partial or total inhibition of SCO-spondin, and stained with hematoxilin-eosin **(A,D,G,J,M)** or with antibodies against SCO-spondin and βIII tubulin and counterstained with TOPRO3 **(B,C,E,F,H,I,K,L,N,O)**. **(A–C)** Control embryos. **(D–O)** Embryos with partial or total inhibition of SCO-spondin expression. **(D–F),** Caudal inhibition; **(G–I)**, central inhibition; **(J–L)**, cephalic inhibition; and **(M–O)**, complete inhibition of SCO-spondin. In **(B,E,H,K,N)** dotted double arrowheads show the inhibited region, while plain double arrowheads show remnants of SCO-spondin expression. Arrows in **(C)** show the basal prolongations of the SCO cells. Arrows in **(F,I)** point at the presence of nuclei between the axonal fascicles, showing that the basal prolongations have been substituted by cell bodies. Asterisk in **(I)** shows the presence of SCO-spondin in contact with the apical membrane of the diencephalon neuroepithelial cells. Asterisks in **(J,M)** show ectopic cellular clusters located at the dorsal diencephalon. Note the absence of a recognizable pineal gland in **(J,M)**. Arrow in **(L)** shows dramatic axonal defasciculation. The inset in **(O)** shows that a normal axonal tract is absent and has been replaced by βIII tubulin positive neurons. PG, Pineal gland; SCO, Subcommissural organ; PC, Posterior commissure; eCSF, Embryonic cerebrospinal fluid. Scale bars represent 250 μm in **(A,B,D,E,G,J,K,M,N)**; 100 μm in **(C,F,I,L,O)**.

least until day 8. We also provide evidence showing that this protein is firmly bound to the apical membrane of neuroepithelial cells at HH24 (fourth day of development), since it is recognized by SCO-spondin antibodies injected to the eCSF *in vivo*. Our results open new questions regarding the biochemical structure of the soluble form of SCO-spondin detected in the eCSF and to the directionality of its diffusion at this stage. By performing western blots on HH23 eCSF, we have detected the presence of four bands

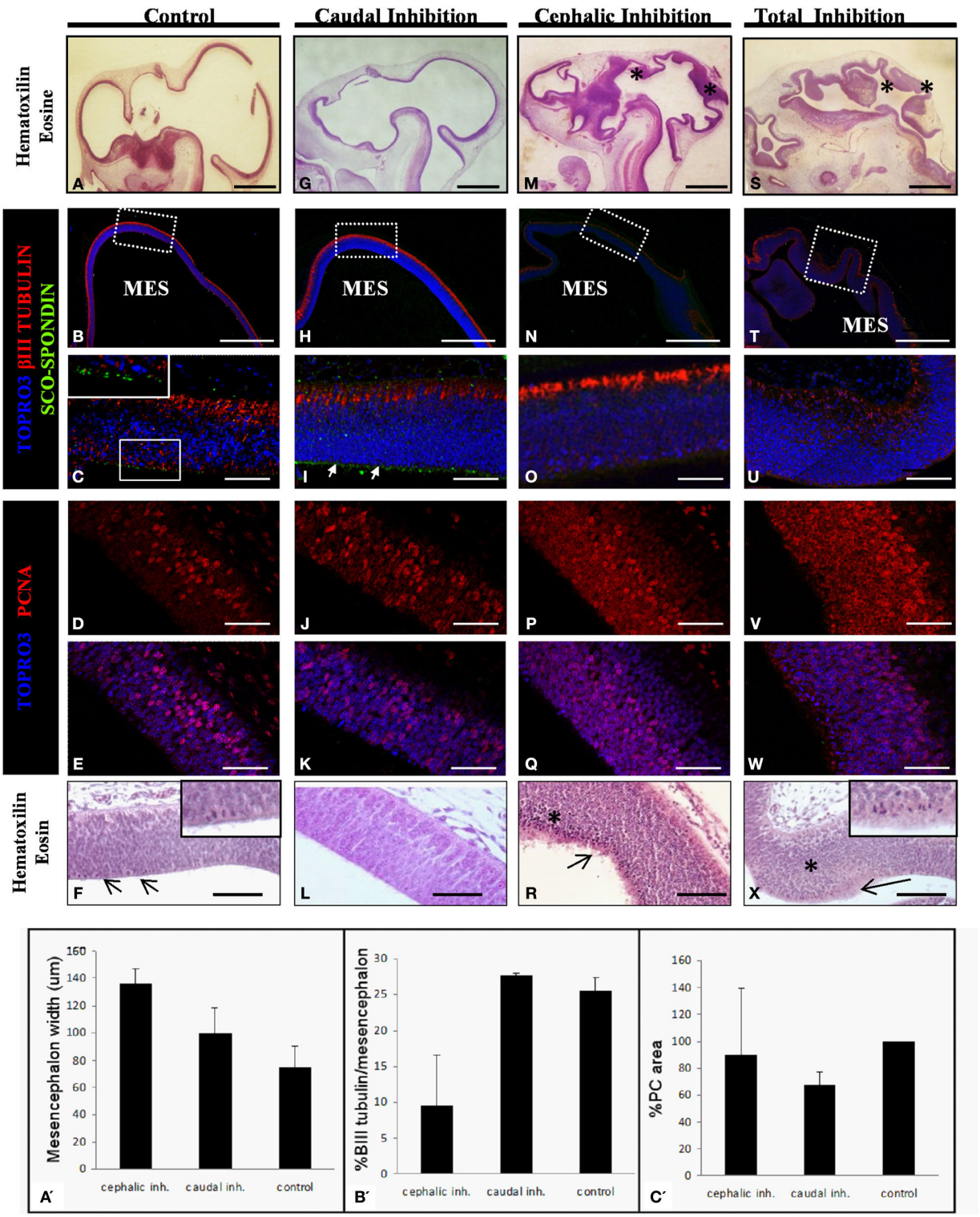

FIGURE 7 | Effect of the SCO-spondin loss of function on mesencephalic development. The panels show sagittal sections of mesencephalon of HH29 chick embryos with partial or total inhibition of SCO-spondin. **(A–F),** Control embryos; **(G–L)**, caudal inhibition; **(M–R)**, cephalic inhibition; **(S–X)**, complete inhibition of SCO-spondin; **(A,F,G,L,M,R,S,X)**, Hematoxilin-Eosin staining; **(B,C,H,I,N,O,T,U)**, Immunohistochemistry for βIII tubulin and SCO-spondin counterstained with TOPRO3. Inset in **(C)** and arrows in **(I)** show the apical localization of SCO-spondin. **(D,E,J,K,P,Q,V,W)** Immunohistochemistry for PCNA counterstained with TOPRO3. Asterisks show ectopic cellular bodies in **(M,S,R,X)**. **(A′–C′)** Quantification of the phenotypes observed in the SCO-spondin inhibited animals revealing differences in mesencephalic width **(A′)**, percentage of βIII-tubulin positive area with respect to total mesencephalic area **(B′)**, and PC area **(C′)**. Scale bars = 2 mm in **(A,B,G,H,M,N,S,T)**; 50 μm in **(C–F,I–L,O–R,U–X)**.

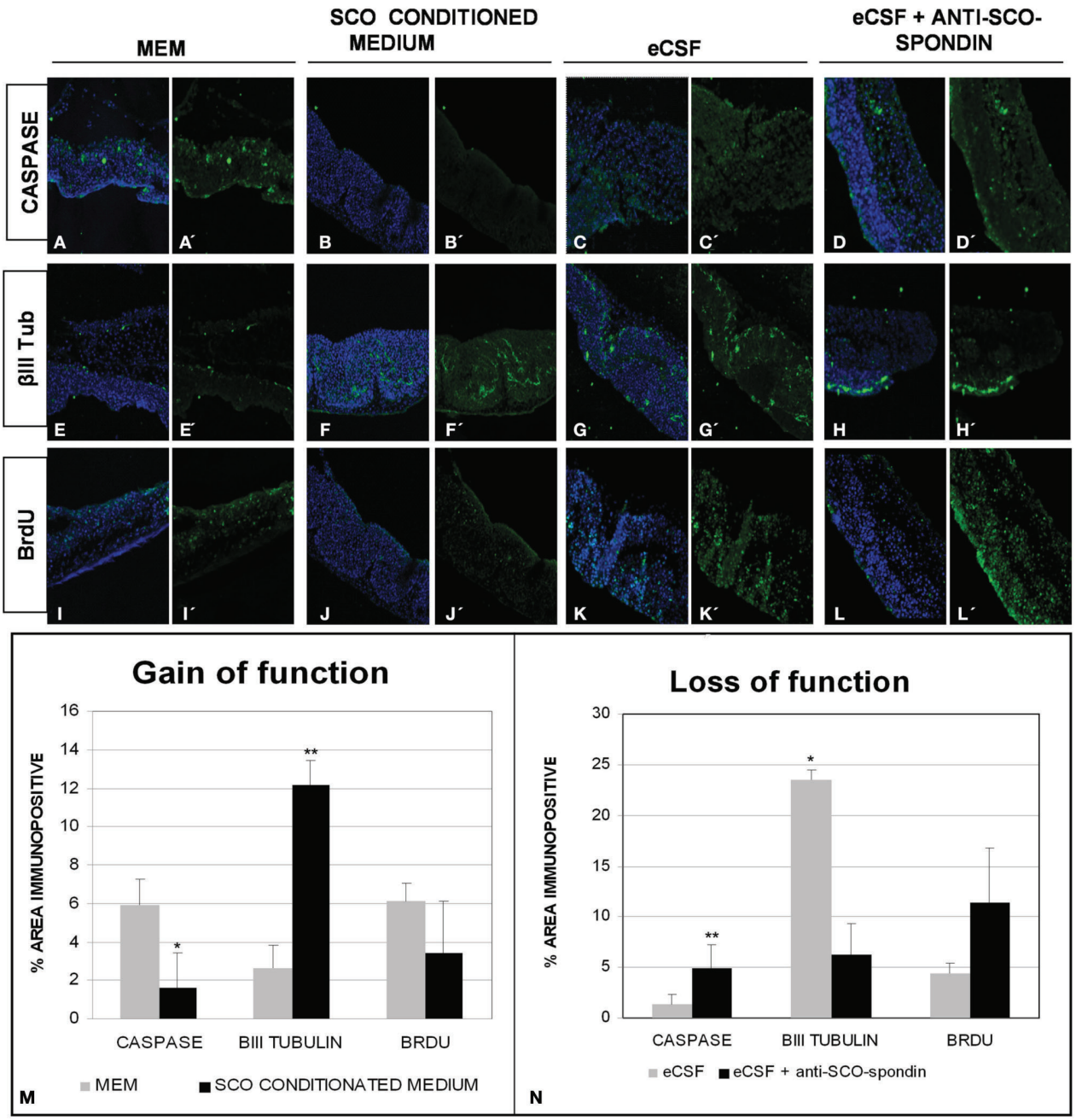

FIGURE 8 | SCO-spondin regulates the behavior of mesencephalic cells *in vitro*. Optic tectum explants from HH20 embryos cultured in presence of DMEM **(A,E,I)**; SCO conditioned DMEM **(B,F,J)**; DMEM supplemented with 20% eCSF **(C,G,K)**; DMEM supplemented with 20% eCSF and incubated with anti SCO-spondin antibody **(D,H,L)**. The explants were analyzed for the presence of activated caspase 3 **(A′–D′)**, βIII tubulin **(E′–H′)** and BrdU incorporation **(I′–L′)**. Panels **(A–L)** show the merge with the TOPRO3 nuclear signal used to counterstain the tissue. **(M–N)** Quantification of the area immunopositive for the different antibodies in each experiment. $^{*}p < 0.05$; $^{**}p < 0.01$. Bars mean ± SEM.

of 175, 140, 65, and 50 kDa; while at later stages additional bands of 350, 300, and 200 kDa appear. The presence of similar molecular weight bands was found in the CSF of 7 days postnatal rats (Vio et al., 2008) using the same antibody (AFRU) as well as the anti-P15 antibody raised against a peptide derived from the bovine SCO-spondin. These observations suggest the existence of several SCO-spondin isoforms generated by alternative splicing and/or by cleavage. This possibility is in agreement with the presence of several transcripts detected by northern-blot using an SCO-spondin-specific probe (Meiniel et al., 2003).

A smaller 138 kDa human SCO-spondin isoform has been reported (A2VEC9-2, Uniprot), containing eight LDLR-A, two EGF-like and three TSP domains, but lacking the CTCK domain, responsible for oligomerization. Therefore, it remains possible that the 140 kDa SCO-spondin isoform detected in the eCSF at early developmental stages (**Figure 2**) correspond to this small

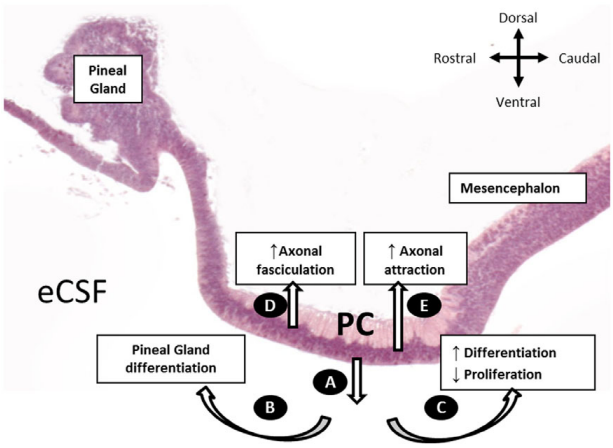

FIGURE 9 | Schematic representation of the SCO-spondin secretory routes and their respective roles, as proposed in the present work. **(A)** Apical secretion of SCO-spondin to the eCSF. **(B)** Rostral spreading of SCO-spondin promoting pineal gland differentiation. **(C)** Caudal spreading of SCO-spondin towards the mesencephalic cavity, inducing neurodifferentiation at the expense of proliferation. **(D)** Basal secretion of SCO-spondin by the rostral SCO cells, promoting axonal fasciculation of the PC. **(E)** Basal secretion of SCO by the caudal SCO cells promoting incorporation of new axons to the PC.

isoform, and that the onset of expression of larger isoforms containing the CTCK triggers polymerization and RF formation after the seventh day of development (Schoebitz et al., 1986; Caprile et al., 2009).

REGION SPECIFIC MORPHOGENETIC ROLE OF SCO-SPONDIN

The present work reveals a strong region-specific effect for SCO-spondin, as complete or cephalic inhibition severely affects mesencephalic development, while animals with caudal inhibition display an almost normal morphology.

These results suggest that the region of SCO-spondin inhibition is more important than the total area of inhibition, since the presence of few SCO-spondin immunopositive cells in the cephalic region is sufficient to sustain a normal mesencephalic development. While it is possible that the SCO-spondin secreted at the caudal and cephalic region may correspond to distinct isoforms with different roles on PC and mesencephalic development, we favor a second hypothesis according to which the SCO-spondin secretion pathway differs between the caudal and cephalic region. Indeed, we found that animals whose SCO-spondin expression is restricted to the cephalic region display an SCO-spondin immunoreactivity in the apical region of mesencephalic cells. In contrast the mesencephalic cells of animals that express SCO-spondin only at the caudal region are devoid of this immunoreactivity.

The secretion of SCO-spondin to the eCSF opens the question about how this protein will spread into the brain cavities The circulation of eCSF at early stages of development is not yet fully understood, since the absence of choroid plexus does not provide the cephalo-caudal directionality of liquid flows observed in the adult. In this respect, recent studies performed in living *Xenopus leavis* embryos report the existence of a semicircular fluid flow in the telencephalic and mesencephalic cavities, acting the cerebral aqueduct (the region that contacts the SCO and where SCO-spondin is secreted) as a bridge between the eCSF of both cavities (Mogi et al., 2012). The diencephalic roof plate is therefore a favored region whose secretions can efficiently spread into the brain cavities, since they will be carried away both anteriorly (e.g., toward the pineal gland) and posteriorly (e.g., toward the mesencephalic cavity).

SCO-SPONDIN AS A MORPHOGEN CARRIER?

One fundamental issue that still remains to be tackled is the molecular mode of action of SCO-spondin. Our *in vitro* experiments show that the addition of a SCO-spondin inhibitory antibody diminishes drastically the ability of native eCSF to promote neurodifferentiation (**Figure 8**). It is known, however, that the eCSF contains a variety of factors involved in brain development such as dystroglycan, retinoic acid, FGF2, or LDL (Gato and Desmond, 2009; Zappaterra and Lehtinen, 2012) suggesting that these factors might influence each other or act redundantly. For instance, more than 60% of the neural differentiation activity exerted by native eCSF requires the presence of LDL (Parada et al., 2008). Interestingly, to fulfill this role, LDL requires the presence of others eCSF components that still remain to be identified (Parada et al., 2008). Considering the presence of several LDLR-A domains in SCO-spondin, it is possible that SCO-spondin is involved in the delivery of lipoproteins to neuroepithelial cells. In addition to their function as lipid carriers, LDLR-A domains can also act as carriers for morphogens of the hedgehog (Hh) and Wnt families (Panakova et al., 2005; Willnow et al., 2007). The association between SCO-spondin and lipoproteins-morphogens could offer an efficient mean to transport them around the whole brain cavities, and to increase the local concentration of such morphogens. Indeed, if this turned out to be the case, each morphogens will be presented as multiple copies on the same lipoprotein particle, generating a multivalent ligand complex able to promote homomeric clustering of their cognate receptors, as well as heterodimeric interaction between different morphogens. Furthermore, the presence of multiple domains in SCO-spondin like TSP, and EFG-like would increase the range of combinatorial interactions between extracellular ligands.

In summary, our work strengthens the idea that SCO-spondin is a multifunctional protein, involved locally in PC development, and also able to exert a long-range function on remotely located regions of the brain. The secretion and diffusion of a soluble form of SCO-spondin into the eCSF allows its binding to the apical surface of the neuroepithelial cells of the diencephalon and mesencephalon, where it triggers signaling events promoting the neuronal differentiation and exit of mitosis. Future challenges will involve deciphering the molecular actors collaborating with SCO-spondin, such as morphogens, receptors, and signaling pathways.

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WNT signaling in neuronal maturation and synaptogenesis

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The Wnt signaling pathway plays a role in the development of the central nervous system and growing evidence indicates that Wnts also regulates the structure and function of the adult nervous system. Wnt components are key regulators of a variety of developmental processes, including embryonic patterning, cell specification, and cell polarity. In the nervous system, Wnt signaling also regulates the formation and function of neuronal circuits by controlling neuronal differentiation, axon outgrowth and guidance, dendrite development, synaptic function, and neuronal plasticity. Wnt factors can signal through three very well characterized cascades: canonical or β-catenin pathway, planar cell polarity pathway and calcium pathway that control different processes. However, divergent downstream cascades have been identified to control neuronal morphogenesis. In the nervous system, the expression of Wnt proteins is a highly controlled process. In addition, deregulation of Wnt signaling has been associated with neurodegenerative diseases. Here, we will review different aspects of neuronal and dendrite maturation, including spinogenesis and synaptogenesis. Finally, the role of Wnt pathway components on Alzheimer's disease will be revised.

Keywords: Wnt factors, neuronal development, dendrite, synapses, Alzheimer disease

INTRODUCTION

The development and specification of different organs and tissues that take place during embryonic development is tightly coordinated by specific signaling molecules known as morphogens including sonic hedgehog (Shh), transforming growth factor β (TGF β), fibroblast growth factor (FGF), and Wnt factors. These ligands bind to specific receptors and activate intracellular cascades that regulate different cellular processes and behavior (Tabata and Takei, 2004).

Wnt factors are secreted morphogens that regulate cell fate decision, cell polarity, and embryonic patterning (Parr and McMahon, 1994; Wodarz and Nusse, 1998; Nusse and Varmus, 2012). In the last years, a great body of evidence has demonstrated that Wnt signaling also plays a key role in the formation and modulation of neuronal circuits. In the nervous system, Wnt proteins are required during development for early patterning by acting as posteriorising signals, for neural crest cell induction, neural precursor, cell proliferation, and neurogenesis (Ciani and Salinas, 2005; Toledo et al., 2008). Moreover, Wnts regulate essential processes including neuronal migration, neuronal polarization, axon guidance, dendrite development, and synapse formation which are required for a proper brain wiring (Ciani and Salinas, 2005; Inestrosa and Arenas, 2010). Wnts are highly conserved molecules among animal species (van Amerongen and Nusse, 2009). Wnt proteins bind to the amino-terminal cysteine-rich domain (CRD) of the seven transmembrane Frizzled (Fz) receptors. In addition to Fz, Wnt ligands can also signal through other receptors with tyrosine kinase activity such as atypical receptor related tyrosine kinase (RYK) and orphan receptor tyrosine kinase (ROR2) (Inoue et al., 2004; Logan and Nusse, 2004). Interestingly, insulin-like growth factor 1 (IGF-1) receptor (IGF-1r) is emerging as a potential Wnt receptor to modulate neuronal events (Hu et al., 2012). After Wnts bind to their receptors, they activate a number of possible intracellular cascades: the Wnt–β-catenin pathway (canonical pathway), the planar cell polarity pathway (PCP pathway) and the calcium pathway (**Figure 1**). In the canonical pathway, the binding of the Wnt ligand to its Fz receptor and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) activate the scaffolding protein Dishevelled (Dvl), which in turn induces the disassembly of the complex formed by axin, adenomatous polyposis coli (APC), and the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β). In the absence of Wnt signaling, β-catenin is synthesized but rapidly degraded due the phosphorylation by GSK-3β (Orford et al., 1997; Salic et al., 2000). In the presence of Wnt, GSK-3β is inhibited leading to the accumulation and the stabilization of β-catenin in the cytosol and its translocation into the nucleus, where it associates with the transcription factor TCF (T cell factor)/LEF (lymphoid enhancing factor) to regulate Wnt target genes (Gordon and Nusse, 2006). In the PCP pathway, Wnt binds to its receptor and activates Dvl which leads to changes in both actin and microtubule reorganization. These alterations involve the activation of the small Rho-GTPases proteins and c-Jun-N-terminal kinase (JNK; Rosso et al., 2005; Gordon and Nusse, 2006). Finally, in the calcium pathway, Wnt increases the intracellular level of Ca^{2+} leading to the activation of calcium sensitive kinases such as protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) and the nuclear translocation of the transcription factor nuclear factor of activated T cells (NFACT).

Importantly, the same ligand can function through different Wnt pathway depending on the receptor and cellular context.

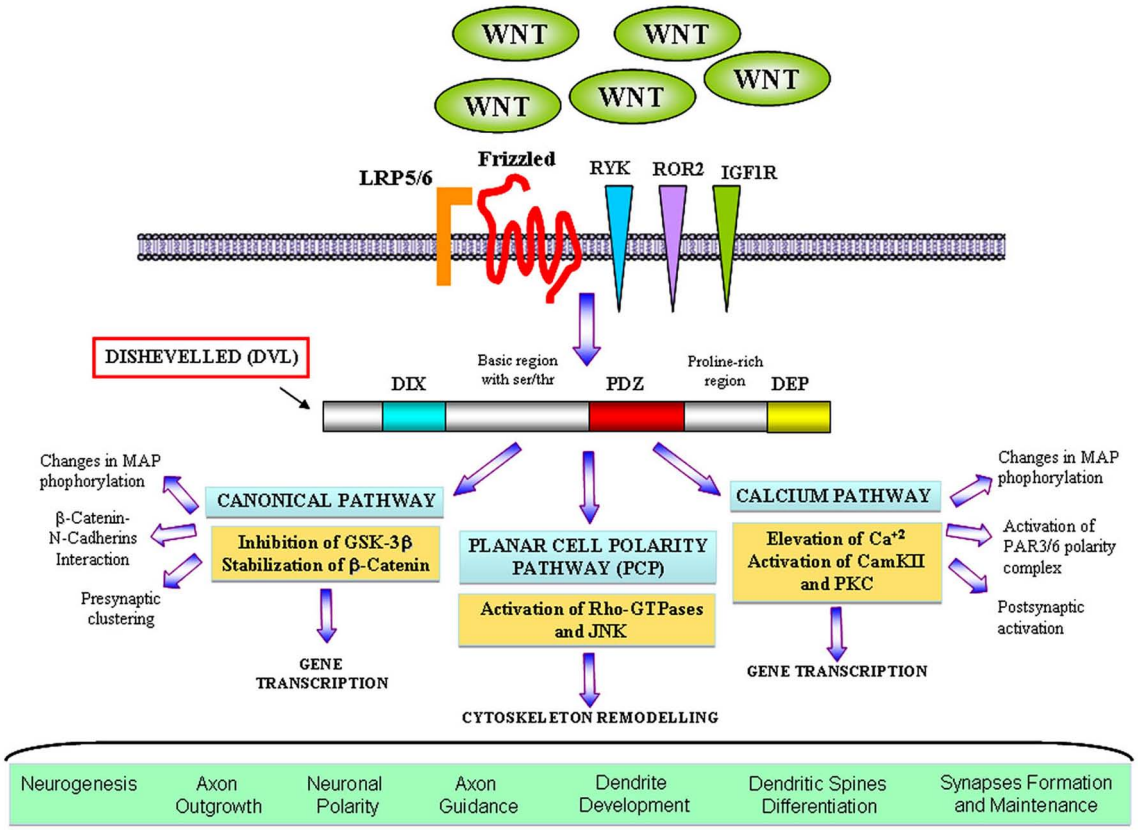

FIGURE 1 | The main branches of Wnt signalling pathways in neuronal development. Wnt factors bind to their classical receptor Frizzleds (Fz) or interact with tyrosine kinase receptors such as RYK, ROR2, and also IGF1R depending on the cellular context. In the *canonical or WNT/β-catenin pathway* Wnt also interacts with the LRP5/6 co-receptor and activates Dvl that contains three domains involving in different neuronal functions. Activation of Dvl mainly results in the GSK3β inhibition and the β-catenin accumulation in the cytosol which translocates to the nucleus where it activates specific gene transcription. Divergent pathways have been shown to control axon and dendrite morphology and presynaptic function. In the *planar cell polarity (PCP) pathway* Wnt binds to Fz and activates Dvl, which in turns signals to small Rho-GTPases proteins. Activation of Rac induces changes in the activity of JNK leading to changes in the cytoskeleton. This cascade was implied in dendrite development and complexity. In the *Wnt/calcium pathway*, activation of Dvl induces the increase of intracellular level of calcium and activation of PKC and CamKII affecting the transcription of NF-AT nuclear factor. However, divergent cascades involving the activation of CamKII have been identified to modulate neuronal polarity, dendritic spines morphology and synapses.

In this review, we will discuss the role of Wnt proteins during neuronal development and maturation. Firstly, we will describe the Wnts function during initial neuronal differentiation and axon behavior. Then, we will focus on the role of Wnt factors on neuronal maturation particularly the formation of dendritic arbors and their function as modulators of synaptic physiology. Finally, we will concentrate on the preventive role of Wnt signaling on neurodegenerative diseases in particular Alzheimer's disease (AD).

Wnt SIGNALING AND NEURONAL DEVELOPMENT

Wnt REGULATE AXON OUTGROWTH AND MORPHOLOGY

The proper function of the nervous system depends on the morphological complexity of the neurons, the participation of non-neuronal cells and the establishment of suitable neuronal connections. Neurons are highly polarized cells that ensure an unidirectional flow of information. After they are born, neurons differentiate and establish two compartments: axon and dendrites which have distinct molecular composition, morphology and functioning (see Dotti et al., 1988). This polarized arrangement is fundamental for neuronal function in order to receive and propagate electrical signals to distinct sites.

A very useful system for studying neuronal polarization and axon growth is the cultured hippocampal neurons, which displays five developmental stages. Firstly, neurons elaborate lamellipodia (stage 1) and then, short neurites or minor processes (stage 2). After that, one of these neurites grows faster and becomes the axon with a large and highly dynamic growth cone (stage 3), leaving the rest of the neurites to form dendrites (stage 4). Finally, dendrite maturation takes place and very complex dendritic arbors are able to initiate synaptic function (stage 5; Dotti et al., 1988; Craig and Banker, 1994).

Neuronal polarization and maturation are controlled not only by intrinsic factors and genes expression programs but also by molecules which come from the extracellular matrix. These extrinsic molecules are potential regulators for axon specification and pathfinding, neuronal maturation and synapses formation and maintenance. Among these molecules there are neurotrophic

factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT3) which function as neuronal polarity and axon development modulators through the activation of tyrosine receptor kinases (Trks; Huang and Reichardt, 2003). However, another family of extracellular factors called Wnt proteins is emerging as a regulator of initial axon differentiation, growth and axonal behavior. Interestingly, Wnt5a is required for NGF-dependent axonal branching and growth (Bodmer et al., 2009). Wnt5a would function as a key NGF downstream effector in developing sympathetic neurons by locally activation of PKC (Bodmer et al., 2009). In addition, NGF enhances the expression of Wnt5a in sympathetic neurons. Furthermore, Wnt5a-null mice neurons show deficits in NGF-dependent axonal development (Bodmer et al., 2009). Another study carried out in *Drosophila* demonstrates that Wnt5a functions as a ligand in the PCP pathway during axonal growth and branching (Shimizu et al., 2011). Importantly, other PCP pathway components such as Fz, strabismus, flamingo, and disheveled are cooperatively required for axonal targeting and branching. Authors propose that Wnt5a and the PCP pathway regulate axonal development in *Drosophila* neurons carefully (Shimizu et al., 2011).

Disheveled is the first downstream effector of Wnt signaling pathways. Many studies have demonstrated that Dvl is a neurite growth and differentiation key regulator. Dvl expression in neuroblastoma 2A cell (N2A cells) promotes neurite outgrowth and induces N2A cells differentiation (Fan et al., 2004). This neuronal remodeling depends on a Dvl N-terminal DVL domain (DIX). The DIX domain is essential for the Dvl effect on neuronally differentiating N2A cells (Fan et al., 2004). Accordingly, other studies have shown that Dvl regulates neurite extension through the microtubule stability regulation. Dvl colocalizes with axonal microtubules and protects stable microtubules from nocodazole depolymerization (Krylova et al., 2000; Rosso et al., 2005). Dvl increases microtubule stability through GSK-3β inhibition and changes in the microtubule-associated protein 1B (MAP1B) activity (Ciani et al., 2004). Additionally, another work reveals that Dvl promotes axon differentiation by regulating atypical PKC (aPKC; Zhang et al., 2007). In cultured hippocampal neurons aPKC is directly regulated by Dvl. Thus, Dvl downregulation abolishes axon differentiation. In contrast, Dvl overexpression induces multiple axons formation (Zhang et al., 2007). Interestingly, the authors show that Dvl associates and activates aPKC in these neurons and the expression of a aPKC dominant negative prevents the Dvl multiple axons formation (Zhang et al., 2007). To add, Dvl forms a complex with PAR3, PAR6, and aPKC, resulting in aPKC stabilization and activation. Furthermore, treatment with Wnt5a, a non-canonical Wnt factor, induces the aPKC activation and promotes axon differentiation in cultured hippocampal neurons (Zhang et al., 2007). These evidences demonstrate the Wnt pathways participation on the initial neuronal differentiation.

Several Wnt signaling effectors are very well known as axonal modulators, such as GSK3β and APC belonging to the canonical pathway and the small Rho-GTPases proteins and associated kinases from the PCP pathway. Many proteins regulate the cytoskeleton dynamics and organization functioning as direct targets of GSK-3β. For example, microtubule-associated proteins (MAPs), such as MAP1B, tau, and MAP2, which are expressed in developing neurons, function as microtubule stabilizers and can be phosphorylated by GSK-3β (Berling et al., 1994; Lucas et al., 1998). Another protein that is directly phosphorylated by GSK-3β is APC, an important player in the Wnt signaling pathway and a microtubule plus-end binding protein that is accumulated at growth cones (Zumbrunn et al., 2001; Zhou et al., 2004). Phosphorylation of these proteins by GSK-3β changes their ability to bind microtubules and their function as modulators of microtubule dynamics (Gonzalez-Billault et al., 2004; Zhou et al., 2004; Baas and Qiang, 2005). Thus, changes in the cytoskeletal proteins phosphorylation might contribute to axon determination and growth. In addition, it has been observed that cultured neurons exposed to Wnts elicits an axonal remodeling, a process characterized by axonal spreading and growth cone enlargement (Hall et al., 2000; Krylova et al., 2002). In the presence of Wnt, microtubules form loops as observed in axonal growth cones of granule cells (Hall et al., 2000). This microtubule reorganization is likely to determine changes in axon behavior. Importantly, loss of Wnt7a or its effector DVL causes severe defects in the terminal remodeling of axons *in vivo* (Ahmad-Annuar et al., 2006; Hall et al., 2000). According to this evidence, another later study showed that exposure to Wnt3a decreases the speed of growth cone advance whilst increasing growth cone size (Purro et al., 2008). The authors propose that Wnt regulates axon behavior through changes in microtubule growth directionality induced by a decrease in the APC level on microtubule plus-ends (Purro et al., 2008).

Taken together, these data clearly demonstrate that Wnt factors and their effectors positively modulate axon outgrowth and growth cone behavior through changes in the cytoskeletal components activity affecting their organization and stability.

Wnt PROTEINS CONTROL DENDRITIC MORPHOGENESIS

A mature neuron elicits a clear architecture characterized by a long distally branched axon which transmits signals, and a very complex dendritic arbor which is specialized to collect and integrate signals. Appropriate connections within the nervous system require the establishment of a polarized morphology to ensure unidirectional signal propagation (Jan and Jan, 2003; Conde and Caceres, 2009). Although a proper dendrite remodeling and shape underlies the normal mammalian brain function, including cognition and memory formation, abnormal dendritic development closely correlates with mental retardation and a number of central nervous system (CNS) disorders including Down's, Rett, and Fragile X syndromes (Comery et al., 1997; Kaufmann and Moser, 2000; Miller and Kaplan, 2003; De Rubeis and Bagni, 2010).

The molecular and cellular mechanisms that regulate dendritic growth and refinement are an area of intense research. Each neuron acquires its precise dendritic pattern through the regulation of its cytoskeleton by activating signaling pathways that change the activity, localization, and stability of cytoskeletal regulators (Conde and Caceres, 2009). Dendrite growth is a very dynamic process and the pattern of dendritic trees is believed to be regulated by an interplay between an intrinsic genetic program, extrinsic factors, and neuronal activity (Cline, 2001; Scott and Luo, 2001; Whitford et al., 2002a; Jan and Jan, 2003). Many extracellular factors have been identified as regulators of dendritic growth and

branching. For example, in vertebrates NT3, BDNF, and NGF can function as extrinsic factors to modulate the neuronal dendritic morphology (McAllister et al., 1995). Furthermore, the same neurotrophic factor can either inhibit or promote dendritic outgrowth depending on the type of neuron (McAllister et al., 1997). Semaphorin-3A (Sema3A) acts through the neuropilin-1 receptor, functions as chemoattractant factor for growing apical dendrites whereas antibody against neuropilin-1 can attenuate this dendrite orientation (Polleux et al., 2000). Consistent with this notion, in Sema3A null mice, many of the cortical pyramidal neurons have misoriented apical dendrites. Several evidences have shown that other extracellular factors such as Notch1 and Slit1 also regulate the emergence and growth of basal dendrites (Threadgill et al., 1997; Redmond et al., 2000; Whitford et al., 2002b). In addition, bone morphogenetic protein 7 (BMP7), a member of the TGF-β superfamily of ligands that are crucial for nervous system development, also affect dendritic morphogenesis in cultured neurons (Whitford et al., 2002a; Miller and Kaplan, 2003; Podkowa et al., 2010).

Another family of proteins that have been extensively involved in dendrite formation, maintenance, and functioning during the last decade is Wnt proteins. Several evidences suggest that Wnt factors modulate axon morphology and branching probably because they affect the activity and localization of many cytoskeletal regulators. These findings lead to consider that Wnt signaling may also regulate the dendritic trees morphology. Several studies have shown that Wnt proteins regulate dendritic architecture through the activation of different cascades. In this context, Yu and Malenka (2003) have postulated to β-catenin as a critical mediator of dendritic morphology. Thus, overexpression of β-catenin increases dendritic arborization in hippocampal neurons through a non-transcriptional mechanism (Yu and Malenka, 2003). Instead, constitutively active β-catenin increases dendritic arborization through its interaction with N-cadherin and αN-catenin (neural-catenin). Conversely, sequestering endogenous β-catenin leads to a decrease in dendritic complexity caused by neural activity (high K+ depolarization) suggesting that the level of endogenous β-catenin is important to the regulation of dendritic branching (Yu and Malenka, 2003). In addition, dickkopf-1 (Dkk-1), an extracellular Wnt antagonist (Glinka et al., 1998), blocks the dendritogenic effect of depolarization by high K^+ suggesting that neuronal activity regulates Wnt expression or release, which in turn modulates dendritic arborization. Importantly, conditioned medium from depolarized neurons contain higher levels of Wnt than does media from non-stimulated cells (Yu and Malenka, 2003). Another study showed that neuronal activity enhances the expression of Wnt-2, which stimulates dendritic complexity in cultured hippocampal neurons (Wayman et al., 2006). Activity-dependent dendritic outgrowth and branching in cultured neurons and slices is mediated through activation of Ca^{+2} depending signaling pathway (Wayman et al., 2006). In agreement with these studies, it has been demonstrated that Wnt signaling through Dvl stimulates dendritic growth and branching in hippocampal neurons (Rosso et al., 2005). Particularly, Wnt7b which is expressed in the hippocampus during dendritogenesis increases dendritic arborization by increasing dendritic length and the formation of complex branches. This effect is blocked by a Wnt scavenger, Sfrp1 (soluble Fz-related protein-1). Sfrp1 blocks endogenous Wnt activity present in hippocampal cultures that contributes to the normal dendritic development (Rosso et al., 2005). The Wnt effect on dendrite development is mimicked by Dvl that localizes along the neurites associated with microtubules and is highly concentrated in the peripheral region of growth cones co-localizing with actin cytoskeleton (Rosso et al., 2005; **Figure 2**). Dvl1 mutant neurons exhibit shorter and less complex dendrite arbors compared to neurons from wild-type mice (Rosso et al., 2005). These results demonstrate that Dvl1 is required for normal dendritic development in hippocampal neuron (Rosso et al., 2005). Further analyses revealed that Wnt7b/Dvl signaling regulates dendritic development through a non-canonical pathway, since activation of GSK-3β or inhibition of β-catenin is not involved. In contrast, Wnt7b and Dvl modulate dendrite development through changes in the activity of Rho GTPases and JNK. Wnt7b and Dvl activate endogenous Rac and its downstream effector JNK, this effect is abolished by Sfrp1.

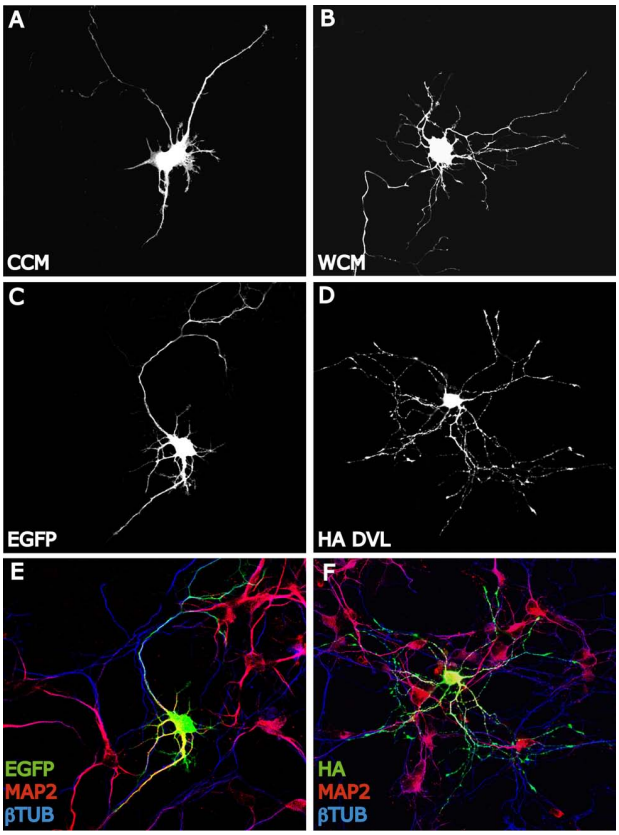

FIGURE 2 | Wnt-Dvl pathway modulates dendrite development. (A,B) EGFP expressing hippocampal neurons of 3 days *in vitro* (DIV) expose to Wnt7b conditioned medium (WCM) show longer and more complex dendritic arborizations compare to EGFP-control cells (CCM). Neurons are staining with GFP antibody to analyze the entire morphology. **(C–F)**: DVL mimics the dendritogenic effect of Wnt7b. EGFP expressing neurons of 3 DIV develop several short and unbranched dendrites (MAP2+) **(C,E)**. However, hippocampal neurons expressing HA-DVL reveal very complex dendritic trees with longer and more number of dendritic branches (MAP2+) **(D,F)**.

Moreover, inhibition of JNK or expression of a Rac dominant negative mutant in neurons blocks the Dvl function in dendritic development. These finding suggested that Dvl functions as a molecular link between Wnt factors and the cytoskeletal modulators Rho GTPases to control dendritic development (Rosso et al., 2005). In addition, a recent study shows that the non-canonical Wnt signaling is necessary for normal morphological maturation of olfactory bulb (OB) interneurons (Pino et al., 2011). Interestingly, traditionally non-canonical Wnt ligands Wnt5a and Wnt7b, but not canonical Wnts, are expressed by OB interneurons themselves (Shimogori et al., 2004). Moreover, evidence from the Wnt5a knockout mouse indicates that normal morphological development of OB interneurons is disrupted in the absence of endogenous Wnt5a, while cell number and OB architecture remain intact (Pino et al., 2011). These data are in accordance with those in hippocampal neurons where Sfrp1 blocks neurite outgrowth and non-canonical Wnt ligand Wnt7b increases dendrite complexity (Rosso et al., 2005; Endo et al., 2008).

The role of Wnt ligands on neuronal maturation have also been described in another model, such as *C. elegans* in which five Wnts were identified (Park and Shen, 2012). A recent study in *C. elegans* shows that dendrite outgrowth can be regulated by distinct processes which are independent of axon formation (Kirszenblat et al., 2011). This work suggests that the Wnt ligand (LIN-44), and its Fz receptor (LIN-17), regulate dendrite development of the oxygen sensory neurons (PQR) (Kirszenblat et al., 2011). In lin-44 and lin-17 mutants, neurons show a delayed growth. Additional experiment revealed that LIN-44 functions as an attractive cue to define the outgrowth of the dendrite (Kirszenblat et al., 2011). LIN-44 acts at very early stages of PQR development by regulating proper formation of the growth cone and its extension.

Taken together, these evidences reveal the important role for Wnt signaling pathways in regulating dendrite development and complexity and how Wnt expression and secretion may be influenced by extracellular cues to modulate neuronal morphogenesis.

Wnt SIGNALING AT CENTRAL SYNAPSES

Wnt ligands have been linked to the assembly of structural components in presynaptic compartments. In the cerebellum, Wnt7a is expressed in granular cells at the same time as the mossy fiber axon, which is the presynaptic contact (Hall et al., 2000). Several changes remodel the connectivity between both areas to increase the contact surface. Wnt7a induces axonal spreading and incremental growth of cone size and branching, leading to the accumulation of synaptic proteins (Hall et al., 2000; Budnik and Salinas, 2011). Wnt7a probably contributes to the formation of active zones because it increases the clustering of synapsin I, a protein located in the presynaptic membrane involved in synapse formation and function (Hall et al., 2000). This effect has been blocked by the Wnt scavenger Sfrp and a mutant mice deficient in Wnt7a shows a delayed synaptic maturation (Hall et al., 2000). Then in the cerebellum, Wnt7a can act as a retrograde signal from granular cells to induce presynaptic differentiation in mossy fiber, working as a synaptogenic factor (Hall et al., 2000; Ahmad-Annuar et al., 2006). Like Wnt7a, Wnt7b, and Wnt3a increase the number of presynaptic puncta suggesting a role for these ligands in presynaptic assembly (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008). Wnt7a also increases the clustering of presynaptic proteins such as synaptophysin, synaptotagmin, and synaptic vesicle protein 2 (SV2), but does not affect postsynaptic clustering of proteins like postsynaptic density protein-95 (PSD-95; Cerpa et al., 2008). Despite Wnt7a clustering induction correlates with β-catenin stabilization, this does not involve Wnt gene target expression – an effect that is also mimicked by Wnt3a. Unexpectedly, GSK-3β is also not required for presynaptic clustering induced by Wnt7a, suggesting that an upstream mechanism is involved (Cerpa et al., 2008). It has been suggested that Wnt7a requires Dvl1 to mediate the normal recycling rate of synaptic vesicles, and the deficiency of both proteins (double null mutant) significantly reduces miniature exitatory postsynaptic current (mEPSCs) frequency, an indication of a defect in neurotransmitter release (Ahmad-Annuar et al., 2006). Additionally, the use of FM dyes has shown that Wnt7a stimulates recycling and accelerates exocytosis of synaptic vesicles (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). Moreover, Wnt7a increases the frequency of mEPSCs, suggesting that Wnt7a increases the dynamic of neurotransmitter release (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). Furthermore, Wnt7a/Dvl1 double mutant mice exhibit reduced mEPSC frequency at the mossy fiber-granule cell synapses, revealing a defect in neurotransmitter release as a consequence of this mutation (Ahmad-Annuar et al., 2006). Electrophysiological recordings on hippocampal rat slices also show that, in the CA3–CA1 synapse Wnt7a, but not Wnt5a, increases the amplitude of field excitatory postsynaptic potentials (fEPSP) and decreases the rate of paired pulse facilitation (PPF; Cerpa et al., 2008), a protocol used to distinguish the involvement of the presynaptic from the postsynaptic terminal. In addition, a similar modulation has been shown with nanomolar concentrations of Wnt3a, which modulates the recycling and exocytosis of synaptic vesicles in hippocampal synapses, increasing the frequency of mEPSC through a mechanism that involves Ca^{2+} entrance from extracellular media (Cerpa et al., 2008; Avila et al., 2010). Most of the ligands that are able to modulate presynaptic differentiation have shown to activate the Wnt/β-catenin signaling pathway.

Wnt7a has been also involved in trafficking of receptors, increasing the number and size of co-clusters of presynaptic α_7-nicotinic acetylcholine receptors (α_7-nAChR) and APC in hippocampal neurons, as well as in the modulation of the α_7-nAChR trafficking to the nerve terminal (Farias et al., 2007), indicating that Wnt pathway components are actively involved in the functional availability of receptors in the synaptic terminal.

Wnt signaling also plays relevant roles in the postsynaptic structure. Wnt5a induced a transient formation of dendritic protrusions, which results in a net increase of mature dendrite spines. Video microscopy revealed that Wnt5a induced *de novo* formation of dendrite spines and also increased the size of the preexistent ones (Varela-Nallar et al., 2010). Interestingly, treatment with the soluble CRD region of Fz2, which act as a Wnt scavenger, decreases spine density in cultured neurons, supporting the idea that Wnt ligands participate in dendrite spine

morphogenesis (Varela-Nallar et al., 2010). Wnt5a also induces an increase of calcium in synaptic puncta of neurons, suggesting the activation of the Wnt/Ca^{2+} signaling pathway in cultured hippocampal neurons through a mechanism that involves fast phosphorylation of CamKII induced by Wnt5a (Varela-Nallar et al., 2010), as we demonstrated previously (Farias et al., 2009). Wnt7a is also able to increase the density and maturity of dendritic spines through a CamKII-dependent mechanism (Ciani et al., 2011). Wnt7a rapidly activates CamKII in spines and inhibition of this kinase abolishes the effects of Wnt7a on spine growth and excitatory synaptic strength. These findings implicate the Wnt/Ca^{2+} signaling cascade in synaptic effects of Wnt ligands (**Figure 3**).

Interestingly, Dvl expressed only in postsynaptic spines and not in innervating presynaptic axons is enough to induce spine growth, suggesting that it is the activation of postsynaptic Wnt signaling which induces spine maturation (Ciani et al., 2011). Moreover, Dvl promotes the assembly of pre- and postsynaptic structures at pre-existing spines because this does not change the number of spines (Ciani et al., 2011). This evidence supports the idea that an extracellular signal such as Wnt7a can generate a divergent intracellular product, using a common molecule such as Dvl to support processes like synaptic differentiation (Gao and Chen, 2010), and a new role for Wnt7a inducing the formation and function of excitatory synapses through CamKII (**Figure 3**).

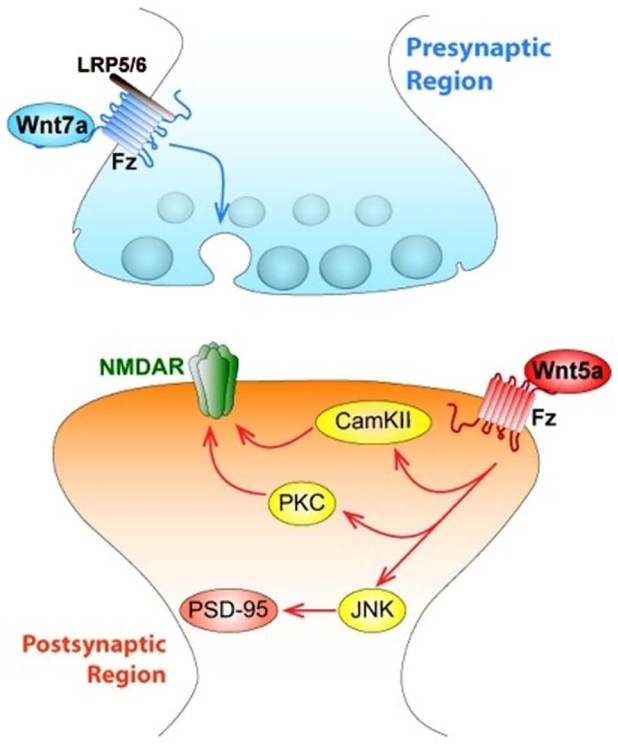

FIGURE 3 | Wnts modulate both pre- and postsynaptic regions of central synapses affecting synaptic wiring and plasticity. The figure indicates a central synapse of the mammalian nervous system. At the *presynaptic region,* canonical Wnt7a ligand activates Fz receptor associated to LRP5/6, and triggers the activation of the synaptic vesicle cycle, which determines the release of the neurotransmitter glutamate. At *the postsynaptic level*, Wnt5a ligand activates both the Wnt/PCP (JNK) and the Wnt/Ca signaling pathways, through activation of JNK, Wnt5a regulates the clustering of PSD-95. Wnt5a also activates both non-canonical pathways that allow an increase in the traffic of NMDA receptors.

Wnt5a modulates postsynaptic assembly increasing the clustering of the PSD-95 via Wnt/JNK signaling pathway (Farias et al., 2009), inducing a fast increase in the number of PSD-95 clusters without affecting total levels of PSD-95 protein or presynaptic protein clustering in hippocampal cultured neurons (Farias et al., 2009; **Figure 3**). PSD-95 is a scaffold protein of the postsynaptic density, which is a multiprotein complex that interacts with key molecules involved in the regulation of glutamate receptor targeting and trafficking and regulatory proteins relevant for neurotransmission (Han and Kim, 2008). When hippocampal neurons were incubated with the formylated hexapeptide Foxy-5, derived from the sequence of Wnt5a and mimics the full Wnt5a action, there was an increase in PSD-95 since 1 h, but after 24 h an increase in the SV2 clustering was also observed. In consequence, there was an increase in the total number of synaptic contacts (Varela-Nallar et al., 2012).

At the neuromuscular junction of vertebrate skeletal muscles, Wnt3 was also able to induce recruitment of AChRs (Henriquez et al., 2008). This effect requires Dvl1 and agrin, a protoglycan released by motoneurons, but does not involve the Wnt/β-catenin pathway. Instead, aggregation is induced through activation of Rac1 (Henriquez et al., 2008). However, Wnt3a inhibits agrin-induced AChR clusters through the activation of the Wnt/β-catenin pathway, suggesting that Wnt signaling dynamically regulates the interaction between postsynaptic components during the establishment of neuromuscular junctions (Wang et al., 2008).

Different Wnts have shown modulatory effects on glutamatergic neurotransmission. Wnt3a modulates the recycling of synaptic vesicles in hippocampal synapses (Cerpa et al., 2008; Varela-Nallar et al., 2009) and is able to induce an increase in the frequency of mEPSCs (Avila et al., 2010). In hippocampal slices, blockade of Wnt signaling impairs long-term potentiation (LTP), whereas activation of Wnt signaling facilitates LTP (Chen et al., 2006). In the case of Wnt5a, acute application of this ligand in hippocampal slices increases the amplitude of fEPSP and upregulates synaptic *N*-methyl-D-aspartate (NMDA) receptor currents facilitating induction of LTP (Cerpa et al., 2010, 2011; Varela-Nallar et al., 2010). Interestingly, Wnt5a produced a two-step increase in the amplitude of NMDA receptor responses, not α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Cerpa et al., 2011). There is a fast PKC-dependent potentiation and a slower JNK-dependent potentiation that does not require previous activation of PKC (Cerpa et al., 2011).

Wnt5a also regulates postsynaptically the hippocampal inhibitory synapses (Cuitino et al., 2010). Wnt5a induces surface expression and maintenance of $GABA_A$ receptor in the membrane of hippocampal neurons, increases the amplitude of gamma-aminobutyric acid (GABA)-currents due to postsynaptic mechanisms, and induces the recycling of functional $GABA_A$ receptors through activation of CamKII (Cuitino et al., 2010). Therefore Wnt5a is able to modulate both, excitatory and inhibitory synapses which must be relevant for neurotransmission.

ROLE OF Wnt SIGNALING IN ALZHEIMER'S DISEASE

Major neurological diseases are all progressive disorders with common symptoms: a range of neuropsychiatric features, massive neuronal degeneration, and neither preventive nor effective long-term treatment strategies available. However, all of them develop in particular brain regions generating a specific phenotype according to the circuit that is being affected. Here, we review recent studies related to the progression of AD in which the Wnt signaling pathway and its components might be relevant. AD is a neurodegenerative disorder characterized by progressive deterioration of cognitive functions, caused by synaptic dysfunction and damage of specific brain regions (Mattson, 2004; Toledo et al., 2008). Distinctive features of AD brains are the presence of senile plaques, composed by extracellular deposits of amyloid-β (Aβ) peptides and neurofibrillary tangles (NFTs), composed by intracellular aggregates of hyper-phosphorylated tau protein (Mayeux and Stern, 2012). Oligomeric forms of $A\beta_{1-42}$ are the physiologically relevant neurotoxic Aβ species and Aβ oligomers isolated from AD brains can damage the memory and alter hippocampal synaptic plasticity in healthy rats, inhibiting LTP, increasing long-term depression and reducing spine density in the hippocampus *in vivo* (Shankar et al., 2007). The presence of Aβ oligomers reduces the fEPSP and EPSCs, but not the PPF in the CA3–CA1 synapse of rat hippocampal slices (Cerpa et al., 2010; Inestrosa et al., 2012). Altogether these evidences suggest that AD cognitive decline might be due to a direct effect of Aβ oligomers on synaptic transmission (Palop and Mucke, 2010).

During more than a decade, a strong relationship between an impaired Wnt signaling pathway activity and neuronal damage in AD has been raised (De Ferrari and Inestrosa, 2000; Inestrosa et al., 2000; Garrido et al., 2002; De Ferrari et al., 2003; Inestrosa and Arenas, 2010). Different studies have shown that Wnt signaling components are altered in AD (Zhang et al., 1998; Inestrosa et al., 2002; Caricasole et al., 2004; Ghanevati and Miller, 2005; De Ferrari et al., 2007; Magdesian et al., 2008). Among the Wnt components that are affected in AD, it was shown that β-catenin levels are reduced in AD patients carrying presenilin-1-inherited mutations (Zhang et al., 1998), while the secreted Wnt antagonist Dkk1 is elevated in postmortem AD brains and brains from transgenic mouse models for AD (Caricasole et al., 2004; Rosi et al., 2010). A variant of the LRP6 has been associated with late-onset AD, which confers low levels of Wnt signaling (De Ferrari et al., 2007). Epidemiological data show an increased risk for AD in populations where the allele 4 of apo-lipoprotein E (apoE4) is present. Interestingly apoE4 causes inhibition of the canonical Wnt signaling in PC12 cells upon stimulation with Wnt7a as determined by luciferase activities and nuclear β-catenin levels (Caruso et al., 2006). A direct Aβ binding to the extracellular CRD-Fz5 receptor at or in close proximity to the Wnt-binding site inhibits the canonical Wnt signaling pathway (Magdesian et al., 2008), linking directly Aβ to Wnt impairment. Moreover, the exposure of cultured rat hippocampal neurons to Aβ results in inhibition of canonical Wnt signaling as determined by destabilization of endogenous levels of β-catenin, increase in GSK-3β activity, and a decrease in the expression of some Wnt target genes (Alvarez et al., 2004). Chronic overexpression of Dkk-1 causes age-related tau phosphorylation and cognitive deficits (Killick et al., 2012). During Aβ exposure there is induction of Dkk-1 expression that depends on p53 (Killick et al., 2012). This generated Dkk-1 can bind LRP5/6 to inhibit their interaction to Wnts. Thus Wnt cannot inhibit GSK-3β facilitating tau hyperphosphorylation and NFT formations, leading to neurotoxicity and apoptosis caused by Aβ peptides (Caricasole et al., 2004). The use of an antibody antiDkk-1 also blocks the synaptic loss induced by Aβ (Purro et al., 2012). In the same report, Purro et al. (2012) documented that Dkk-1 can reversibly reduce the amount of synaptic proteins and the number of active presynaptic sites, by inducing synaptic disassembly at pre- and postsynaptic sites and not by degrading proteins.

Recently, new evidence supports a link between Aβ toxicity and the Wnt pathway. Aβ substantially increases the intracellular protein clusterin, which induces Dkk-1 expression (Killick et al., 2012). The C-terminal of the Dkk-1 protein, which antagonizes canonical Wnt pathway binding LRP5/6, later activates gene transcription involved in AD-like pathology (Killick et al., 2012). Because Dkk-1 blocks Wnt/β-catenin, Dkk-1 activates the Wnt/JNK pathway, as has been shown from the increase in c-Jun activity (Killick et al., 2012). Thus, the transcriptional Aβ effects occur because Dkk-1 activates Wnt/JNK pathway. Clusterin has been recently identified as a susceptibility factor in late-onset AD (Harold et al., 2009; Lambert et al., 2009) and several genes from Wnt/JNK have been found in the AD human brain (Killick et al., 2012).

Synaptic failure is an early event in AD, and soluble Aβ oligomers are proposed to be responsible for the synaptic pathology that occurs before the plaque deposition and neuronal death (Serrano-Pozo et al., 2011). Electrophysiological analysis of Schaffer collaterals-CA1 glutamatergic transmission in hippocampal slices demonstrated that Wnt5a prevents the decrease in the amplitude of fEPSP and EPSCs induced by Aβ oligomers, indicating that Wnt5a prevents the synaptic damage triggered by Aβ (Cerpa et al., 2010). Moreover, Wnt5a prevents the decreases in PSD-95 and synaptic loss in cultured hippocampal neurons (Farias et al., 2009) (Cerpa et al., 2010), supporting that Wnt5a improves synaptic function in the presence of Aβ.

Several studies have shown neuroprotective properties of the Wnt signaling activation against the toxicity of Aβ peptide (**Figure 4**). The protective effect of Wnt3a against the toxicity of Aβ oligomers was shown to be mediated by the Wnt Fz1 receptor, since this effect is modulated by the expression levels of Fz1 in both, PC12 cells and hippocampal neurons (Chacon et al., 2008). Overexpression of Fz1 significantly increased cell survival induced by Wnt3a and diminished caspase-3 activation, while knocking-down the expression of the receptor by antisense oligonucleotides decreased the stabilization of β-catenin induced by Wnt3a and decreases the neuroprotective effect elicited by this Wnt ligand (Chacon et al., 2008). These studies support the evidence that alterations in Wnt/β-catenin are involved in AD.

One of the hallmarks of AD brains is the abnormal phosphorylation of the tau protein which accumulates as intraneuronal NFT (Serrano-Pozo et al., 2011). Cultured neurons exposed to Aβ show an increase in GSK-3β activity (Takashima et al., 1993; Alvarez et al., 2004; Inestrosa et al., 2005). Importantly, active

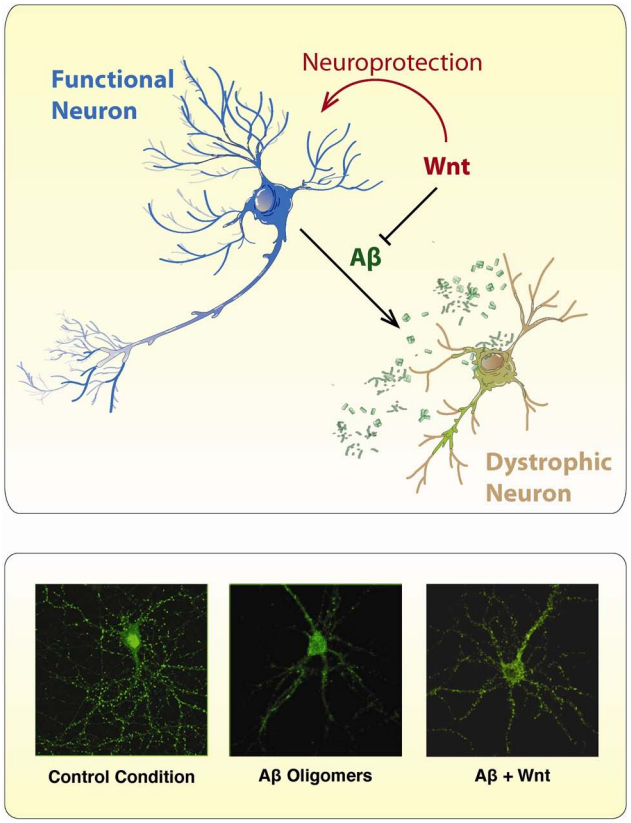

FIGURE 4 | Wnt ligands protect neurons from the toxic effects of Aβ Oligomers. The scheme illustrates the neurotoxic effect of Aβ oligomers on neurons which became dystrophic showing neurite fragmentation and eventual cell death. However, in the presence of a canonical Wnt ligand, neurons became neuroprotected and no neuropathological changes are evident. The immunofluorescence photographs presented below, were taken against the *GFP* expressing protein present in hippocampal neurons incubated under different conditions: (1) control conditions, (2) Aβ oligomers, and (3) Aβ oligomers plus Wnt7a. In the presence of Aβ oligomers, treated neurons showed typical neurodegenerative alterations including loss of somatodendritic arborization and neurite fragmentation. After co-incubation with Wnt7a, the neurotoxic effect of Aβ oligomers is not longer present, and hippocampal neurons look pretty normal.

GSK-3β has been found in brains staged for AD neurofibrillary changes with a concomitant decreases in β-catenin levels and an increase in tau hyperphosphorylation (Pei et al., 1999). Also, neurodegeneration and spatial learning deficits have been observed in GSK-3β conditional transgenic mice (Lucas et al., 2001; Hernandez et al., 2002). Moreover, over-expression of GSK-3β in mice prevents the induction of LTP and reduces spatial learning (Hernandez et al., 2002; Hooper et al., 2007), linking the characteristic memory failure in AD to the increase in GSK-3β. In cultured neurons the toxicity mediated by Aβ depends on increased GSK-3 activity, and reverses when GSK-3β expression or activity is blocked (Busciglio et al., 1995; Alvarez et al., 1999). We previously demonstrated that activation of Wnt signaling inhibits GSK-3β and leads to neuroprotection in both hippocampal cultured neurons and *in vivo* transgenic model of AD (Alvarez et al., 2004; Quintanilla et al., 2005; Toledo and Inestrosa, 2010). Moreover, Li et al. (2007) found that tau phosphorylation, which inhibits competitive phosphorylation of β-catenin by GSK-3β, protects neurons from apoptosis. This results support a role of β-catenin as a survival element in AD. Finally, the activation of several signaling pathways that crosstalk with the Wnt pathway, including the nicotinic and muscarinic ACh receptors, peroxisome proliferator-activated receptor (PPAR) α and γ, anti-oxidants, and anti-inflammatory pathways, all support the neuroprotective potential of the Wnt cascades in AD (Inestrosa and Toledo, 2008; Inestrosa and Arenas, 2010; Inestrosa et al., 2012).

CONCLUSION

This review focused on the role of Wnt proteins on neuronal development and their participation on the synaptic function. Wnt signaling regulates neuronal maturation by stimulation of dendrite formation and complexity. Particularly, it has been reported that neurons exposed to Wnt elicit longer dendrites and more complex dendritic branches. In addition, several works have demonstrated that the non-canonical Wnt pathways would modulate dendrite formation. Thus, Wnt effectors as JNK and CaMKII may control dendrite architecture and neuronal maturation (Threadgill et al., 1997; Rosso et al., 2005; Wayman et al., 2006). Importantly, these effectors have been implicated in cytoskeletal remodeling by controlling MAPs phosphorylation and microtubule dynamics and, they also bind and modulate actin filopodia extension (Shen et al., 1998; Chang et al., 2003; Fink et al., 2003; Bjorkblom et al., 2005). Changes in the organization and stability of cytoskeletal components by Wnt pathways likely affect dendritic growth and dynamics. Furthermore, neuronal activity plays a central role in dendrites formation and maintenance. Several studies have shown that the stimulation of neuronal activity leads to increase the expression and/or secretion of Wnt proteins and also can modify the MAPs activity and microtubule stability (Vaillant et al., 2002; Yu and Malenka, 2003; Wayman et al., 2006). Dendrites morphologies influence on synaptic function and neuronal circuits formation. Thus, the timing of synapse formation coincides with the period of dendritic growth and branching. A great body of evidences extensively suggests that Wnt signaling modulate synaptic function and plasticity. Thus, the pre- and postsynaptic terminal assembly is modulated by Wnt signaling to maintain the central connectivity (Hall et al., 2000; Packard et al., 2002; Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Henriquez et al., 2008; Farias et al., 2009; Cuitino et al., 2010). Taken together, these compiled findings provide important insights about the involvement of Wnt signaling pathways on the formation and functioning of neuronal circuits.

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Thyroid hormone treated astrocytes induce maturation of cerebral cortical neurons through modulation of proteoglycan levels

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Proper brain neuronal circuitry formation and synapse development is dependent on specific cues, either genetic or epigenetic, provided by the surrounding neural environment. Within these signals, thyroid hormones (T3 and T4) play crucial role in several steps of brain morphogenesis including proliferation of progenitor cells, neuronal differentiation, maturation, migration, and synapse formation. The lack of thyroid hormones during childhood is associated with several impair neuronal connections, cognitive deficits, and mental disorders. Many of the thyroid hormones effects are mediated by astrocytes, although the mechanisms underlying these events are still unknown. In this work, we investigated the effect of 3, 5, 3′-triiodothyronine-treated (T3-treated) astrocytes on cerebral cortex neuronal differentiation. Culture of neural progenitors from embryonic cerebral cortex mice onto T3-treated astrocyte monolayers yielded an increment in neuronal population, followed by enhancement of neuronal maturation, arborization and neurite outgrowth. In addition, real time PCR assays revealed an increase in the levels of the heparan sulfate proteoglycans, Glypican 1 (GPC-1) and Syndecans 3 e 4 (SDC-3 e SDC-4), followed by a decrease in the levels of the chondroitin sulfate proteoglycan, Versican. Disruption of glycosaminoglycan chains by chondroitinase AC or heparanase III completely abolished the effects of T3-treated astrocytes on neuronal morphogenesis. Our work provides evidence that astrocytes are key mediators of T3 actions on cerebral cortex neuronal development and identified potential molecules and pathways involved in neurite extension; which might eventually contribute to a better understanding of axonal regeneration, synapse formation, and neuronal circuitry recover.

Keywords: astrocyte, thyroid hormones, neurite outgrowth, extracellular matrix, brain morphogenesis

INTRODUCTION

Central nervous system (CNS) development is characterized by differentiation of neural progenitors into different cell types, proper neuronal migration and maturation, and axonal growth in order to innervate specific targets. Accuracy in neuronal circuitry formation and synapse development is dependent on specific cues provided by the surrounding neural environment, either genetic or epigenetic (Powell et al., 1997). Astrocytes, the most abundant glial cell in the CNS, are source of most the extracellular matrix components (ECM) and neurotrophic factors involved in these events, including 3, 5, 3′-triiodothyronine (T3), the biological active form of thyroid hormones, which are essential morphogens of the nervous system (Garcia-Abreu et al., 1995; Trentin et al., 1995; Ullian et al., 2001, 2004; Martinez and Gomes, 2002; Christopherson et al., 2005; Lie et al., 2005; Martinez and Gomes, 2005; Kornyei et al., 2007; Spohr et al., 2008; Barker and Ullian, 2010; Stipursky et al., 2011, 2012; Tc et al., 2011).

Thyroid hormones, thyroxin (T4) and T3, influence critical events in brain morphogenesis including neuronal migration and differentiation, glial cells maturation, synaptogenesis, and myelination (Alvarez-Dolado et al., 1999; Auso et al., 2004; Cuevas et al., 2005; De Escobar et al., 2007; Dezonne et al., 2009; Portella et al., 2010). Hypothyroidism leads to impaired cerebral cortical layering and altered callosal connections (Gravel et al., 1990; Berbel et al., 1993; Calikoglu et al., 1996). Moreover, it has been associated with clear reductions on axonal and dendritic outgrowth (Eayrs, 1955; Ruiz-Marcos et al., 1979) as well as disorganization of dendritic spines and fewer synaptic connections (Ruiz-Marcos et al., 1988) in experimental models. In humans,

Abbreviations: ACM, astrocyte conditioned medium; C-CM, control astrocyte conditioned medium; CM, conditioned medium; CNS, central nervous system; CSPG, chondroitin sulfate-conjugated proteoglycan; D2, type II iodothyronine 5′-deiodinase; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMEM/F-12, Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12; E14, embryonic day fourteen; ECM, extracellular matrix; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; GPC-1, Glypican 1; HSPG, heparin sulfate conjugated-proteoglycan; PBS, phosphate-buffered saline; SDC-3, Syndecans 3; SDC-4, Syndecan 4; T3, 3, 5, 3′-triiodothyronine; T3-CM, T3-primed astrocyte conditioned medium; T4, Thyroxin; TBS-T, Tris-buffered saline-Tween 20; TRs, thyroid hormones receptors; TRα, isoform α of TR; TRβ, isoform β of TR.

insufficient levels of thyroid hormones during brain development are associated with severe neurological deficits that might result in the cretinism syndrome (Joffe and Sokolov, 1994).

T4 is the predominant hormone form found in the nervous tissue, where it is converted to the active form, T3, by the type II iodothyronine 5′-deiodinase (D2), mainly expressed by astrocytes and tanycytes (Guadano-Ferraz et al., 1997; Santisteban and Bernal, 2005). Activation of major signaling pathways by T3/T4 involves binding of T3 to nuclear receptors, although extranuclear pathways have also been described (Santisteban and Bernal, 2005; Bernal, 2007). Thyroid hormones nuclear pathway is triggered by activation of thyroid hormone receptors alpha (TRα) and beta (TRβ) widely expressed by neuronal and glial cells (Puymirat, 1992; Carlson et al., 1996; O'Shea and Williams, 2002; Bernal, 2007).

Despite thyroid hormones effects on neurons are well known, several works have shown that some of these effects might be indirectly controlled by astrocytes (Trentin and Moura Neto, 1995; Gomes et al., 1999; Martinez and Gomes, 2002, 2005).

Thyroid hormones have been reported to modulate astrocyte morphology, differentiation, and proliferation (Lima et al., 1997; Trentin et al., 1998; Trentin, 2006), and to regulate ECM organization and synthesis (Farwell and Dubord-Tomasetti, 1999a,b; Calloni et al., 2001; Martinez and Gomes, 2002; Mendes-De-Aguiar et al., 2008). *In vivo*, thyroid hormones regulate radial glia-astrocyte transition and the vimentin- glial fibrillary acidic protein (GFAP) switch, a hallmark of astrocyte differentiation, in the basal forebrain and hippocampus (Gould et al., 1990; Martinez-Galan et al., 1997, 2004). Although astrocytes are evident targets for thyroid hormones *in vitro* and *in vivo*, the precise effects of these hormones in neuron-astrocyte interactions are still under investigation.

We previously demonstrated that thyroid hormone-treated cerebellar astrocytes induce cerebellar progenitor proliferation (Gomes et al., 1999) and neurite outgrowth (Martinez and Gomes, 2002, 2005). These events result from the synthesis and secretion of soluble factors, such as epidermal growth factor (EGF), and the ECM molecules laminin (LN), fibronectin (FN) and syndecans, producing a substrate for neuronal maturation (Mendes-De-Aguiar et al., 2008, 2010).

In the present work, we investigated the role of cortical astrocytes as mediators of thyroid hormone T3, on neuronal maturation, using an *in vitro* system consisting of astrocyte-neuron cocultures. Here we report that thyroid hormone-primed astrocytes increase neuronal differentiation and neuritic arborization, mainly by modulation of chondroitin and heparan sulfate proteoglycans (HSPG).

MATERIALS AND METHODS

ETHICAL APPROVAL

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

ASTROCYTE PRIMARY CULTURE

Astrocytes primary cultures were prepared from cerebral cortex derived from newborn Swiss mice, as previously described (Spohr et al., 2008). Briefly, after mice decapitation, brain structures were removed and the meninges were carefully stripped off. Tissues were washed in phosphate-buffered saline (PBS), 0.6% glucose (Merck, Darmstadt, Hessen, DE) and cortical structures were dissociated into single cells in a medium consisting of Dulbecco's modified Eagle's medium supplemented with nutrient mixture F-12 (DMEM/F-12, Invitrogen Life Technologies, Carlsbad, California, USA), enriched with glucose (3.3×10^{-2} M), glutamine (2×10^{-3} M) and sodium bicarbonate (0.3×10^{-2} M). Dissociated cells were plated onto plastic culture flask or glass cover slips (24 wells plates, Techno Plastic Products, Trasadingen, CH) previously coated with polyornithine (1.5 μg/mL, molecular weight 41,000; Sigma Chemical Co., St Louis, Missouri, USA) in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (Invitrogen). The cultures were incubated at 37°C in a humidified 5% CO_2, 95% air chamber. After 24 h, cell cultures were washed and media were replaced by DMEM/F-12 supplemented with 10% FBS. The medium was changed every second day until reaching confluence.

T3 TREATMENT AND CONDITIONED MEDIUM (CM) PREPARATION

After reaching confluence, glial monolayers were washed three times with serum-free DMEM/F12 medium, and incubated for an additional day in serum-free medium. After this period, cultures were treated with 50 nM of T3 (Sigma Aldrich) in DMEM/F12 for 3 days with daily medium change. Control astrocyte carpets were maintained in DMEM/F12 without serum. After that, glial monolayers were washed three times with serum-free DMEM/F12 and maintained for an additional day with serum-free medium. CMs derived from T3-treated (T3-CM) or control cultures (C-CM) were recovered, centrifuged at 1500 g for 10 min, and used immediately or stored at −70°C for further use.

ENZYMATIC TREATMENT OF ASTROCYTE MONOLAYERS

To analyze a possible influence of glycosaminoglycans, astrocyte monolayers were enzymatically digested with chondroitinase AC (5.0×10^{-7} U/μL) (that specifically digests chondroitin sulfate glycosaminoglycan chains) or heparanase III (5.0×10^{-7} U/μL) [that specifically digests heparan sulfate glycosaminoglycan (HSG) chains] (Sigma Aldrich) in DMEM/F12, for 2 h at 37°C, prior to addition of progenitor cells. After, cultures were extensively washed with medium without serum to remove all residual enzymes, followed by addition of neuronal progenitors to astrocyte monolayers.

NEURAL PROGENITOR CULTURE AND ASTROCYTE-NEURAL PROGENITOR COCULTURE

Pregnant Swiss females with 14-gestational days were killed by halothane followed by cervical dislocation, and embryos (E14) were removed. Cortical progenitors were prepared as previously described (Spohr et al., 2008). Briefly, for coculture assays cells were freshly dissociated from cerebral cortex and 5×10^4 cells were plated onto control, thyroid hormones-treated glial monolayer carpets or onto astrocyte-carpets previously digested with chondroitinase or heparanase III. Cocultures were kept for 24 h at 37°C in a humidified 5% CO_2, 95% air atmosphere. For pure neural progenitor cultures 1×10^5 dissociated cells were plated

onto glass cover slips previously coated with polyornithine, and incubated with C-CM or T3-CM. Cultures were kept for 24 h at 37°C in a humidified 5% CO_2, 95% O_2 air atmosphere.

IMMUNOCYTOCHEMISTRY

Cells were fixed with 4% paraformaldehyde for 5 min for ECM protein analyses or 15 min, for cytoskeleton protein analyses. For cytoskeleton proteins analysis, cells were additionally permeabilized with 0.2% Triton-X (Vetec Química Fina Ltda, Rio de Janeiro, Rio de Janeiro, BR) for 5 min at room temperature. Subsequently, cells were blocked with 5% bovine serum albumin (Invitrogen) and 3% normal goat serum (Invitrogen) in PBS (block solution) for 1 h and incubated overnight at 4°C with the specified primary antibody diluted in block solution. Primary antibodies were mouse anti−βTubulin III antibody (Promega Corporation; Madison, Wisconsin, USA; 1:1000); rabbit anti-GFAP (Dako, Glostrup, DK; 1:500); rabbit anti-Fibronectin (Sigma Aldrich; 1:200); rabbit anti-Laminin (Sigma Aldrich; 1:100). After primary antibodies incubation, cells were extensively washed in PBS and incubated with the following secondary antibodies diluted in block solution for 2 h: goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-rabbit IgG and anti-rat IgG conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, Oregon, USA; 1:400, 1:500, and 1:1000, respectively). Cell nuclei were labeled with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and cell preparations were mounted directly on N-propyl gallate (Sigma Aldrich). Negative controls were obtained by omitting primary antibodies; in all cases, no reactivity was observed. After immunostaining, cell cultures were visualized and counted using a TE300 Nikon microscope. At least 10 fields were counted per well.

NEURONAL MORPHOMETRY

To analyze neurite outgrowth, neuronal cells cultured either onto astrocyte monolayers or onto glass cover slips, were measured using the NeuronJ plug-in of Image J 1.36 b software. At least 10 fields were measured per well. In all cases, at least 100 neurons randomly chosen were observed per well. All neurites emerged from neuronal soma were considered. Neurite length was analyzed by 3 different methods either considering only the major process per neurons, the sum of all neurite measurements per neuron and the sum of all neurite measurements divided by the number of process per neuron.

WESTERN BLOT

Protein concentration on cell extracts was measured by the BCA™ Protein Assay Kit (Pierce, IL, USA). Fifty micrograms of protein per lane were eletrophoretically separated in 5–15% gradient sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After separation, proteins were electrically transferred onto a Hybond-P polyvinylidene difluoride transfer membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) for 3 h. Membranes were blocked overnight in Tris-buffered saline-Tween 20 (Merck) (TBS-T) containing 10% BSA. Primary antibodies were added for 2 h at room temperature. After several washes in TBS-T, peroxidase-conjugated secondary antibodies were added to membrane and incubated for 2 h at room temperature. Proteins were visualized using the enhancing chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate/Pierce, Milwaukee, Wisconsin, USA), and PVDF membranes were exposed to autoradiographic films (Kodak, São José dos Campos, São Paulo, BR). Primary antibodies were mouse anti−α-Tubulin (Sigma Aldrich; 1:5000); rabbit anti-Fibronectin (Sigma Aldrich; 1:1000); rabbit anti-Laminin (Sigma Aldrich; 1:1000). Secondary peroxidase-conjugated antibodies were goat anti-rabbit IgG and goat anti-mouse IgG (Amersham Biosciences; 1:5000). After protein detection, densitometric analysis of autoradiographic films was done using Image J 1.36 b software.

RT-PCR

Total RNA was isolated from cells using TRIZOL (Invitrogen) according to the protocol provided by the manufacturer. After DNAse treatment (RQ1 RNAse-free DNAse, Promega Wisconsin, USA), RNA samples (up to 1.5 μg) were reverse-transcribed into cDNA using oligo (dT) and Super ScriptTM II Reverse transcriptase (Invitrogen). cDNA was amplified by Taq DNA Polymerase in 103 PCR Buffer using Invitrogen's protocol. Sense and antisense specific oligonucleotides were in **Table 1**. Amplification was performed in 35 cycles, and PCR products were size-fractionated by electrophoresis using a 2% agarose gel and visualized by ethidium bromide staining. Negative controls for genomic DNA contamination were carried out. Densitometries were done using Image J 1.36 b software.

QUANTITATIVE RT-PCR

Total RNA was Trizol® (Invitrogen, USA) extracted from astrocytes monolayers were used for RNA extraction and RNA was quantified using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). Two micrograms of total RNA were reverse-transcribed with Oligo (dT) 15 Primer and ImProm-II Reverse Transcription System with Recombinant RNasin® Ribonuclease inhibitor (Promega, USA) and MgCl2. The primer sequences were verified to be specific using GenBank's BLAST (Altschul et al., 1997). The primers used in this assay were in **Table 1**. Quantitative real-time RT-PCR was performed using SYBR®-Green PCR Master Mix, including AmpliTaq-GOLD polymerase (Applied Biosystems, USA). Reactions were performed on ABI PRISM 7500 Real Time PCR System (Applied Biosystems). The relative expression levels of genes were calculated using the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001). The amount of target genes expressed in a sample was normalized to the average of the endogenous control. This is given by ΔCT, where ΔCT is determined by subtracting the average endogenous gene CT value from the average target gene CT value [CT target gene—CT average (endogenous gene)]; where $2-\Delta CT$ is the relative expression of the target gene compared to the endogenous gene. The calculation of ΔΔCT was done by subtracting ΔCT value for the controls from the ΔCT value for a given treatment [ΔCT target gene (treated)—ΔCT target gene (control)]; where $2-\Delta\Delta CT$ is the relative expression of the target gene at T3-treated astrocytes compared to controls.

ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

A quantitative indirect immunoenzyme assay was performed after the protein levels were measured. Polystyrene microtiter plate

Table 1 | Specifications of the oligonucleotides used in conventional and real time RT-PCR.

Gene	Sequence	Product (bp)
GFAP	5′ CGA TTC AAC CTT TCT CTC CAA ATC CAC ACG 3′ 5′ CTT TGC TAG CTA CAT CGA GAA GGT CCG CTT 3′	339
TRα1	5′ GGT GCT GCA TGG AGA TCA TG 3′ 5′ GGA ATG TTG TGT TTG CGG TG 3′	225
TRβ1	5′ CGG AGG AGA AGA AAT GTA AAG G 3′ 5′ GCT TCG GTG ACA GTT TTG AT G 3′	421
Dio2	5′ CTT GAC TTT GCC AGT GCA GA 3′ 5′ GCA CAC ACG TTC AAA GGC TA 3′	351
GAPDH	5′ AAG AAG GTG GTG AAG CAG GCA TCT 3′ 5′ ACC CTG TTG CTG TAG CCG TAT TCA 3′	200
Syndecan-3	5′ TCG TTT CCT GAT GAT GAA CTA GAC 3′ 5′ GTG CTG GAC ATG GAT ACT TTG TT 3′	301
Syndecan-4	5′ AGA GCC CAA GGA ACT GGA AGA GAA 3′ 5′ ATC AGA GCT GCC AAG ACC TCA GTT 3′	147
Glypican-1	5′ ACT CCA TGG TGC TCA TCA CTG ACA 3 5′ TTT CCA CAG GCC TGG ATG ACC TTA 3′	151
Versican	5′ TCC AGG AGA AAC AGT TGG GAT GCT 3′ 5′ AAG GAA GGA AAG GTT GGC CTC TCA 3′	192

wells (Maxisorp, Nunc, and Roskilde, Denmark) were coated with 50 μL of protein (5 μg/mL in PBS) by passive adsorption overnight at 4°C. The plates were then washed with PBS containing 0.05% Tween 20 and 0.1% BSA (PBS-Tween). Non-specific binding was blocked by incubating the plates for 2 h with 1% BSA in PBS, pH 7.4 at 37°C. After an additional PBS-Tween washing, the plates were incubated with the primary antibodies, rabbit anti-Fibronectin (Sigma Aldrich; 1:1000); rabbit anti-Laminin (Sigma Aldrich; 1:1000) and mouse anti−α-Tubulin (as control) (Sigma, USA; 1:3000) for 24 h at 4°C, followed by incubation with a goat anti-rabbit or mouse IgG peroxidase-linked conjugated antibody (1:8000 Amersham Biosciences, UK). The plates were washed with PBS-Tween, and the reaction was developed with the substrate o-phenylenediamine (0.5 mg/mL and 0.005% H_2O_2 in 0.01 M sodium citrate buffer, pH 5.6) (Vetec, Brazil). The reaction was stopped with 0.2 M H_2SO_4 (Vetec, Brazil), and the absorbance was measured using an automated reader (BioRad ELISA Reader, Hercules, CA, USA). The experimental groups consisted of triplicate samples from three independent experiments.

STATISTICAL ANALYSES

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

RESULTS

CEREBRAL CORTEX ASTROCYTES PRIMED BY T3 INDUCE NEURONAL FATE COMMITMENT AND ENHANCE NEURONAL MATURATION

In order to evaluate the role of astrocytes as mediators of T3 in cerebral cortex neuron development, astrocyte monolayers derived from newborn cerebral cortex were primed by 50 nM of T3 for 3 days, followed by coculture with embryonic cerebral cortex progenitors for 24 h (**Figures 1A**,**B**).

RT-PCR assays of control astrocyte monolayers demonstrated that astrocytes express both TRα1 and TRβ1 receptors, as well type II deiodinase (D2) mRNAs, thus, ensuring that they might respond to thyroid hormone (**Figure 1C**).

Culture of progenitor cells onto T3-primed astrocyte monolayers yielded a 44% increase in neuronal population as revealed by immunostaining for the neuronal marker βTubulinIII (**Figure 1D**). Morphometric analysis revealed an 80% increase in the number of neurons with three or more processes, followed by a 62% decrease of aneuritic cells, when neurons were cultured onto T3-primed astrocyte carpets (**Figure 1E**).

In order to evaluate the effect of thyroid hormones-treated astrocytes on axonal growth, neurite length was analyzed by three parameters: either considering the sum of total neurite length per neuron; the longest neurite per neuron or the sum of all neurite measurements divided by the number of process per neuron. Treatment of astrocytes by T3 promoted a 75% increment on neurite outgrowth (**Figure 1F**). Moreover, around 33% of neuronal cells plated over astrocyte monolayers treated with thyroid hormones developed neurites with an

average size around 100–150 μm whereas less than 15% of neurons plated over control astrocytes displayed these characteristics (**Figure 1G**). Treatment of astrocytes by T3 greatly decreased the number of neurons with neurites under 50 μm (control: 23% × T3-treated: 1%) and increased those bigger than 150 μm (control: 2% × T3-treated: 10–18%) (**Figure 1G**).

Together, these results show that thyroid hormone induce astrocytes to adopt a permissive and/or inductive phenotype that favors neuronal fate specification of cortical progenitors followed by further neuronal maturation and arborization.

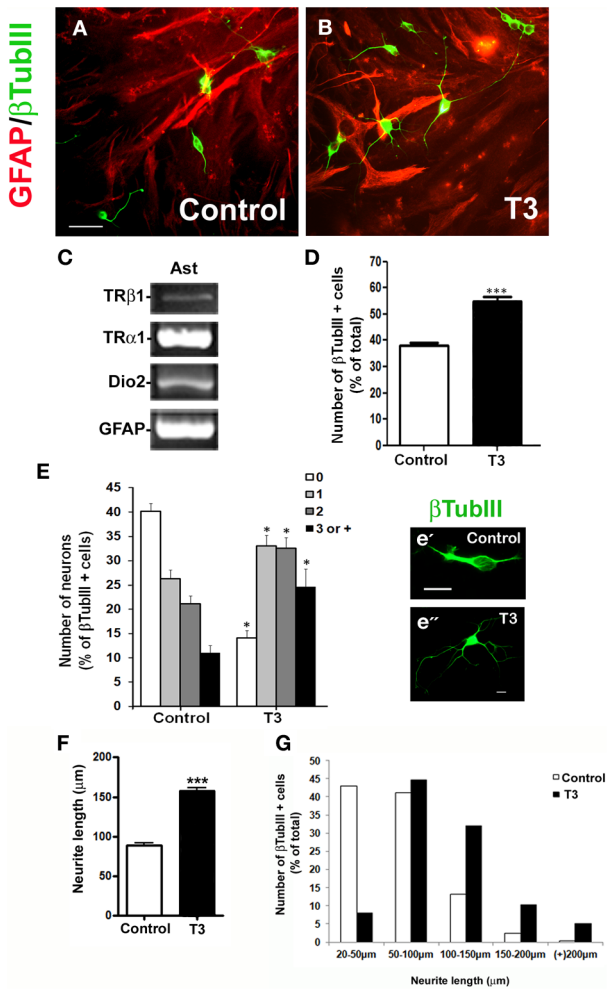

FIGURE 1 | Thyroid hormone-primed astrocytes induce neuronal differentiation and arborization: Cortical progenitors obtained from E14 mice were cultured onto untreated (**A**; Control) and T3 **(B)** astrocyte monolayers. After 24 h of coculture, neurons were morphologically characterized by βtubulin III (βtubIII+) immunostaining for number of neurons **(D)**, number of neurites **(E)** and length of neurites **(F,G)**. In all cases, at least 100 neurons randomly chosen were observed. RT-PCR assays identified expression of members of thyroid hormone signaling pathway in cultured cortical astrocytes: TRα1, TRβ1, and D2 mRNAs **(C)**. Note the complexity of neuronal cells plated over T3-astrocytes **(e")** compared with control-astrocytes **(e')**. Increase in arborization complexity was also followed by an increment in neurite length **(G)**. Scale bars: 50 μm **(A)**, 10 μm **(e')**. $*P < 0.05$; $***P < 0.001$.

EFFECT OF ASTROCYTES SOLUBLE FACTORS ON NEURONAL DIFFERENTIATION AND MATURATION

Astrocytes constitute the main source of neurotrophic factors during nervous system development (Banker, 1980; Martinez and Gomes, 2002; Kornyei et al., 2007). In order to investigate if neuronal arborization induced by T3-primed astrocytes was mediated by soluble factors, embryonic cerebral cortex progenitors were cultured in the presence of conditioned medium obtained from control astrocytes (C-CM) or T3-treated astrocytes (T3-CM), for 24 h (**Figures 2A,B**) and number of neurons quantified after immunostaining for the neuronal marker, βTubulin III. T3-CM had no effect in the number of βTubulin III positive cells compared to C-CM (**Figure 2C**). On the other hand, T3-CM enhanced neuronal arborization as revealed by a decrease

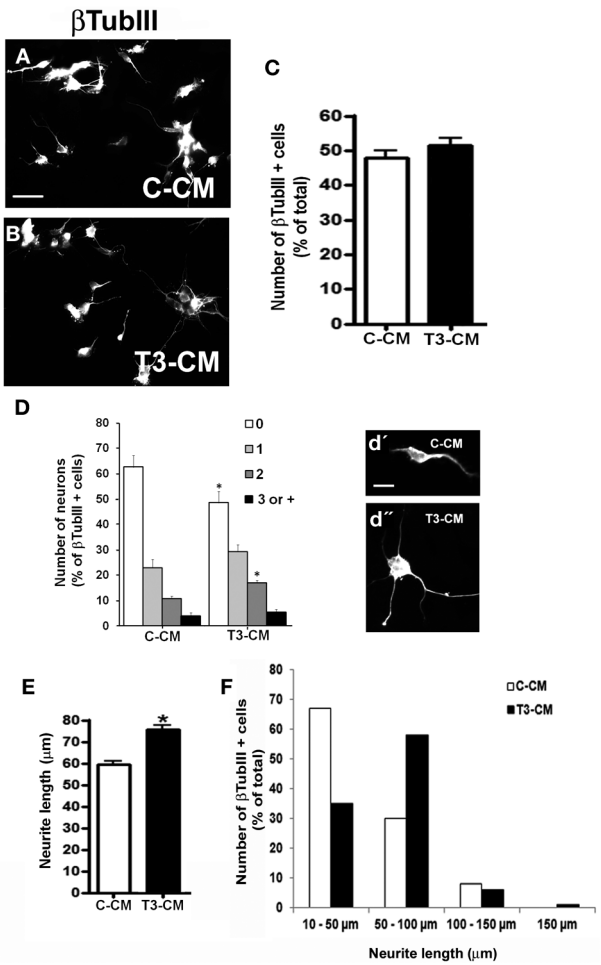

FIGURE 2 | Astrocytic soluble factors secreted in response to thyroid hormones affect neuronal differentiation. E14 cerebral cortex progenitors were cultured in the presence of control conditioned medium obtained from non-treated astrocytes (**A**; C-CM) and T3-treated astrocytes (**B**; T3-CM) for 24 hr. Neurons were morphologically characterized by βtubulin III (βtubIII+) immunostaining for number of neurons **(C)**, number of neurites **(D)** and length of neurites **(E,F)**. Note that although T3-CM enhanced neuronal arborization and neurite outgrowth **(d")**, it did not affect number of neurons **(C)**. Scale bars: 50 μm **(A)**, 10 μm **(d')**. $*P < 0.05$.

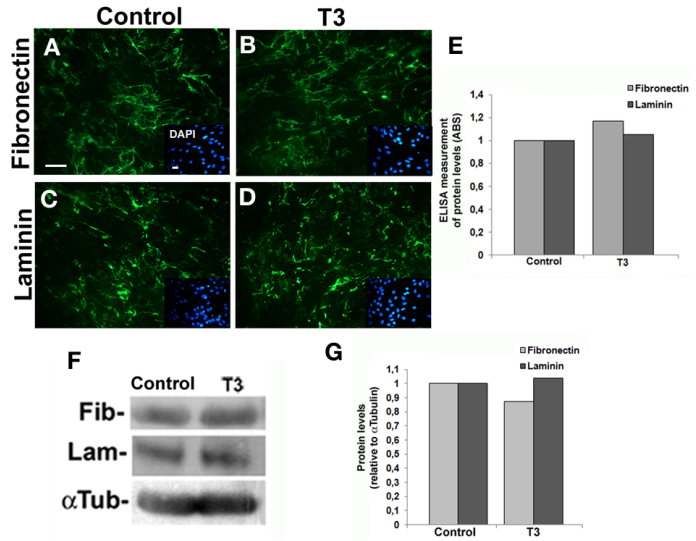

FIGURE 3 | Thyroid hormones do not affect levels and organization of laminin and fibronectin on cerebral cortex astrocytes. After reaching confluence, cerebral cortex primary astrocyte cultures were maintained for 3 days in DMEM-F12 medium alone (Control) or supplemented with T3 (T3). Cells were immunostained for Fibronectin **(A,B)** and Laminin **(C,D)**. Thick fibrils characteristics of ECM networks are shown throughout the extracellular membrane surface. DAPI stained nuclei (blue) reveal astrocyte monolayers localized underneath Fibronectin and Laminin matrix (green). No significant differences in the organization and distribution of both ECM proteins were observed **(A–D)**. ELISA assays of total protein extracts from cortical astrocyte cultures, did not reveal any difference in Fibronectin or Laminin levels in control or T3-treated cultures **(E)**. Western blot assays of total protein extracts from cortical astrocyte cultures did not reveal differences in the protein levels after T3 treatment of cortical astrocytes **(F,G)**. Immune reaction for α-Tubulin was used as loading control. Scale bar: 30 μm **(A)**.

in the number of aneuritic cells (T3-CM: −23%), followed by an increase in those cells with 2 neurites (T3-CM: +58%) (**Figure 2D**). Likewise, neurite outgrowth was improved by conditioned medium derived from T3-treated astrocytes (**Figure 2E**). In the presence of T3-CM, most of the neurons presented neurites with average size between 50 and 100 μm, whereas most of those in C-CM exhibited an average size between 10 and 50 μm (**Figure 2F**). Nevertheless, T3-CM was not able to fully mimic the effects of cocultures on neuronal differentiation and arborization (compare **Figures 1**, **2**), suggesting, a role for astrocyte-neuron contact factor in this process.

THYROID HORMONES-TREATED ASTROCYTES INDUCE NEURONAL DIFFERENTIATION AND NEURITE OUTGROWTH THROUGH MODULATION OF PROTEOGLYCANS COMPONENTS

ECM proteins are key regulators of neuronal differentiation, migration, axonal projection, neurite outgrowth, synaptogenesis, and regeneration (Faivre-Bauman et al., 1984; Carri et al., 1988; Hammarback et al., 1988; Chamak and Prochiantz, 1989; Martinez and Gomes, 2002). Recently, we demonstrated that astrocytes cultured under hypothyroidism-like conditions, present FN decreased level (Dezonne et al., 2009). Moreover, we also described that thyroid hormone treatment induces FN and LN reorganization in cerebellar astrocytes (Martinez and Gomes, 2002). We then sought to investigate if thyroid hormone treatment affects synthesis and organization of cerebral cortex astrocytic ECM. To accomplish that, the content of LN and FN of cortical astrocytes was evaluated by immunocytochemistry, western blot, and ELISA assays (**Figure 3**). In all conditions, immunolabeling showed the thick and fibrous networks staining pattern of these two proteins, with no clear difference between control and treated-astrocytes (**Figures 3A–D**). Equally, neither ELISA nor western blot assays revealed any obvious differences in LN or FN protein levels (**Figures 3E–G**), suggesting that other components of ECM might be involved in the neurite outgrowth induced by T3-primed astrocytes.

A large number of proteoglycans have been implicated in regulation of neurite outgrowth. These molecules provide signals that either stimulate or inhibit axonal growth during CNS morphogenesis (Williamson et al., 1996). HSPG are generally correlated with neuronal maturation and neurite outgrowth (Bespalov et al., 2011; Mammadov et al., 2012). In order to investigate if thyroid hormone treatment affects the expression of HSPGs in cerebral cortex astrocytes, we performed a quantitative RT-PCR for the two family of HSPG: syndecan (SDC) and glypican (GPC). T3-primed astrocytes presented an increase of 100%, in the expression of SDC4 and GPC1, and 98% of SDC3 (**Figure 4A**).

We then sought to analyze the involvement of the HSPG components in cerebral cortex glia-induced neuronal fate commitment and axonal growth. To do that, astrocyte monolayers were treated with heparanase III, which specifically digests HSG chains, after T3 treatment and previously to coculture assays. Enzymatic digestion of HSG chains completely abolished the increase in neuronal population induced by thyroid hormones treatment (**Figures 4B–F**). It also reversed neurite outgrowth (**Figure 4G**), but not the increase in neurite number (**Figure 4H**) induced by T3-treated astrocytes.

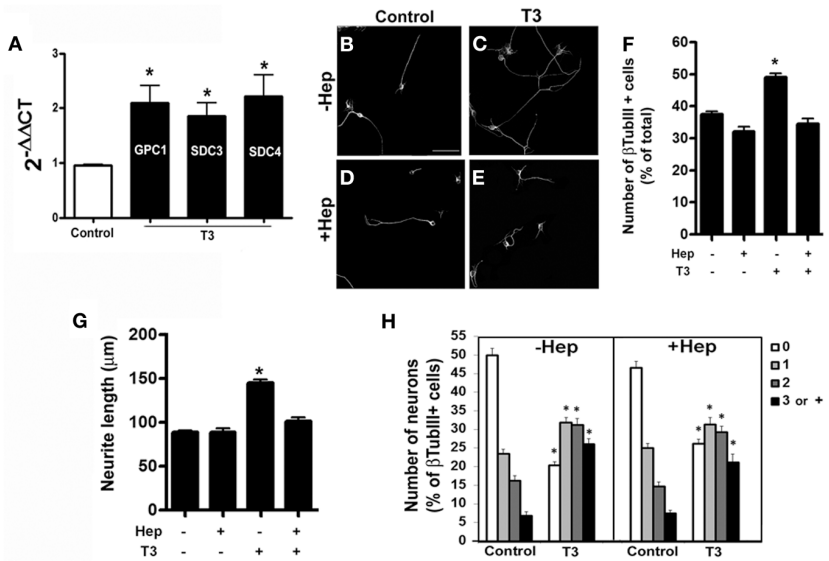

FIGURE 4 | Thyroid hormone-primed astrocytes induce neuronal differentiation, arborization and neurite outgrowth through heparan-sulfate proteoglycans. Cortical astrocytes cultures were evaluated for Glypican 1 (GPC1) and Syndecans 3 and 4 (SDC3, SDC4) heparan-sulfate proteoglycans mRNA expression by real time RT-PCR. Representative graph analysis shows that T3 promoted increase in GPC1, SDC3, and SDC4 expression **(A)**. Cortical control astrocytes **(B,D)** or treated with T3 **(C,E)** were enzymatically treated with Heparanase III **(D,E)** previously to addition of embryonic cortical progenitors. After 24 hr of coculture, number of neurons **(F)**, length of neurites **(G)** and number of neurites **(H)** were evaluated. Digestion of heparan chains completely impaired the effects produced by T3 in neuronal number, arborization and neurite outgrowth. Scale bars: 50 μm **(B)**, $^*P < 0.05$.

Growing axons navigate complex environments thus, integrating with local structural and chemical cues in order to make net growth decisions. This process is driven by positive and negative clues present in neuronal neighborhood. Among the inhibitoriest molecules to axonal growth are chondroitin sulfate proteoglycans (CSPG) (Nakamae et al., 2009). We thus analyzed the levels of Versican, the major CSPG in the brain and which has been shown to inhibit neurite outgrowth *in vitro* and *in vivo* following injury (Morgenstern et al., 2002). Quantitative RT-PCR assays revealed a 50% decrease in the expression of Versican in T3-treated astrocyte monolayers (**Figure 5A**).

To evaluate if CSPG might be also responsible for the neuronal maturation induced by T3-treated astrocytes, we enzymatically treated astrocyte monolayers with chondroitinase AC, prior to addition of E14 cerebral cortex progenitors. The digestion of chondroitin sulfate glycosaminoglycan chains completely abolished the increase in neuronal population induced by thyroid hormones treatment (**Figures 5B–F**). Besides, chondroitinase AC completely inhibited neurite outgrowth induced by T3-treated astrocytes (**Figure 5G**). Unexpectedly, the absence of inhibitor clues, enhance the extension of neurite by neurons (**Figure 5H**).

Taken together, these data show that T3 induces differentiation and axonal growth of cerebral cortex neurons by modulation of positive and negative signals: through enhancement of HSPG levels and decreasing versican expression by astrocytes.

DISCUSSION

Here we report that T3-primed astrocytes induce neuronal maturation and neurite outgrowth of embryonic neural progenitors. These events are mediated by signals derived from HSPG and CSPG, specifically GCP1, SDC3, SDC4, and versican. Our work provides evidence of a novel mechanism, through astrocytes, underlying thyroid hormone effects in cerebral cortex development. The fact that astrocytes highly express several isoforms of TRs makes them strong targets for thyroid hormones action during nervous system development (Carlson et al., 1996; Morte et al., 2004). Our findings support experimental observations that correlate thyroid hormones insufficiencies with clear reductions in axonal and dendritic growth (Eayrs, 1955; Ruiz-Marcos et al., 1979, 1988), and they strengthen the role of astrocytes in these processes, suggesting a thyroid hormone-indirect action on neuronal cells.

Thyroid hormone-conditioned medium also increased astrocyte permissivity to neurite outgrowth, although in less scale than coculture systems, leading to the hypothesis that either astrocyte conditioned medium (ACM) contains a soluble factor with redundant effects of cell contact on these events; or the effects of the ACM are due to soluble proteoglycans. The identity of the putative astrocyte-derived soluble factor should await further investigation.

Congenital hypothyroidism characterized by morphological brain alterations results in disturbed neuronal migration, deficits in axonal projection and synaptogenesis (Eayrs, 1955; Ruiz-Marcos et al., 1979, 1988; Gravel et al., 1990; Berbel et al., 1993; Calikoglu et al., 1996; Cuevas et al., 2005). It is well known that signals derived from ECM are essential to these events, especially those triggered by FN and LN (Carri et al., 1988; Hammarback et al., 1988; Chamak and Prochiantz, 1989; Martinez and Gomes,

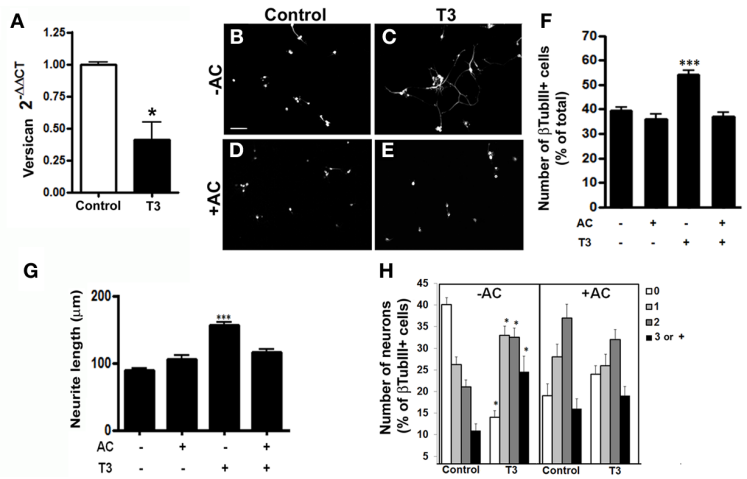

FIGURE 5 | Thyroid hormone-primed astrocytes induce neuronal differentiation, arborization, and neurite outgrowth through chondroitin-sulfate proteoglycans. Cortical astrocytes cultures were evaluated for Versican chondroitin-sulfate proteoglycan mRNA expression by real time RT-PCR. Representative graph analysis shows that T3 promoted decrease in the Versican expression **(A)**. Cortical control astrocytes **(B,D)** or treated with T3 **(C,E)** were enzymatically treated with chondroitinase AC **(D,E)** previously to addition of embryonic cortical progenitors. After 24 hr, number of neurons **(B,F)**, length of neurites **(G)** and number of neurites **(H)** were evaluated. Digestion of chondroitin chains completely impaired the effects produced by T3-astrcoytes in neuronal number and neurite outgrowth. Scale bars: 50 μm **(B)**, $^{*}P < 0.05$; $^{***}P < 0.001$.

2002). We previously demonstrated that thyroid hormones induce neurite outgrowth of cerebellar granular neurons through synthesis and secretion of FN and LN *in vitro* (Martinez and Gomes, 2002). These data contrast with those shown here since we did not observe any alteration in both ECM proteins, either at their levels or organization in the extracellular matrix. These apparent discrepancies might be due to species specificity, since in previous work rats were used and here we used cerebral cortex derived from mice. An alternative possibility is that these differences reflect astrocyte heterogeneity within CNS, since those works analyzed hormone effects in cerebellar development instead of cerebral cortex. It is known that astroglial cells derived from distinct brain regions markedly vary in their responsiveness to thyroid hormone (Lima et al., 1997). It has also been speculated that spatial differences in the expression of T3 receptors account for the variety of T3 response elicited in brain structures (Lima et al., 1997; Gomes et al., 1999).

Another possible explanation is that thyroid hormones exert an indirect action on LN and FN mediated by secondary growth factors secreted in response to hormone treatment. This is supported by the fact that a direct T3-regulation has not been undoubtedly reported for LN and FN, whereas several growth factors secreted by astrocytes in response to thyroid hormone, like EGF and FGF2, have been shown to modulate ECM components (Calloni et al., 2001; Martinez and Gomes, 2002; Mendes-De-Aguiar et al., 2010). Our data agree with those obtained from Farwell and Dubord-Tomasetti who demonstrated that T4, but not T3, increases LN expression (Farwell and Dubord-Tomasetti, 1999a,b).

Enzymatic digestion of glycosaminoglycan chains, with chondroitinases AC and heparanase III, completely abolished the effects of thyroid hormone primed-astrocytes on neuronal phenotype acquisition observed here, although a basal neurite extension was observed in all conditions. Since the optimum concentration of HSPG to elicit neurite outgrowth *in vitro* is unknown, one possibility is that the remaining HSPG, either in control or T3-treated cultures, is sufficient to induce a basal neurite extension. In fact, our data show that both heparan sulfate and chondroitin sulfate glycosaminoglycan chains were important for neurite outgrowth, since we only disrupted glycosaminoglycan chains instead of core proteins. However, it might be related with an alteration in HSPG and CSPG expression by T3-treated astrocytes. In mammalian tissues, most of glycosaminoglycans are covalently linked to proteins, forming the proteoglycans. These macromolecules exert important roles during morphogenesis and homeostasis of the CNS (Carey, 1997; Song and Filmus, 2002; Matsui and Oohira, 2004). Although specific receptors have not been identified for these molecules, they mainly act by interacting with cell adhesion molecules and growth factors (Wang et al., 2008; Mythreye and Blobe, 2009). The CNS presents multiple species of proteoglycans in the ECM and at cell surface, including the two families of HSPG, syndecans and glypicans (Bandtlow and Zimmermann, 2000; Matsui and Oohira, 2004), and many CSPGs, such as neurocan, versican and aggrecan (Carey, 1997; Bandtlow and Zimmermann, 2000; Oohira et al., 2000; Song and Filmus, 2002; Akita et al., 2004; Matsui and Oohira, 2004).

The study of the role of proteoglycans in CNS development and pathology has provided contradictory results regarding their permissive or inhibitory effects on axonal growth. In the adult CNS, CSPGs are upregulated after CNS injury and constitute the main component of the glial scar, which impairs axonal regeneration (Jones et al., 2003; Wang et al., 2008). The 4-sulfated chondroitin chains have been shown to repeal growing axons of several cell types including cerebellar granular cells and dorsal root ganglion neurons (Oohira et al., 2000; Matsui

and Oohira, 2004; Wang et al., 2008). However, Wang and coworkers have also shown that in reactive astrocyte monolayers, 6-sulfated chondroitin did not show any inhibitory action on axonal guidance. In addition, ascending sensory axons regenerate into areas where CSPG is expressed after spinal cord injury (Pasterkamp et al., 2001; Inman and Steward, 2003). Versican is an ECM CSPG, and its isoforms are aberrantly expressed in CNS injuries. Diverse works suggest that versican plays key inhibitory role in regulating neurite outgrowth (Zhang et al., 2011). In the present work, we showed that T3-treated astrocytes decreased versican expression, which might contribute to neurite outgrowth.

HSPG are permissive to neurite outgrowth and among them is the transmembrane family of HSPG, SDC. Here, we demonstrated that enhanced expression of SDC 3 and 4 by T3-treated astrocytes is associated with neuronal maturation. These data are in concert with reduced expression of SDC 1, 2, and 4, and increased expression of SDC3 in hypothyroid cerebellum (Mendes-De-Aguiar et al., 2008). Furthermore, a reduced expression of SDC2 and 3 in T3-treated astrocytes has been previously associated with T3 action mediated by FGF2 in cerebellar astrocytes. In this case, proteoglycans were suggested to affect the formation of the trimeric signaling receptor complex composed by SDC/FGF/FGF-receptor, which is essential for FGF receptor dimerization, activation, and cell signaling (Mendes-De-Aguiar et al., 2008). It is known that SDC3 are involved in biological functions such as cell adhesion, cell migration and neurite outgrowth (Akita et al., 2004; Choi et al., 2011). N-syndecan (SDC3) is abundantly expressed in the major axonal pathways and in the migratory routes of the developing brain. They might associate with heparin-binding growth-associated molecule (pleiotrophin), and mediates cortactin-Src kinase-dependent neurite outgrowth (Hienola et al., 2006). In addition, SDC3 might bind to neurocan, a major CSPG expressed by neurons, thus, promoting neurite outgrowth (Akita et al., 2004). Thus, it is not completely unpredicted that CSPG might be permissive to certain neuronal types under peculiar conditions. Besides, SDC family might have a chondroitin sulfate modification, thus, presenting a hybrid class of proteoglycans (Bernfield et al., 1999). Yet, a balance between inhibitor and permissive molecules, rather than individual molecules, might be a determinant factor to neurite outgrowth.

Glypicans are another important family of HSPG. GPCs are linked to extracellular surface of plasmic membrane through a covalent glycosyl-phosphatidylinositol (GPI) anchor. Six GPC were described in mammals so far (GPC1-6). In general, they are expressed predominantly during development when they control tissues morphogenesis (Song and Filmus, 2002). GPC1, the major HSPG of the brain, displays a higher expression level throughout CNS, mainly in the developing neuroepithelium surrounding ventricles (Litwack et al., 1998; Song and Filmus, 2002). Previously works reported that two members of the Slit family, Slit1 and 2, bind to GPC1, and have an overlapping patter of expression in the brain (Ronca et al., 2001). Slits proteins are expressed by neurons and glial cells and play key role in axonal guidance (Brose and Tessier-Lavigne, 2000). In addition, we found an increment of amyloid precursor protein (APP) $_{m}$RNA in T3-treated astrocytes (data not shown). It is well known that SDC3 GPC1 also binds to APP and this interaction promotes neurite outgrowth (Williamson et al., 1996; Clarris et al., 1997; Cheng et al., 2011; Hoe et al., 2012). In this case, neuronal SDC3 and GPC1 might also require astrocyte APP for neurite outgrowth. Here, we showed for the first time that the thyroid hormone, T3, induces neurite outgrowth through upregulating GPC1 expression in astrocyte.

We demonstrated that thyroid hormone induces neuronal maturation and neurite outgrowth of cerebral cortex neurons *in vitro* and implicated SDC3 and 4, GPC1 and versican produced by astrocytes, in these events. Moreover, we shed light on glial cells as potential targets of thyroid hormone actions during cerebral cortex neuronal development. Upon hormone influence, astrocytes secrete soluble factors, downregulate ECM proteins, specifically, versican CSPG; and upregulate HSPG (SDC3-4, GPC1) thus affecting neuronal pool: either inducing neuronal commitment of progenitor cells and, further, neuronal maturation and neurite outgrowth (**Figure 6**).

Our work not only increases the knowledge about thyroid hormone role in brain development but helps to identify potential molecules and pathways involved in neurite extension which might eventually contribute to a better understanding of axonal regeneration, synapse formation, and neuronal circuitry recover.

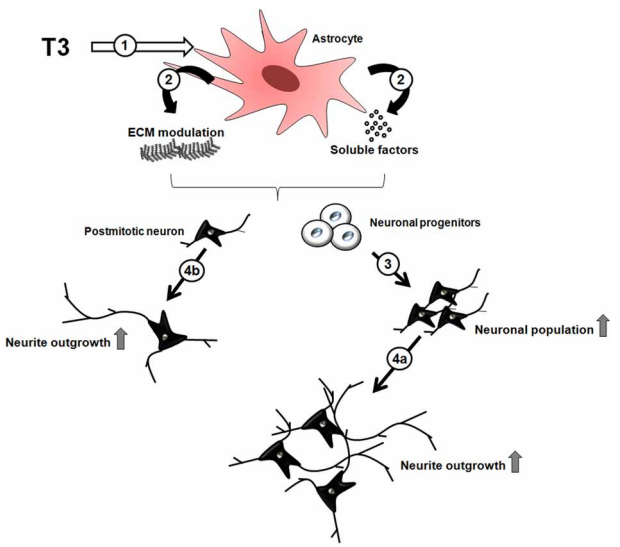

FIGURE 6 | Indirect effects of thyroid hormone on neuronal differentiation through astrocytes. The thyroid hormone, **T3**, induces astroglial cells **(1)** to modulate **ECM** molecules expression, such as heparan sulfate and chondroitin sulfate glycosaminoglycans and **soluble factors (2)**, which in turn induce neuronal commitment of progenitor cells followed by their differentiation **(3)**, and maturation with neurite outgrowth **(4a)**. In parallel, postmitotic neurons can be direct targets of astrocyte-derived molecules, thus, promoting maturation and neurite outgrowth **(4b)**.

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BMP signaling in telencephalic neural cell specification and maturation

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Bone morphogenetic proteins (BMPs) make up a family of morphogens that are critical for patterning, development, and function of the central and peripheral nervous system. Their effects on neural cells are pleiotropic and highly dynamic depending on the stage of development and the local niche. Neural cells display a broad expression profile of BMP ligands, receptors, and transducer molecules. Moreover, interactions of BMP signaling with other incoming morphogens and signaling pathways are crucial for most of these processes. The key role of BMP signaling suggests that it includes many regulatory mechanisms that restrict BMP activity both temporally and spatially. BMPs affect neural cell fate specification in a dynamic fashion. Initially they inhibit proliferation of neural precursors and promote the first steps in neuronal differentiation. Later on, BMP signaling effects switch from neuronal induction to promotion of astroglial identity and inhibition of neuronal or oligodendroglial lineage commitment. Furthermore, in postmitotic cells, BMPs regulate cell survival and death, to modulate neuronal subtype specification, promote dendritic and axonal growth and induce synapse formation and stabilization. In this review, we examine the canonical and non-canonical mechanisms of BMP signal transduction. Moreover, we focus on the specific role of BMPs in the nervous system including their ability to regulate neural stem cell proliferation, self-renewal, lineage specification, and neuronal function.

Keywords: BMP, neural differentiation, morphogen, synaptogenesis, neural development, signal transduction

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily (Derynck and Zhang, 2003; Shi and Massague, 2003; Miyazono et al., 2010). BMPs were originally identified as factors that induce bone formation when implanted at ectopic sites (Urist, 1965). Later, it was shown that BMP functions exist in vertebrates as well as in invertebrates and that they perform a wide range of biological action in various cell types and tissues (Chen et al., 2004). BMPs control many indispensable steps in embryogenesis, including the formation and differentiation of the vertebrate nervous system (Mehler et al., 1997; Kishigami and Mishina, 2005; Liu and Niswander, 2005). At initial steps of development, BMP inhibition is required to establish neuroectoderm from ectoderm, although certain levels of BMP signaling are later required for neural crest induction, neural crest cell migration, and spinal cord patterning. At later development stages, BMP signaling promotes astrogliogenesis and inhibition of neuronal or oligodendroglial lineage commitment. Given the functions of BMPs in nervous system development and maintenance, BMP signaling dysfunction and modulation could have a strong impact on various nervous system pathologies as well as their repair processes (Matsuura et al., 2008; Sabo et al., 2009; Ma et al., 2011). In this review, we highlight the main aspects of BMP signaling and BMP's involvement in neural induction and patterning, embryonic and postnatal neuronal differentiation, and the establishment of neuronal connections.

SIGNALING BY BMPs

Bone morphogenetic proteins are the largest subfamily of the TGF-β superfamily, which includes 33 members in mammals. BMPs can be further classified into at least four subgroups: BMP-2/4 group, BMP-5/6/7/8 group, growth and differentiation factor-5,-6,-7 (GDF-5/6/7) group, and BMP-9/10 group (Little and Mullins, 2009; Miyazono et al., 2010; Wagner et al., 2010). BMPs are known to be involved in many developmental and homeostatic processes throughout life. However, the exact function of individual BMPs in a particular tissue and at a specific time during development is far from clear. Due to their pleiotropic roles, genetic manipulation often leads to embryonic lethality, thus precluding analysis of their later embryonic or postnatal functions in multiple tissues and organs (Bragdon et al., 2011). In addition, compensatory functional overlaps between BMPs make interpretation complicated. Furthermore, although we may have a rough estimate of the place and the timing of expression of a particular BMP, many factors present in the extracellular environment are able to modify its exact diffusion rate, morphogen range, and its bioavailability for a target cell (Eldar et al., 2002; Peluso et al., 2011).

BMP SECRETION AND EXTRACELLULAR REGULATION

Bone morphogenetic proteins are synthesized as large precursor proteins comprising an N-terminal signal peptide, which directs the protein to the secretory pathway, a prodomain for proper folding and a C-terminal mature peptide (Sieber et al., 2009). BMPs

are first secreted and then proteolitically cleaved upon dimerization by serine proteases such as furin or PC6, releasing the mature peptide (Cui et al., 1998). Active BMPs consist of two monomers stabilized by three intramolecular disulfide bonds, known as cysteine knots, and an inter-dimer disulfide bond (Scheufler et al., 1999). Mature forms may be either homo- or hetero-dimers consisting of different BMP gene products. It has been shown *in vivo* and *in vitro* that some hetero-dimerization could lead to increased functional activity (Valera et al., 2010). In some cases, the cleaved prodomain remains attached to the mature form, as in the case of TGF-β, leading to reduced bioavailability and retention in the extracellular matrix (Ramel and Hill, 2012).

This restricted availability to their membrane receptors is mostly emphasized by the existence of highly regulated diffusible and cell surface-bound antagonists. There are more than a dozen diffusible antagonists that include chordin, noggin, follistatin, and chordin-like proteins (Rider and Mulloy, 2010; Walsh et al., 2010; Zakin and De Robertis, 2010). Binding of antagonists physically prevents BMPs from binding to their cognate receptors by masking the epitopes involved in ligand–receptor interactions (Groppe et al., 2002; **Figure 1**). Subsequent cleavage of chordin by tolloid zinc metalloproteinases triggers the release of active BMPs from the chordin/BMP complex (Peluso et al., 2011). Twisted gastrulation (Tsg) has a dual role in distinct model systems, acting as either a BMP antagonist or as an agonist. In the case of chordin, the stability of the chordin/BMP complex is greatly increased by *Tsg* (Chang et al., 2001; Ross et al., 2001).

Finally, regulation of BMP transport is crucial for its role as a morphogen. It has been shown that BMP-2 has the ability to link directly to heparan sulfate proteoglycans (HSPGs).

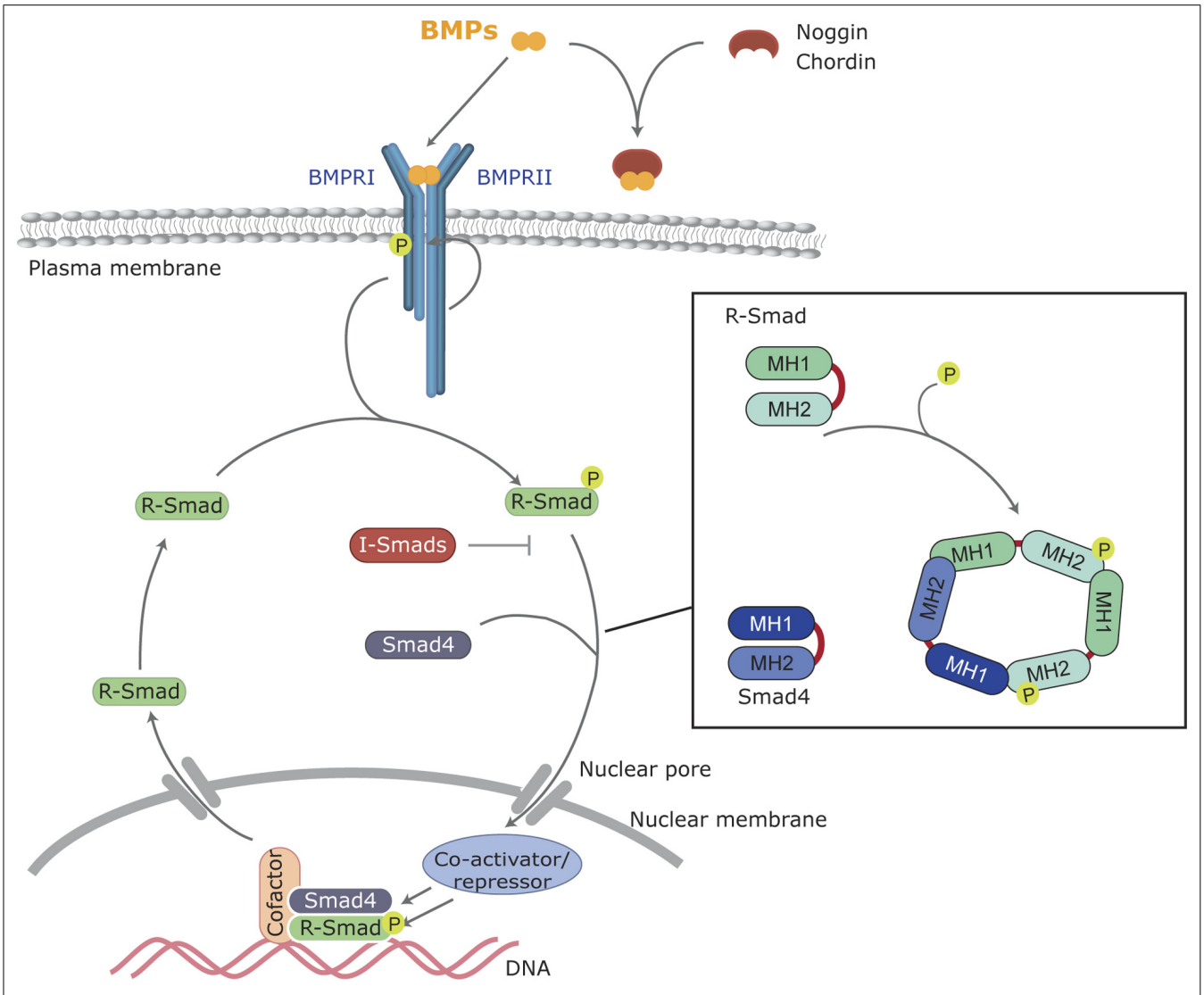

FIGURE 1 | Canonical BMP signaling. BMPs bind to the BMP receptors type I and II, and then type II receptor phosphorylates and activates the type I BMP receptor. Activated type I receptor phosphorylates R-Smads, which associate with the common Smad (Smad4) and enter the nucleus, where they regulate transcriptional processes. BMP signaling can be inhibited by extracellular antagonists, such as Noggin and Chordin, or intracellularly by I-Smads.

In several experimental models, extracellular HSPGs and collagen IV have been involved in the regulation of BMP transport (Ruppert et al., 1996; Osses et al., 2006). Collagen IV binds to BMP-4 and chordin homologs, sequestering them in the extracellular matrix. As mentioned above, Tsg, acting as a BMP agonist, releases chordin/BMP complexes from the collagen IV matrix, allowing their diffusion (Sawala et al., 2012). Thus, all these events of binding of BMPs to the extracellular matrix and/or to antagonists allow the formation of specific gradients through regulated solubility and bioavailability and constitute the first level of signal modulation.

BMP RECEPTORS AND RECEPTOR ACTIVATION

Bone morphogenetic proteins bind to a heterotetrameric complex of transmembrane receptors known as type I and II serine/threonine kinase receptors (Mueller and Nickel, 2012). Both types of receptors contain an N-terminal extracellular binding domain, a single transmembrane region, and an intracellular serine/threonine kinase domain (Shi and Massague, 2003; Feng and Derynck, 2005; Miyazono et al., 2010). Strong evidence confirms that both type I and II receptors, acting in coordination, are required for complete signal transduction. BMPs can bind to type I in the absence of type II receptors but when both types are present in the membrane of target cells their binding affinity is highly increased (Hinck, 2012; **Figure 2**). Based on their structures and functions, type I BMP receptors can be divided into the Bmpr1A and Bmpr1B group (also known as Alk3 and Alk6) and the Acvrl1 and Acvr1 group (also known as Alk1 and Alk2; Hinck, 2012). These groups show slight preferences for binding to specific BMPs. For instance, BMP-2 and -4 bind preferentially Bmpr1A and Bmpr1B whereas BMP-5, -6, and -7 additionally bind to Acvr1 (Liu et al., 1995). It is also well established that BMPs bind to three distinct type II receptors, namely Bmpr2, Acvr2A, and Acvr2B (**Figure 2**). Bmpr2 shows a unique, long C-terminal extension that allows specific recruitment of intracellular transducers. A question arises as to how such a limited number of signaling receptors allows binding of a large number of ligand members. One simple answer would rely on promiscuous interactions between shared ligands and several receptors (Hinck, 2012; Mueller and Nickel, 2012). However, combinatorial interactions of different type I and II receptors should allow selectivity and specificity of ligand binding as well as intracellular signaling in target cells. Numerous *in vivo* examples confirm the unique functions for an individual BMPs that are not shared even for closely related ligands with similar receptor binding affinities *in vitro* (Saremba et al., 2008; Meynard et al., 2009; Perron and Dodd, 2011, 2012). Further study into the molecular mechanisms that drive such functional specificity in living organisms is needed.

Moreover, co-receptors have been shown to modulate ligand binding and downstream signaling events. Members of the repulsive guidance molecule (RGM) family are

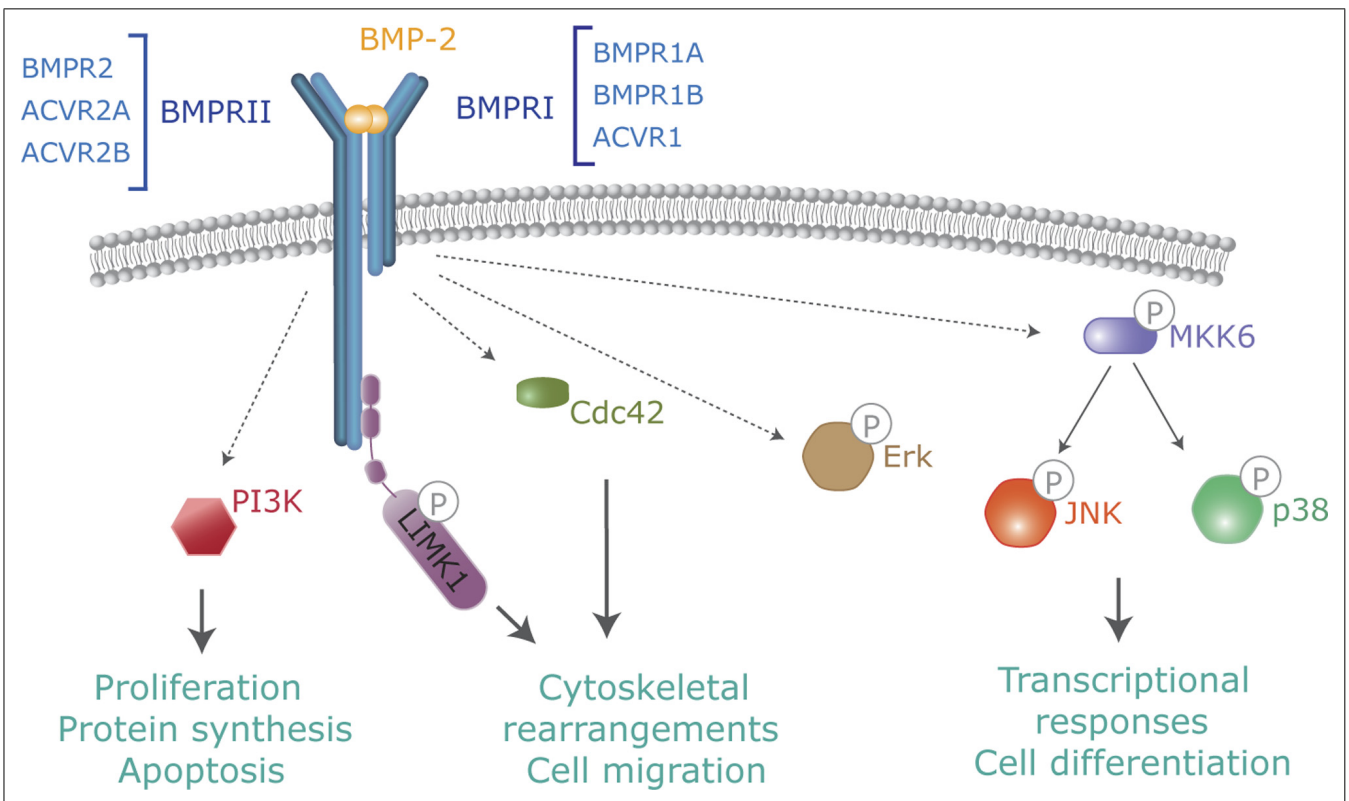

FIGURE 2 | Non-canonical BMP signaling. In addition to Smads, activated BMP receptors activate several intracellular pathways which modulate BMP-dependent cellular responses. Pathways include TAK-p38, PI3-kinase, Cdc42 or activation of LIMK by binding to the BMPR-II cytoplasmic tail.

glycophosphatidylinositol (GPI)-anchored co-receptors for BMP-2 and -4 and enhance responses at low BMP concentrations (Xia et al., 2007). Dragon and hemojuvelin (RGMb and c, respectively) also interact with BMP receptors and ligands and enhance responses for BMP-2 and -6. It has been shown that hemojuvelin plays a major role in regulation of iron metabolism in hepatocytes by increasing the binding affinity of BMP receptors for BMP-6 (Babitt et al., 2006). In addition to facilitating co-receptors, other membrane proteins function as suppressors of BMP signaling. For instance, Bambi is a transmembrane protein, structurally related to type I receptors that act as pseudoreceptor. Bambi forms a stable receptor complex but, since it lacks the intracellular kinase domain, inhibits transduction by titrating available type II receptors (Onichtchouk et al., 1999).

Type I and II BMP receptors show some ligand-independent affinity for each other. In the absence of a ligand, small amounts of pre-existing homo- and hetero-dimer receptor complexes are present at the cell surface (Ehrlich et al., 2012). However, ligand binding dramatically increases oligomerization involving type I and II complexes. Ligand-induced oligomerization promotes type II-dependent phosphorylation of a specific domain of type I receptors (known as the GS domain; **Figure 1**). Upon phosphorylation of the GS domain the type I receptor kinase is converted to its active conformation (Wrana et al., 1994). Thus, the kinase activity of type I receptors is the major intracellular transducer: whereas mutated or kinase-deficient type I receptors block most of the cellular responses to the ligand, constitutively active type I receptors (induced by pathological mutations or artificially designed) are able to signal most of the responses in the absence of type II receptors or ligands (Wieser et al., 1995).

INTRACELLULAR Smad SIGNALING FROM BMP RECEPTORS

Smads are the main and best-known intracellular signal transducers for BMP receptors. According to their structural and functional properties, three different types of Smads have been defined: the receptor-regulated Smads (R-Smad) Smad1, -2,-3,-5, and -8; a common mediator Smad, Smad4; and the inhibitory Smads Smad6 and -7 (Shi and Massague, 2003; Sieber et al., 2009; Miyazono et al., 2010). Active type I kinases phosphorylate R-Smads at serine residues located in their C-terminus. Specific phosphorylation of different R-Smads depends on the L45 loop of the type I receptor (Feng and Derynck, 1997). All the BMP type I receptors mentioned above (BMPR1A, BMPR1B, Acvr1, and Acvrl1) phosphorylate Smad1, Smad5, and Smad8, which are thus defined as BMP-activated Smads. Phosphorylation and activation of R-Smads disrupts the autoinhibitory interaction between the N-terminal (MAD homology 1, MH1) and C-terminal (MH2) domains of Smad monomers (Shi et al., 1997). This favors the formation of a multimeric complex composed of two molecules of R-Smad and one molecule of Smad4 interacting through their MH2 domain (**Figure 1**). On the new conformation, the nuclear import signal is exposed and the complexes translocate into the nucleus where they execute distinct transcriptional regulatory functions (Feng and Derynck, 2005; Hill, 2009).

Intracellular BMP signaling, as shown for transduction of all other morphogens described so far, is subjected to a growing number of cross-talk mechanisms with other extracellular ones, as well as regulation by internal cues, in order to integrate a final cell response. For instance, the inhibitory Smads, Smad6, and Smad7, block BMP signaling by preventing phosphorylation of R-Smads by type I receptors in a dominant negative fashion by binding to active receptor complexes (Derynck and Zhang, 2003). Another known mechanism is the degradation of Smads through the ubiquitin–proteasome pathway. Several homologous to E6-associated protein C-terminus (HECT)-type E3 ligases, such as Smurf-1,-2, or Nedd4-2, interact and ubiquitinate Smads and, when complexed with I-Smads, BMP receptors (Wicks et al., 2006). Degradation of Smads is also regulated by mitogen-activated protein (MAP) kinase and Gsk-3 phosphorylation allowing specific interaction with MAP kinase and Wnt signaling cascades (Fuentealba et al., 2007).

NON-CANONICAL SIGNALING FROM BMP RECEPTORS

In addition to Smads, BMPs activate other intracellular signaling pathways relevant to their cell functions. Non-canonical BMP signaling includes Rho-like small GTPases, phosphatidylinositol 3-kinase/Akt (PI3K/Akt) or various types of MAP kinases (Derynck and Zhang, 2003; Zhang, 2009; **Figure 2**). Mechanistically, it is well established that BMPs regulate the Tak1/p38 pathway through recruitment and ubiquitylation of Traf6 by activated receptor complexes (Sorrentino et al., 2008; Yamashita et al., 2008). BMPs trigger canonical and non-canonical pathways simultaneously, driving to a specific cellular output (Ulsamer et al., 2008; Xu et al., 2008; Susperregui et al., 2011). Several studies indicate that BMP-mediated cell migration or axon and dendrite growth requires activation of the small GTPase Cdc42 and Limk activities. Most of these effects are Smad-independent, but depend on Limk binding to the long cytoplasmatic tail of Bmpr2 complexes (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Furthermore, additional reports indicate that activation of Limk also requires the activation of Paks through Cdc42 and PI3K, as well as p38 MAP kinase activities (Gamell et al., 2008, 2011). The specific abilities of distinct receptors or receptor combinations to activate these non-canonical pathways and promote specific signaling outcomes need to be studied.

BMPs IN NERVOUS SYSTEM DEVELOPMENT AND MAINTENANCE

In recent years, there has been exponential progress in the clarification of the role of BMPs at different stages of nervous system development. BMP ligands and receptors are expressed in very complex patterns throughout neural development in all regions of the central and peripheral nervous system (Ebendal et al., 1998; Miyagi et al., 2011). Evidence clearly demonstrates that these pleiotropic cytokines have a very dynamic role: from the early steps of neuralization and patterning to an instructive role in neural precursor fate commitment and neuronal wiring (Liu and Niswander, 2005). Evidence also extends their role in postnatal brain as well as peripheral nervous system development and maintenance (Bond et al., 2012; **Figure 3**).

EXPRESSION AND ROLE OF BMPs AND RECEPTORS IN TELENCEPHALIC TISSUES

Bone morphogenetic proteins and their antagonists are expressed throughout neural development with a complex chronological and

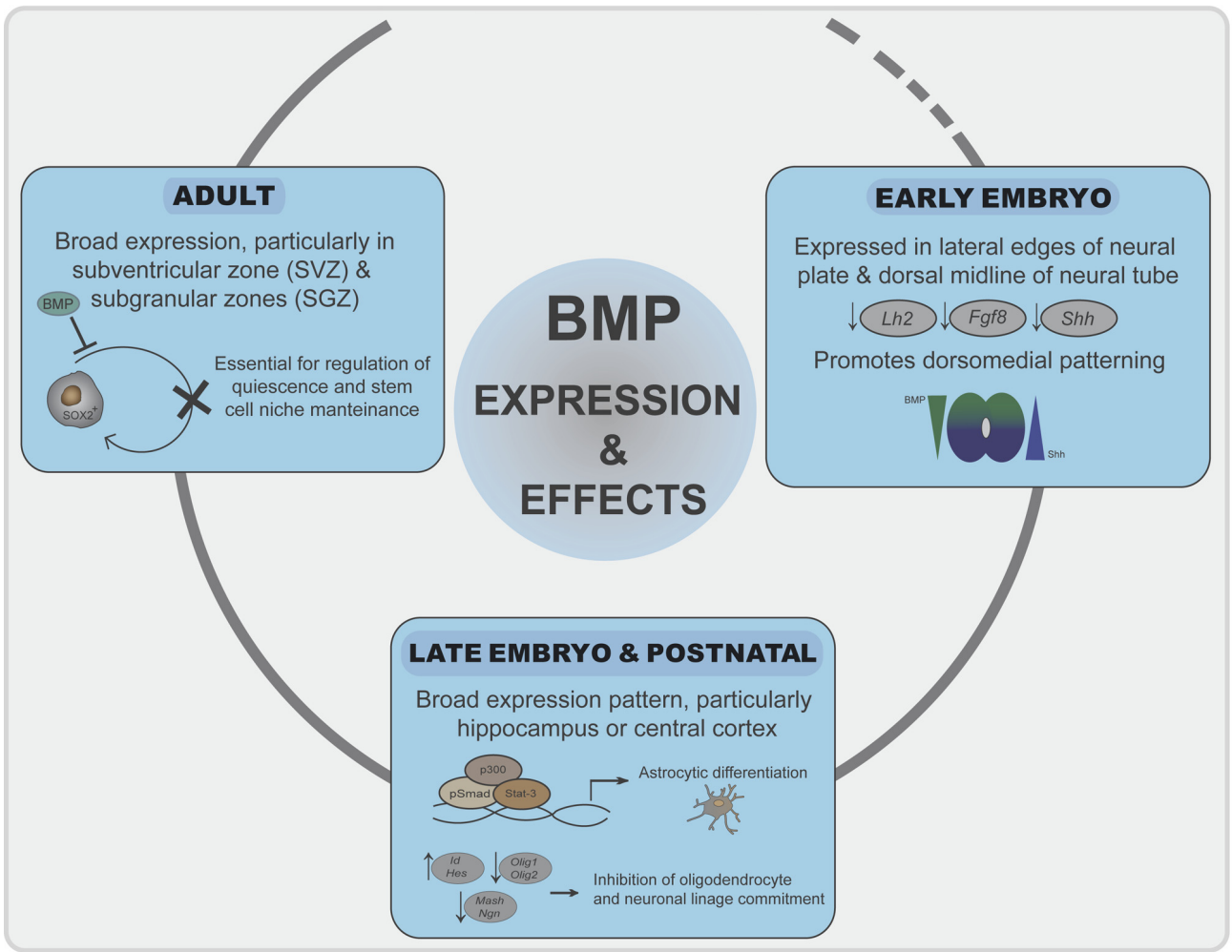

FIGURE 3 | Roles of BMP signaling in neural development. Initially, inhibition of BMP signaling is required for neuroectoderm induction. BMPs generate a signaling gradient and promote dorsomedial identity. BMP signaling is prominent at the dorsal telencephalic midline and in the marginal zone neurons, where it correlates negatively with expression of the cortical selector *Lhx2*, *Fgf8*, or *Shh*. In late embryogenesis and postnatally, BMP signaling promotes astroglial commitment by activation astrocyte-specific promoters through a Stat3-p300/CBP-Smad1 complex. At the same time represses commitment to the other two neural lineages by inducing Ids and Hes proteins. In adulthood, a balanced BMP signaling is required for regulation of quiescence and differentiation of neural stem cell populations in the SVZ and SGZ.

overlapping location that is only known to a limited extent. In early-developing neural tissue, BMPs are expressed particularly at the lateral edges of the neural plate and later on in the dorsal midline of the neural tube (Mehler et al., 1997; Chen and Panchision, 2007). During vertebrate development, the rostral–dorsal part of the neural plate gives rise to the telencephalon, the most complex region of the nervous system. The two signaling centers in the dorsal midline of the telencephalon, the roof plate and the cortical hem, secrete several BMPs (Furuta et al., 1997). These BMPs generate a signaling gradient and promote dorsomedial identity. Mice that are transgenic for specific BMP-dependent reporters indicate that, at embryonic stages, high BMP signaling is prominent at the dorsal telencephalic midline and in the marginal zone neurons, where it correlates negatively with expression of the cortical selector *Lhx2* (Doan et al., 2012). BMPs also reduce the expression of the anterior forebrain markers *Fgf8* or *Shh* (Anderson et al., 2002; **Figure 3**). *Chordin* and *noggin* double mutant embryos further emphasize the importance of the appeasement of BMP signaling for proper ventral forebrain development (Anderson et al., 2002). During further embryo development, brain expression of BMP-2, -4, -5, -6, and -7 peaks around postnatal day 4 with a broad expression pattern (Mehler et al., 1997; Sabo et al., 2009). Particular areas of the telencephalon, such as the hippocampus or cerebral cortex, show strong postnatal BMP activation (Doan et al., 2012). In adult telencephalon, there is also broad expression of BMPs at most locations and cell types, suggesting also a pivotal role in the adult brain. Interestingly, expression of BMPs and their antagonists specifically remain in the two areas where neurogenesis continues in the adult [the subventricular zone (SVZ) and subgranular zones (SGZ); Bond et al., 2012]. Expression of BMPs, from stem cells and neural progenitors, and noggin, secreted from ependymal cells, is essential for stem cell niche maintenance and

neuroblast survival (Lim et al., 2000; Mira et al., 2010; **Figure 3**). Moreover, increased expression of BMPs occurs after distinct brain and spinal cord injuries and suggest a role of BMP signaling in neural cell survival and repair (Sabo et al., 2009).

Bone morphogenetic protein receptors are expressed at high levels throughout all stages of embryonic development but show different expression patterns. *Bmpr1A* is expressed earlier than *Bmpr1B*. *Bmpr2* is mostly restricted to proliferative regions of the nervous system, whereas *Acvr2* and *Acvr1* have also been detected in early neural development and are expressed at high levels in adult brain. Bmpr1A and Bmpr1B are required separately in some development processes although in several studies of neural development, each receptor could, at least partially, compensate for the loss of the other. For instance, deletion of *Bmpr1B* causes no obvious forebrain phenotype (Yi et al., 2000). However, the requirement of BMP signaling for dorsal telencephalic development is shown after forebrain-specific ablation of *Bmpr1A* or the double knock-out of *Bmpr1A* and *Bmpr1B*, which leads to holoprosencephaly (Fernandes et al., 2007). However, later ablation only affects formation of the dentate gyrus of the hippocampus (Caronia et al., 2010). In adult telencephalon, expression of all these receptors remains, with *Bmpr1A* the most abundant and broadly expressed one (Miyagi et al., 2011). All cell types (neurons, astroglia, oligodendroglia, or ependymal cells) express combinations of these receptors (Chalazonitis et al., 2011; Bond et al., 2012). Interestingly, some reports suggest that their expression pattern is differentially distributed within a single neuron, with Bmpr1A mainly expressed in cell bodies and Bmpr1B in dendrites (Miyagi et al., 2011).

BMPs IN EMBRYONIC AND ADULT NEURAL CELL SPECIFICATION

The central and peripheral nervous systems originate from neural progenitor cells, which proliferate and give rise to the three main neural cell types: neurons, astrocytes, and oligodendrocytes. The specification and differentiation of the distinct cell types require interactions between cell-autonomous molecular mechanisms and external signaling cues (Mehler et al., 1997; Mehler, 2002; Liu and Niswander, 2005). Remarkably, BMPs are critical for progenitor cell specification and maintenance of a particular phenotype through dynamic transcriptional regulation (Bond et al., 2012). BMPs decrease proliferation of neural progenitors in cell culture models as well as *in vivo* in combination with other signaling molecules and internal cues (Chmielnicki et al., 2004; Sun et al., 2011; Benraiss et al., 2012).

BMP role in neurogenesis

Neurogenesis is promoted by proneuronal basic helix-loop-helix (bHLH) transcription factors including Mash1, Neurogenin, or NeuroD, which form hetero-dimers with ubiquitously expressed bHLH E proteins, such as the E2A gene products, E12 and E47, through their HLH domain (Ross et al., 2003; Hsieh, 2012). Hetero-dimers bind to DNA through their basic domain and activate the transcription of genes that have E boxes in their promoter region (**Figure 4A**). One subfamily of HLH factors, known as Id proteins, lacks this basic region. Hetero-dimerization of Id with bHLH is sufficient to block their DNA binding and function (Norton, 2000; Ruzinova and Benezra, 2003). Moreover, Ids not only inhibit transcriptional function but also promote the degradation of neurogenic bHLH by sequestering ubiquitous E proteins (Vinals et al., 2004; **Figure 4B**). Similarly, the Hes family of bHLH transcriptional repressors blocks neuronal differentiation through a dominant negative function on E protein availability as well as through direct binding on specific promoters and recruitment of members of the Grouch family of transcriptional repressors (**Figure 4B**). BMPs have been shown to be strong inducers of both *Id* and *Hes* family members (Ross et al., 2003). Thus, Id and Hes family members are thought to be major molecular players in the negative effects of BMPs on commitment and differentiation of neuronal precursors (Takizawa et al., 2003; Imayoshi et al., 2008). Furthermore, BMPs have been proved to increase the expression of the transcriptional repressor *Rest/Nsrf*. Expression of *Rest/Nsrf* allows continuous repression of the neuronal markers in cells committed to other lineages (Kohyama et al., 2010). In addition to their effects on embryonic neurogenesis, BMPs are widely accepted as relevant molecules in adult neurogenesis. In adult telencephalon, neural stem cell populations are maintained in two niches: the SVZ and the SGZ of the dentate gyrus. Colak et al. (2008) showed, through conditional deletion of *Smad4* or noggin infusion, that BMP signaling was required for adult neurogenesis. Noggin can also expand hippocampal progenitors in the SGZ (Bonaguidi et al., 2008). Infusion of Noggin and genetic deletion of either *Bmpr1A* or *Smad4* further demonstrate the role of BMP signaling in regulation of quiescence of neural stem cells from SGZ, restraining their proliferation and allowing these niches to maintain long-term neurogenic ability (Mira et al., 2010). BMP signaling is also required for positional identity and neuronal subtype specification. Endogenously produced BMPs inhibit the expression of a telencephalic gene profile, which was revealed by addition of noggin or other pharmacological signaling inhibitors (Bertacchi et al., 2013). BMPs are also involved in regulation of a transcriptional network to generate forebrain cholinergic neurons (Bissonnette et al., 2011). They also determine a temporally and spatially restricted requirement for generation of glutamatergic neurons in cerebellum (Fernandes et al., 2012).

BMP role in oligodendrogenesis

Oligodendrocyte specification and maturation depend on the function of transcription factors that include Olig1, Olig2, and Sox10. Olig2 directs early oligodendrocyte specification and Olig1 promotes oligodendrocyte maturation and is required for repair of demyelinated lesions (Lu et al., 2002; Ligon et al., 2006; Li and Richardson, 2008). BMPs inhibit the development of several stages of oligodendrocyte differentiation as well as the timing of myelination as shown by expression analysis markers of oligodendrocyte differentiation such as A2B5, galactocerebroside, or myelin protein expression (Hall and Miller, 2004; Samanta and Kessler, 2004; Chen and Panchision, 2007; See and Grinspan, 2009; Weng et al., 2012). Conversely, noggin blocks production of astrocytes from oligodendrocyte precursors in culture (Sim et al., 2006). BMP-4 stimulation increases oligodendrocyte progenitor proliferation in a model of induced demyelination. More importantly, addition of noggin increases the number of mature oligodendrocytes and increased the remyelination of injured axons

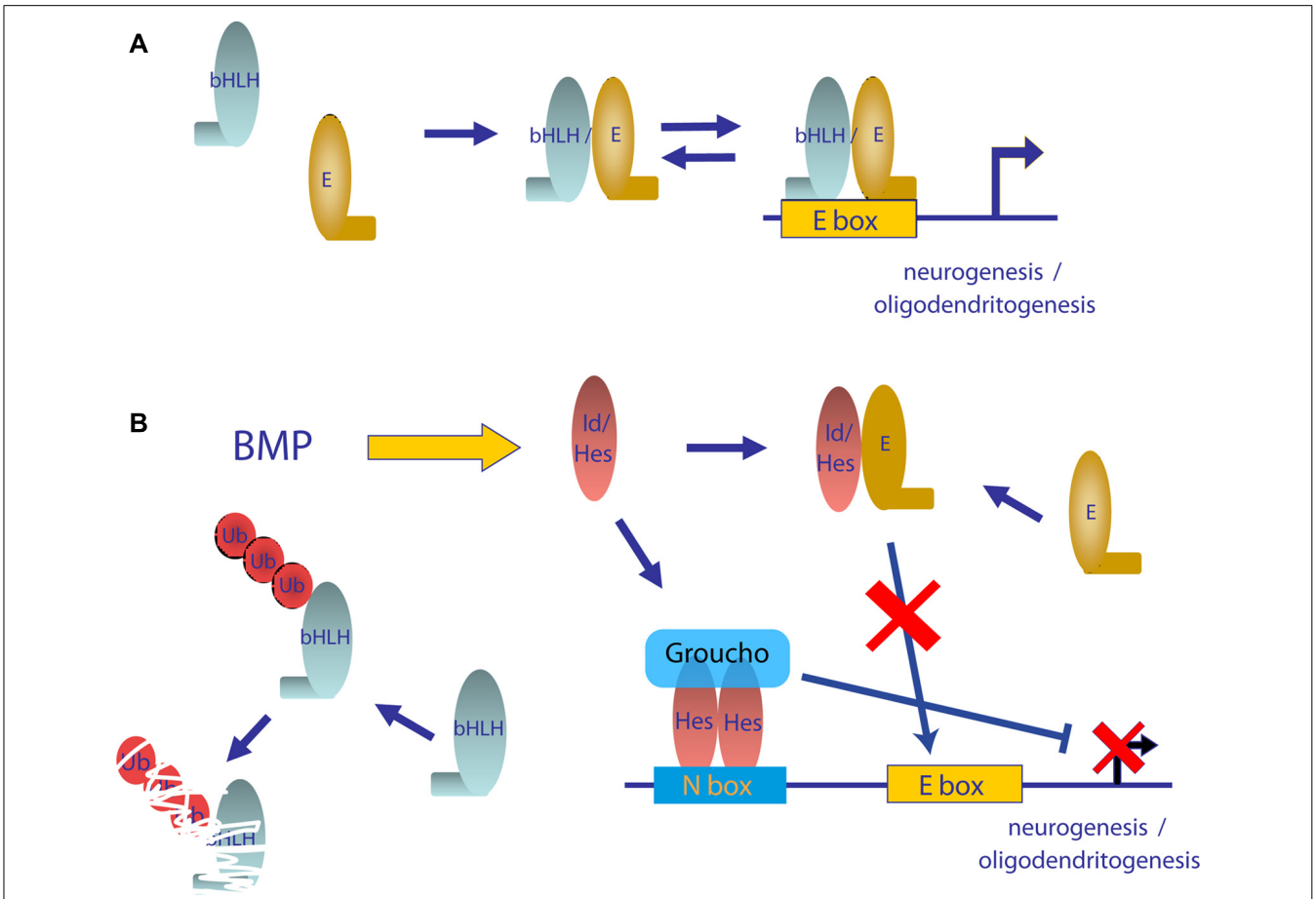

FIGURE 4 | Model of mechanism of repression of neurogenic bHLH transcription factors by BMPs. (A) In differentiating neurogenic precursors, bHLH transcription factors hetero-dimerizes with E proteins, which bind to E boxes and promote expression of neuronal genes. **(B)** Activation of the BMP signaling pathway leads to increased levels of Id and Hes proteins. Id and Hes proteins sequester E proteins away from bHLH transcription factors, which leads to transcriptionally inactive complexes and also enhance degradation of bHLH monomers. Furthermore Hes proteins can bind to N boxes sequences in neural promoters and recruit the Groucho family of transcriptional repressors.

in the corpus callosum (Sabo et al., 2011; Wu et al., 2012; Sabo and Cate, 2013). At the molecular level, BMP-induced expression of Id and Hes proteins seems to be also relevant in such a process by sequestering Olig1 and Olig2 which also belong to the bHLH family of transcription factors (Cheng et al., 2007; Bilican et al., 2008). Additional targets of Notch, such as *Jag1*, *Hey1*, and *Hey2*, are upregulated by BMP in oligodendrocytes through increased epigenetic modification at these loci (Wu et al., 2012). Inhibition of *Olig2* expression by direct binding of Smad4 to the *Olig2* promoter has also been demonstrated (Bilican et al., 2008; **Figure 3**). Recently, it has also been shown that the Smad-interacting protein-1 (Sip1) is an essential *in vivo* modulator of myelination. Sip1 antagonizes BMP signaling acting as a transcriptional repressor of Smad activity, while promoting activation of Olig1/Olig2 transcriptional activity and induction of I-Smads (Weng et al., 2012).

BMP role in astrocytogenesis

In opposition to the repression of neuronal and oligodendrocyte development, during the late embryonic and postnatal periods, BMP signaling strongly induces astrocyte differentiation (Gross et al., 1996; Mehler et al., 1997; Fukuda et al., 2007; See et al., 2007). BMPs promote the generation of astrocytes from precursors in a variety of embryonic neural cells (Gross et al., 1996; Mehler et al., 1997; Mehler, 2002; Bonaguidi et al., 2005). Implantation of noggin-producing cells induced the appearance of increased numbers of oligodendrocyte precursors whereas high BMP signaling inhibits oligodendrocyte precursors and promote astrogliogenesis (Mabie et al., 1999; Mekki-Dauriac et al., 2002; Wu et al., 2012). BMP signaling in the SVZ promotes astroglial lineage commitment and block differentiation of neurons and oligodendrocytes, whereas noggin suppresses astrocyte determination (Chmielnicki et al., 2004; Colak et al., 2008). LIF and BMPs synergize to promote astrocytic differentiation by activating astrocyte-specific promoters through a Stat3-p300/CBP-Smad1 complex (Nakashima et al., 1999; **Figure 3**). The ability of BMPs and Stat3 to promote astrogliogenesis has been shown to be dependent on the histone acetylation/deacetylation machinery (Scholl et al., 2012). Another study indicated that Smad action is not required for gliogenesis but is promoted by BMPs through

mammalian target of rapamycin/FKBP12-rapamycin-associated protein (mTor/Frap) phosphorylation of Stats (Rajan et al., 2003). Interestingly, Bmpr1A or Bmpr1B signaling exerts opposing effects on initial astrocytic hypertrophy after injury, suggesting that distinct BMPs engaging different receptor complexes exert separate activities on gliogenesis. The ability of BMPs to promote maturation of astroglia is further emphasized by the fact that activation of BMP signaling inhibits the tumorigenic potential of human glioblastoma-initiating cells (Piccirillo et al., 2006). Furthermore, Ezh2-dependent epigenetic silencing of *Bmpr1B* desensitizes tumor-initiating glioblastoma cells to differentiation and contributes to their tumorigenicity (Lee et al., 2008).

BMP SIGNALING IN NEURITE AND AXON OUTGROWTH, GUIDANCE AND SYNAPSE FORMATION

After neurogenesis has been completed, several sequential events are needed to establish neuronal circuits: polarized outgrowth of axons and dendrites, axon path finding toward the appropriate synaptic partner cell and establishment and maintenance of the synapse. Growing evidence in several experimental models points to BMP regulation of all these events. For instance, BMP ligands display positive regulation of the number, length, and branching of neurites in neurons from diverse neuronal origins, including cortical and hippocampal neurons (Le Roux et al., 1999; Withers et al., 2000; Lee-Hoeflich et al., 2004; Podkowa et al., 2010). Similarly, BMPs have been included as inductive signals promoting growth cone guidance as well as axonal orientation and path finding. Most of the data arise from studies of the sensory projection neurons of the spinal cord. BMP-7 and GDF-7 supplied by the roof plate orient axons of commissural neurons away from the roof plate and regulate their rate of extension through the spinal cord toward the floor plate (Augsburger et al., 1999; Butler and Dodd, 2003; Yamauchi et al., 2008, 2013; Sanchez-Camacho and Bovolenta, 2009). Guideposts are discrete groups of glial or neuronal cells that provide discontinuous information in intermediate positions along the path of growing axons (Sanchez-Camacho and Bovolenta, 2009). The corpus callosum represents the major forebrain commissure connecting the two cerebral hemispheres. Midline crossing of callosal axons is controlled by several glial and neuronal guideposts specifically located along the callosal path. BMP-7 has been shown to be required at different steps of organization and differentiation of these guidepost cells, which allows formation of the corpus callosum (Sanchez-Camacho et al., 2011). Additional evidence also indicates that BMP-7, secreted from the meninges, is involved in a morphogenic cascade, including Wnt3a and GDF-5, allowing correct corpus callosum development and prevents premature axon projection (Choe et al., 2012). It is not clear which signaling mechanisms are activated by BMP in these processes. In contrast to the slow timing of neural and glial specification, the very rapid time course of BMP-induced axonal orientation suggests transcription-independent pathways, probably depending on cytoskeletal actin remodeling and c-Jun N-terminal kinase (Jnk)-mediated microtubule stabilization (Augsburger et al., 1999; Wen et al., 2007; Podkowa et al., 2010; Perron and Dodd, 2011). Regulation of the cytoskeleton by BMPs has been linked to activation of Limk1 activation. Limk1 and Limk2 are closely related kinases that phosphorylate and inactivate actin-depolymerizing proteins such as cofilin or ADF. Limk1 has been shown to be activated by several BMPs in neural cells through specific binding to the Bmpr2 cytoplasmic tail and a further activation mechanism that involves Rho GTPases and PI3-kinase (Foletta et al., 2003; Lee-Hoeflich et al., 2004; Eaton and Davis, 2005; Gamell et al., 2008). Limk phosphorylation of cofilin/ADF is counteracted by the action of the Slingshot phosphatase, which is activated at later times after BMP addition and enables chemotactic responses to change from attraction to repulsion (Wen et al., 2007).

An intriguing functional and mechanistic question is that, whereas the closely related BMP-6 and -7 both induce the differentiation of commissural neurons, only BMP-7 is able to orients its axons *in vitro* and is required for appropriate axon projection *in vivo* (Perron and Dodd, 2011, 2012). Both ligands have been reported to bind hetero-dimers consisting of Acvr2A or Bmpr2 with Acvr1, Bmpr1A or Bmpr1B in numerous cellular models (Mueller and Nickel, 2012). However, the facts that a single amino acid swapping allows BMP-6 to orient axons and *vice versa* and that binding of BMP-6 to type I receptor depends on N-glycosylation, suggest that distinct receptor recruitment is involved in these functional differences (Saremba et al., 2008; Perron and Dodd, 2012). Changes in expression of BMP receptor subunits at growth cones have been shown after motor neuronal differentiation (Benavente et al., 2012). Moreover, whereas neuronal specification is a redundant function of Bmpr1A and Bmpr1B, axon outgrowth and regulation of cofilin activity only depend on *Bmpr1B* (Yamauchi et al., 2008, 2013).

BMP role in synaptogenesis

Once axons reach their corresponding target, two-way communication between presynaptic and postsynaptic cells is needed for synaptic establishment and homeostasis during development and for proper synaptic plasticity. The *Drosophila* larval neuromuscular junction (NMJ) is a useful model for genetic and biochemical studies of synaptic function (Collins and DiAntonio, 2007). Development of the synapse requires an anterograde as well as retrograde input from the synaptic terminal and target cell. BMP signaling is an indispensable component of retrograde signaling in the NMJ (Henriquez et al., 2011). The muscle-secreted BMP ligand *glass bottom boat* (*Gbb*), signals through presynaptic *wishful thinking* (*Wit*), the Bmpr2 homolog, and the receptor type I homologs *thick veins* and *saxophone* (*Tkv* and *Sax*, respectively; Aberle et al., 2002; Marques et al., 2002, 2003; Rawson et al., 2003). *Wit* mutant larvae show complete presynaptic detachment, which could be rescued by its expression in presynaptic cells (Aberle et al., 2002; Marques et al., 2002). Similarly, *Gbb* expression in muscle, but not in neurons, rescues NMJ defects observed in *Gbb* mutants (Marques et al., 2003). Retrograde *Gbb* activation of synaptic receptors has two parallel effects. One is activation of Limk1 that allows stabilization of the synapse. In the absence of *Limk1*, synaptic footprints are observed in the NMJ. In these footprints the presynaptic components are missing (Eaton and Davis, 2005). Since presynaptic development precedes postsynaptic development, synaptic footprints have been interpreted as being the remnants of mature synaptic contacts that have formed and then retracted. The other effect involves activation of the Smad homologs *Mad* and *Medea*

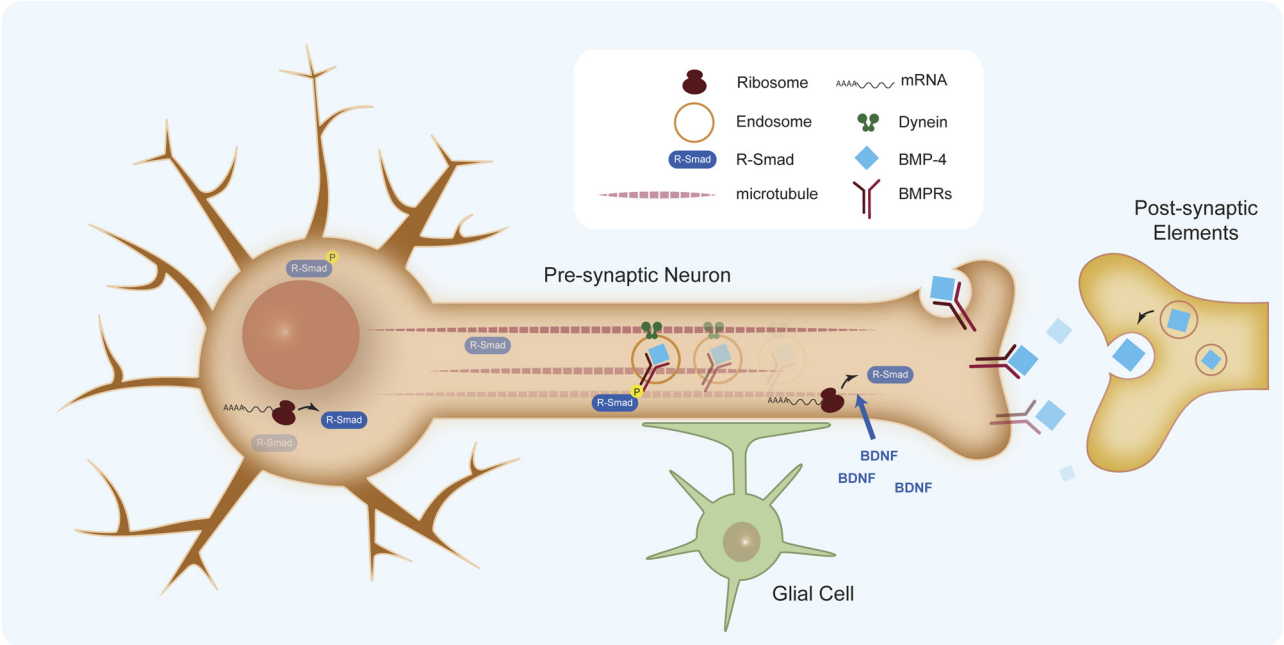

FIGURE 5 | Retrograde signaling of BMPs in synapse formation. BMP retrograde transport of endocytosed BMP receptors by dynein motors. Effective retrograde BMP signal also involves specific translation of Smad protein in the axons.

and their transport to the neuronal soma to regulate transcription at the nucleus (**Figure 5**). There, Mad-dependent transcription of the Rac-guanine nucleotide exchange factor (GEF) *Trio* is relevant for proper NMJ growth and branching since transgenic expression of *Trio* partially rescues BMP signaling mutant larvae (Ball et al., 2010). Therefore, canonical and non-canonical pathways are involved in coordination of the synaptic growth and stability of NMJ. Interestingly, genes related to several motor diseases such as hereditary spastic paraplegia or amyotrophic lateral sclerosis, have been shown to regulate BMP retrograde signaling in model systems (Bayat et al., 2011; Henriquez et al., 2011).

An important aspect in BMP retrograde communication is how signals at the synaptic terminal are conveyed to the nucleus to regulate transcription. It has been shown that BMP retrograde signaling along the axon requires dynein retrograde motors (McCabe et al., 2003). Recently, retrograde transport of endocytosed BMP receptors has been demonstrated, which suggests two populations of phosphorylated Smad transducers, one at the synaptic terminal and one at the cell body (Smith et al., 2012; **Figure 5**). However, studies of the murine trigeminal sensory system indicate that an effective retrograde BMP signal also involves specific translation of Smad protein in the axon, which is transported to the cell body by dynein motors (Ji and Jaffrey, 2012). Axonal translation of Smads is activated by brain-derived neurotrophic factor (BDNF), thus coupling both morphogens in the homeostasis of synapses. Some questions remain about the exact place of Smad activation and the role of the additional Smads translated in the axon when it is commonly accepted that the intracellular pool of unphosphorylated Smads is relatively high. Another relevant aspect is the role of glial cells in the regulation of synaptogenesis. Glia, associate intimately with synaptic terminals and are required for synaptogenesis. Recent work on NMJ points to a new member of the BMP ligand family, *Maverick* (*Mav*), in controlling synthesis and release of *Gbb*. Mav is released in glial cells surrounding the synapse and reinforces BMP retrograde signaling by transcriptional regulation of the synthesis of *Gbb* in postsynaptic cells (Fuentes-Medel et al., 2012). Altogether, evidence points to a very relevant role of BMP ligands in the coordination of several cell types and signaling mechanisms during synaptogenesis.

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Bone morphogenetic protein signaling in vertebrate motor neurons and neuromuscular communication

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An accurate communication between motor neurons and skeletal muscle fibers is required for the proper assembly, growth and maintenance of neuromuscular junctions (NMJs). Several signaling and extracellular matrix molecules play stimulatory and inhibitory roles on the assembly of functional synapses. Studies in Drosophila have revealed crucial functions for early morphogens, such as members of the Wnt and Bone Morphogenetic Proteins (BMP) signaling pathways, during the assembly and maturation of the NMJ. Here, we bring together recent findings that led us to propose that BMPs also work in vertebrate organisms as diffusible cues to communicate motor neurons and skeletal muscles.

Keywords: BMP, motor neuron, muscle, NMJ, synapse

INTRODUCTION

The establishment of functional neuromuscular synapses is essential for the coordinated movement of the organisms. The accurate communication between motor neurons and skeletal muscle fibers is crucial for proper synaptic assembly, growth and maintenance. In this regard, it has been demonstrated that the embryonic formation of the vertebrate neuromuscular junction (NMJ) is driven by signaling and extracellular matrix molecules derived from presynaptic motor neurons and from postsynaptic muscle cells that are intermingled to assemble functional synapses. For instance, the early assembly of postsynaptic densities, characterized by the clustering of acetylcholine receptors (AChRs) on the surface of developing muscle cells, does not require neuronal inputs (Flanagan-Steet et al., 2005; Lin et al., 2008; Jing et al., 2009). However, neuronal cues refine this postsynaptic pattern by, in one hand, eliminating AChRs from extrasynaptic regions via a neurotransmitter-dependent mechanism and, simultaneously, by maintaining and maturing postsynaptic densities in neuromuscular contacts through the action of Agrin (Lin et al., 2005; Misgeld et al., 2005; An et al., 2010). The MuSK activator Agrin has a separate role essential for postnatal maintenance of neuromuscular synapses (Tezuka et al., 2014). Also, presynaptic differentiation is controlled by the coordinated action of extracellular molecules (Nishimune et al., 2004; Fox et al., 2007; Carlson et al., 2010). For instance, members of the fibroblast growth factor family are required for the initiation of presynaptic terminals, laminins act as crucial molecules for presynaptic maturation, and collagens positively affect the maintainance of proper NMJs (Fox et al., 2007). Therefore, multiple signaling pathways are interplayed to orchestrate the correct differentiation and positioning of functional NMJs.

An emerging concept in the field is the participation of signaling molecules of the early development on the wiring of synaptic contacts, including the NMJ. These molecules, named morphogens, have the ability to induce distinct cellular responses in a concentration-dependent manner. Major players of this effect are ligands of the Wnt pathways (for reviews, see Henríquez and Salinas, 2012; Koles and Budnik, 2012; Park and Shen, 2012). Different Wnt pathways are able to induce the early aneural assembly of postsynaptic densities by binding the Agrin-interacting protein MuSK (Jing et al., 2009), and, at the same time, they also regulate the formation of well positioned NMJs, possibly acting through their cognate Frizzled receptors (Packard et al., 2002; Mathew et al., 2005). Growing evidence, mainly obtained from *Drosophila*, reveals that pathways activated by members of the bone-morphogenetic proteins (BMP), a family of secreted ligands belonging to the transforming growth factor-β (TGF-β) superfamily of signaling proteins, also play key roles on neuromuscular synaptogenesis (Marqués, 2005). The role of TGF-β/BMP pathways in invertebrate and vertebrate motor neurons has been reviewed elsewhere (Katsuno et al., 2011). In this review, we focus in highlighting recent insights into the possibility that BMPs also work in vertebrate organisms as diffusible signals to ensure proper communication between motor neurons and skeletal muscles.

SIGNALING PATHWAYS ACTIVATED BY BMP LIGANDS

Based on their high sequence identity, the BMP family members (>20) have been classified in multiple subgroups that are conserved between vertebrate and invertebrate species (Kishigami and Mishina, 2005). Even though BMPs were first named based on their ability to induce ectopic bone formation (Urist, 1965;

Wozney et al., 1988), different studies have shown that they are multifunctional proteins affecting a diversity of biological responses. These include early developmental processes, such as dorso-ventral patterning, patterning of the body axes, early patterning of the central nervous system, as well as the specification of several tissues and organs (Zhao, 2003; De Robertis and Kuroda, 2004; Kishigami and Mishina, 2005; Sieber et al., 2009).

Early events in BMP signaling involve the formation of heteromeric complexes of two types of transmembrane receptors with serine/threonine kinase activities, named type I and type II. Receptors of both types are needed to form a functional complex for signal transduction (**Figure 1**; Yamashita et al., 1994; Liu et al., 1995). BMPs can interact with three distinct type I (ActRIA, BMPRIA and BMPRIB) or type II receptors (BMPRII, ActRIIA and ActRIIB). This interaction relies on their affinity, but also on the specific expression pattern of the different BMP receptors (Sebald et al., 2004; Lin et al., 2006). Detailed analyses of BMP pathways came from studies of cell responses to BMP-2 (Sieber et al., 2009). BMP-2 binding to a preformed heteromeric complex of BMPRII and BMPRI initiate a classical Smad-dependent signaling pathway (see **Figure 1**; Gilboa et al., 2000; Nohe et al., 2002). Upon BMP-2 binding, BMPRI is phosphorylated by BMPRII. Activated BMPRI initiates the phosphorylation of specific receptor-regulated Smad proteins, namely R-Smad-1, -5 or -8, which form heteromeric complexes with the common mediator Smad-4. Such complexes translocate to the nucleus to regulate the transcription of specific target genes in cooperation with co-repressors or co-activators (Shi and Massagué, 2003; Nohe et al., 2004; Miyazono et al., 2005).

In addition, BMP-2 is able to induce non-Smad pathways, such as activation of p38 mitogen-activated protein kinase (MAPK) (Nohe et al., 2004; **Figure 1**). Activation of p38 MAPK is the consequence of BMP-2 binding to its high affinity receptor BMPRI which induce the recruitment of BMPRII, thus leading to the formation of the signaling heteromeric complex (Gilboa et al., 2000; Nohe et al., 2002). Also, BMPs have the ability to trigger other Smad-independent pathways, which are mediated by different intracellular mediators, including ERK, the nuclear factor kappa beta (NFkB), and phosphoinositide 3-kinase (PI3 Kinase; Sieber et al., 2009; Bragdon et al., 2011). The activation of different BMP signaling pathways defines their wide ranges of different biological effects. These signaling cascades are finely regulated by the temporospatial expression of regulatory factors, such as antagonists that bind and inactivate the ligands, co-receptors of the cell surface having stimulatory or inhibitory roles, intracellular regulatory proteins, and specific co-repressors or co-activators that regulate the transcription of specific target genes (Miyazono et al., 2005; Sieber et al., 2009).

BMPs have a very dynamic role in the nervous system including their ability to regulate early steps of neuralization to patterning, neural stem cell proliferation, selfrenewal, lineage specification and neuronal function (dendritic and axonal growth as well as synapse formation and stabilization) by either Smad-dependent or Smad-independent signaling (see Gámez et al., 2013, Front. Cell. Neurosci.; this issue).

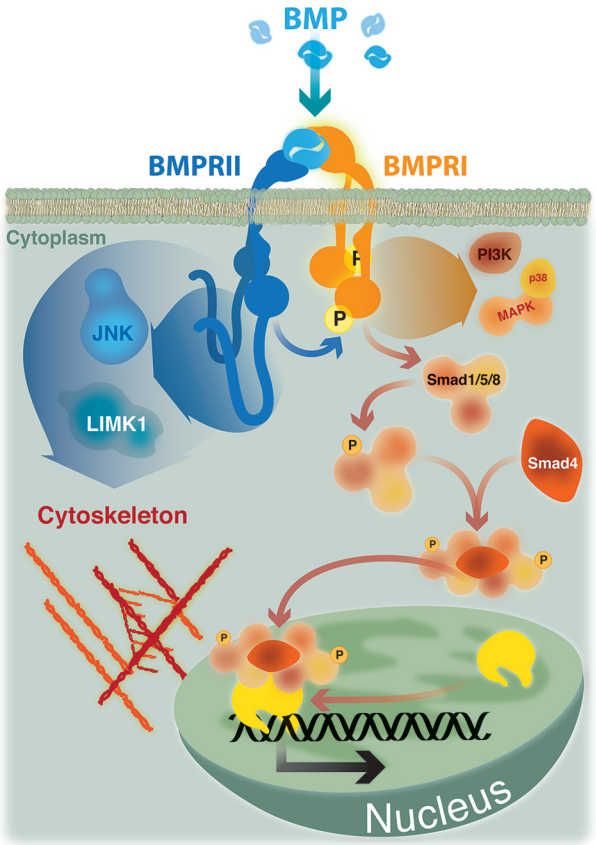

FIGURE 1 | BMP signaling. Smad signaling is initiated upon phosphorylation of Smad-1, -5 or -8 by a heteromeric complex of BMPRII, BMPRI and BMP ligand. Phosphorylated Smads recruit Smad-4 and translocate to the nucleus where, in association with co-repressors or co-activators, regulate the transcription of specific target genes. Non Smad pathways, such as p38 MAP kinase and PI3 kinase, are also initiated by heteromeric complexes. The activity of cytoskeleton regulators mediating actin remodeling (LIMK1) and microtubule stabilization (JNK) are dependent on its ability to bind to the long cytoplasmic tail of BMPRII.

BMPRII ROLE IN NEURONAL OUTCOME

All receptors from the TGF-β superfamily are arranged in three conserved moieties: an extracellular ligand binding domain, a transmembrane segment, and an intracellular serine/threonine kinase domain (Sebald et al., 2004). Among receptors of the TGF- β superfamily, an outstanding feature of BMPRII is the presence of a long Carboxy terminal tail following its kinase domain, which corresponds to ∼500 amino acids, representing the 50% of the protein (Waite and Eng, 2003). The long C-terminal tail of BMPRII is also present in its corresponding homologs in various vertebrate and invertebrate species (Estevez et al., 1993; Ishikawa et al., 1995; Aberle et al., 2002).

An essential role of BMP signaling linked to synaptic growth, synapse stability, and homeostatic plasticity has been shown in Drosophila. Remarkably, these studies have demonstrated that a key component of BMP signaling affecting the NMJ is wishful thinking (Wit), the BMPRII homolog. Wit mutant larvae

show a significant impairment of the morphology and function of the neuromuscular synapse (Aberle et al., 2002; Marqués et al., 2002). The expression of Wit in these mutants rescued the normal phenotype, demonstrating a key role for the signaling mediated by this receptor on the behavior of presynaptic neurons at the invertebrate NMJ (Aberle et al., 2002; Marqués et al., 2002). Impaired motor neuron synaptic growth and function has also been demonstrated in loss of function mutants either for the BMP-7 homolog Glass bottom boat (Gbb), the BMPRI Saxophone (Sax) or the Smad homologs Mad and Medea (McCabe et al., 2003; Rawson et al., 2003; Eaton and Davis, 2005; McCann et al., 2005). Gbb is expressed in presynaptic neurons and postsynaptic muscle (James and Broihier, 2011). A role of Gbb and Wit in the expression of presynaptic proteins that are required for synaptic homeostasis independent of BMP-dependent regulation of synaptic growth or stability has been proposed (Goold and Davis, 2007), whereas muscle-derived Gbb is involved in retrograde signaling related to synaptic growth (James and Broihier, 2011) and synapse maturation (Berke et al., 2013). Hence, in invertebrates Wit is required in cell soma of motor neurons as well as for retrograde signaling to activate canonical Smad-dependent BMP signaling that works through transcriptional mechanism to control motor neuron behavior.

Additionally, a role of BMP signaling in synaptic stability has been shown. In this regard, mutants of Wit receptor showed a strong increase of synaptic footprints (regions of the NMJ where the terminal nerve once resided and has retracted) as compared to those observed in mutants of other canonical BMP signaling molecules. A potential mechanism accounting for this effect is related to the ability of the C-terminal domain of Wit to interact with the actin cytoskeleton modulator LIM Kinase1 (DLIMK1) in motor neurons to stabilize the NMJ. Remarkably, this interaction is not required for Smad-mediated synaptic growth (Eaton and Davis, 2005). In line with this observation, rapidly generated presynaptic varicosities, referred as ghost boutons, require retrograde Gbb signal and are locally regulated by Wit that, through an interaction with LIMK, regulate the synaptic actin cytoskeleton and inhibits bouton budding (Piccioli and Littleton, 2014). Therefore, Smad-dependent and independent signaling pathways via the Wit receptor expressed by invertebrate motor neurons are involved in synaptic outcomes at the NMJ. Supporting a central role of BMPRII in vertebrate species, it has been shown that mice spinal motor neurons expressed detectable mRNA levels of all type I and type II receptors, but BMPRII is at least 26 times more abundant than the other receptors (Wang et al., 2007a).

Different studies have shown that Smad-independent BMP induced pathways are related to local signals that involve cytoskeleton arrangements. In this regard, BMP-mediated cell migration, axon and dendrite growth, or axon guidance require activation of the small GTPase Cdc42, PI3-K, p38 MAPK, c-Jun N-terminal kinase (JNKs) or LIMK (Foletta et al., 2003; Lee-Hoeflich et al., 2004; Eaton and Davis, 2005; Wen et al., 2007; Gamell et al., 2008, 2011; Podkowa et al., 2010; Hiepen et al., 2014). Interestingly, BMPRII has been involved in most of the studies localizing or coordinating this local signaling. For instance, upon BMP-2 stimulation, the p38 downstream effector Hsp25, that regulates cytoskeletal dynamics, colocalizes with BMPRII (Gamell et al., 2011). In addition, BMP-2 induced planar cell polarity and actin dependent lamellipodia formation mediated by PI3-K involve the binding of the PI3K regulatory subunit p55γ to BMPRII, irrespective of the presence of the C-terminal tail (Hiepen et al., 2014). Also, BMPRII lacking the C-terminal tail is able to activate Smad pathways in response to BMP-2 (Nohe et al., 2002), suggesting that this domain of BMPRII could play other regulatory roles.

The first described function of BMPRII C-terminal tail was the demonstration that the activity of LIM kinase 1 (LIMK1), a regulator of actin dynamics, was reduced by its binding to this terminal region of the BMPRII (Foletta et al., 2003). Afterwards, it was showed that the positive effect of BMP-7 on dendritogenesis of mouse cortical neurons required the association of LIMK1 with the BMPRII C-terminal tail, which synergized with the Rho GTPase Cdc42 to activate the LIMK1 catalytic activity (Lee-Hoeflich et al., 2004). Similarly, the binding of JNK proteins to the C-terminal tail of BMPRII was also required both for microtubule stabilization and for the BMP-7 induced dendritogenesis in primary cortical neurons (Podkowa et al., 2010). BMP-7 also induces an attractive response of spinal neuron axons by eliciting asymmetric actin polymerization/stabilization through the spatial regulation of ADF/cofilin activity via interaction of LIMK1 with the BMPRII C-terminal tail (Wen et al., 2007). Hence, BMP induced Smad-independent signals elicited by type I receptors may have cytoskeleton effects depending on the BMPRII relationship with adaptor proteins (**Figure 1**).

In the following sections, we will summarize recent findings revealing that signaling pathways activated by BMP ligands could regulate the behavior of two of the main constituents of the neuromuscular synapse in vertebrate species, muscle cells and motor neurons, and propose a model of how these BMP-mediated effects could combine at the neuromuscular connection.

BMP SIGNALING IN SKELETAL MUSCLE

During embryonic development, skeletal muscles derive from mesodermal precursor cells by successive steps. The first event involves specification of precursor cells to the myogenic lineage. These committed proliferating cells, named myoblasts, withdraw from the cell cycle and begin to express muscle-specific genes. Finally, differentiating myoblasts fuse to generate multinucleated muscle fibers. During the process, a subpopulation of myoblasts does not differentiate and remains associated to the fiber as quiescent satellite cells (Chargé and Rudnicki, 2004). After myofiber damage, satellite cells are activated to generate committed myoblasts that proliferate and differentiate to repair damaged fibers and generate new multinucleated fibers (Chargé and Rudnicki, 2004). *In vivo* models of damage-induced muscle regeneration show the activation of pSmad1/5/8 in Tibialis anterior muscle lysates after 1 day of injury, which is still visible after 3 days, and became undetectable at day seven, suggesting that activated satellite cells express pSmad1/5/8 (Clever et al., 2010). In agreement, pSmad1/5/8 is detected in satellite

cells and myoblasts after 3 days in models of regenerating Gastrocnemius or Tibialis anterior muscle of adult mice (Clever et al., 2010; Ono et al., 2011). These findings suggest that the induction of Smad-dependent BMP signaling is a generalized response to muscle injury and seems to be related to activation and expansion of myogenic precursor cells. Accordingly, isolated myofibres with satellite cells show a strong nuclear immunostaining of phosphorylated Smad1/5/8 in activated and proliferating, but not quiescent, satellite cells (Ono et al., 2011). Importantly, in satellite cells induced to differentiate, BMP-4 causes an increase in total cell number of committed cells but a significant fall in the proportion of differentiating cells and their fusion into myotubes. Consistently, blocking the interaction of BMP-4 with its receptors, as well as down-regulation of the BMPRIA or inhibiting the intracellular BMP-Smad signal, induces a faster differentiation (Ono et al., 2011). The aforementioned studies indicate that the BMP pathway inhibits muscle differentiation, but also has the ability to stimulate satellite cell proliferation (**Figure 2A**). Accordingly, it has been proposed that BMP signaling is important to stimulate the amplification of committed myoblasts and to prevent precocious differentiation during muscle regeneration (Ono et al., 2011).

New insights are also emerging related to the role of BMP signaling in skeletal muscle. Recent findings have demonstrated the role of BMP Smad-dependent signaling in the control of muscle mass, by promoting hypertrophy and counteracting atrophy (Sartori et al., 2013; Winbanks et al., 2013). Gdf6 (encoding BMP13) and Gdf5 (encoding BMP14) are induced in mouse skeletal muscle subjected to denervation, used as a model of muscle atrophy (Sartori et al., 2013; Winbanks et al., 2013). Accordingly, an autocrine signal is proposed as responsible of the increased Smad1/5/8 phosphorylation in muscle that is essential to limit atrophy in denervated muscles (Sartori et al., 2013; Winbanks et al., 2013). Importantly, several BMP genes and BMP receptors are expressed in innervated muscles (Sartori et al., 2013; Winbanks et al., 2013) suggesting that BMP induced signaling is regulated in adult muscle by a mechanism dependent on motor nerve activity. In agreement, phosphorylation of Smad1/5 declined markedly from 1 week after birth until 6 months in mouse skeletal muscle (Winbanks et al., 2013), indicating that BMP dependent signaling in muscle cells is repressed during postnatal maturation.

Agrin, a main motor neuron-derived postsynaptic organizer (Bowe and Fallon, 1995), binds BMP-2 and -4 and decreases their rate of association to the extracellular domain of BMPRIA, thereby inhibiting BMP-induced signaling (Bányai et al., 2010). Thus, it is possible that Agrin plays a role in the control of BMP activity in muscle fibers, by modulating the extracellular distribution and availability of BMPs for receptor binding. Consistent with this idea, BMP-4 has been immunodetected in Soleus muscle fibers, localized in close vicinity to postsynaptic densities at the NMJ (Chou et al., 2013). Indeed, denervated Soleus muscle loose BMP-4 immunoreactivity, reinforcing the idea that this specific localization of BMP-4 in muscle fibers is regulated by a factor derived from motor neurons (Chou et al., 2013). Interestingly, BMP-4 surrounds the postsynaptic density at the NMJ neurons (Chou et al., 2013). This localization of BMP-4 could be the consequence of a local increase of BMPRII, which becomes associated to protein adaptors, such as LIMK1. It is interesting to note that peripheral regions of postsynaptic densities at the NMJ are characterized by the absence of AChR clusters but also by the accumulation of polymerized F-actin; indeed, the actin binding protein cofilin is likely to play a crucial role on the active disaggregation of AChR clusters (Lee et al., 2009). Also, these regions contain the phosphoinositide-binding protein LL5β, which promotes postsynaptic maturation at the NMJ by delimiting AChR-rich areas (Kishi et al., 2005). Therefore, it is tempting to speculate that the specific localization of BMP-4 at the border of postsynaptic densities of the NMJ could have a dual effect on muscle fibers: on one hand, it may represent a sequestering of this BMP ligand to preclude activation of BMP signaling in innervated muscles. On the other hand, it may activate local BMP-dependent pathways involved in the disassembly of AChR clusters at the NMJ (**Figure 2B**). Remarkably, this notion is supported by recent evidence showing that the BMP-2 induced chemotaxis of mesenchymal progenitor cells depends on the effect of LL5β, likely mediated by the BMPRII, on cortical actin rearrangements (Hiepen et al., 2014).

Collectively, information from models of muscle induced regeneration and neurogenic atrophy lead us to propose that BMP signaling plays local roles on skeletal muscle that are like dependent on motor neuron activity. Probably, the source of BMP is the skeletal muscle; indeed, BMP-4 is expressed in myogenic cells (Kumar et al., 2009; Clever et al., 2010; Chou et al., 2013). Furthermore, as mentioned before, several BMP genes and BMP pathway components are expressed in adult muscle, and the transcript expression levels of some BMPs are induced in skeletal muscle after denervation (Sartori et al., 2013; Winbanks et al., 2013). In addition, Agrin increases mRNA expression and the immunoreactivity of BMP-4 in differentiated C2C12 muscle cells (Chou et al., 2013). Thus, BMPs expressed by muscle cells could play autocrine effects but, considering the contiguous relationship between skeletal muscle and motor neurons, they could also affect motor neuron behavior. This idea is consistent with the aforementioned data pointing to the crucial role that the retrograde secretion of the BMP ligand Gbb plays in the proper nerve terminal development and establishment of the NMJ in Drosophila.

BMP SIGNALING IN MOTOR NEURONS

In the adult neuronal tissue, BMPs regulate several features of cell behavior. For instance, dendritogenesis, number of neurites, length of neurites and branch points have been shown to be stimulated or inhibited by different BMPs in diverse neuronal types, including cultured sympathetic, cerebral cortical, hippocampal, postnatal cerebellar and peripheral neurons (Iwasaki et al., 1999; Gratacòs et al., 2001; Horbinski et al., 2002; Yabe et al., 2002; Lee-Hoeflich et al., 2004; Matsuura et al., 2007; Anitha et al., 2010).

In motor neurons of vertebrates, a distinctive role of BMPs has not yet been described. However, some evidence came from experimental models of injury. For instance, BMP-2 mRNA is

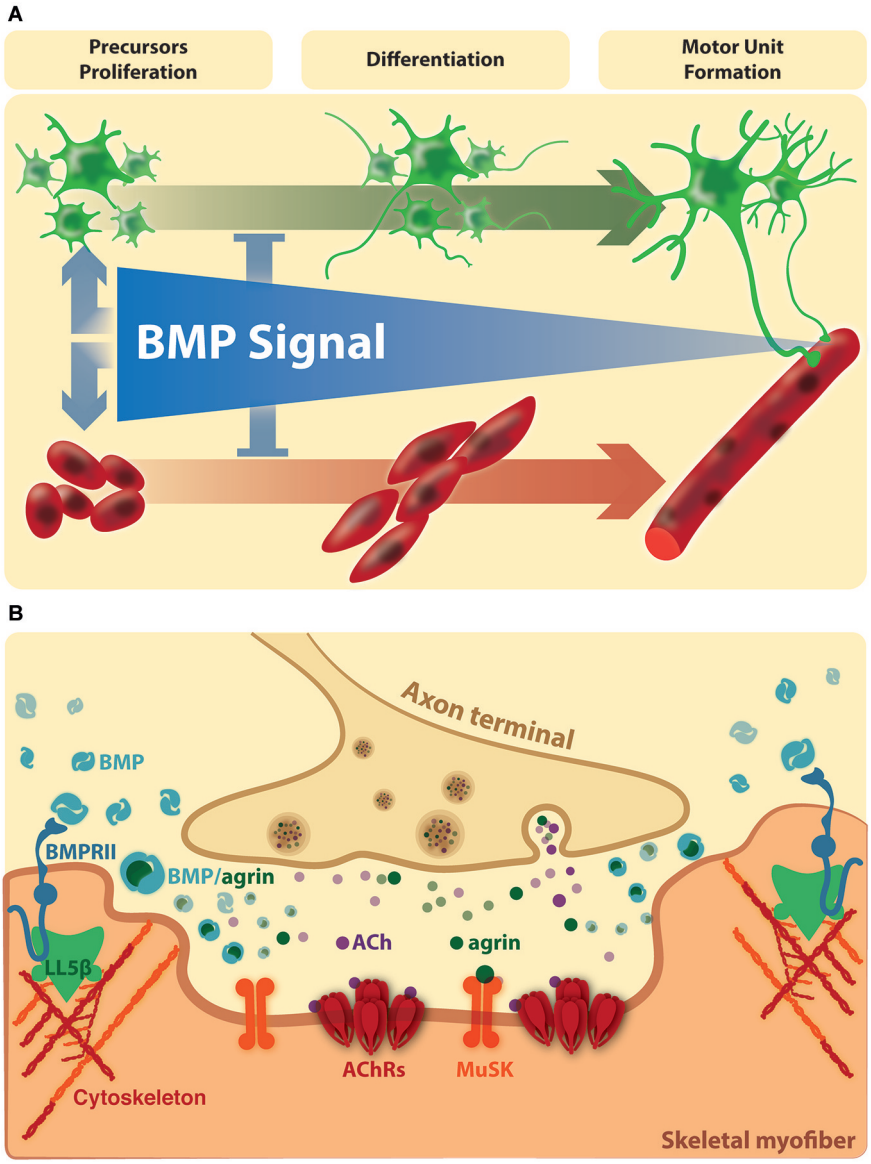

FIGURE 2 | BMP signaling on the connectivity of the vertebrate neuromuscular synapse. **(A)** In vertebrates, the evidence suggests that BMPs stimulate the amplification of muscle and motor neurons precursors and repress precocious differentiation. At this stage, the BMP dependent effects are mainly Smad dependent. At later stages, BMP signaling becomes restricted to the site of innervation. **(B)** Here, activation of BMP pathways could be involved in NMJ formation, maturation and/or maintenance. Agrin and BMPs could modulate the extracellular distribution and availability of each other for receptor binding in synaptic domains. In turn, local BMP-dependent pathways could affect cortical actin rearrangements at extrasynaptic domains (see text for details).

expressed in motor neurons after crush injury of the facial nerve in rabbits (Wang et al., 2007b). Similarly, traumatic injury of the rat spinal cord results in a remarkable up-regulation of BMP-7 and BMP-2 around the injury site during recovery (Setoguchi et al., 2001; Matsuura et al., 2008). It has also been considered that some BMPs induce astroglial differentiation and, according to this, the manipulation of BMP signaling at the injured spinal cord has been mainly intended to evaluate possible functional recovery (Sahni et al., 2010). In this regard, enhanced locomotor activity and axonal regrowth is observed when BMP binding to their receptors is inhibited by administration of noggin—a high affinity soluble antagonist of BMP-2—into the injured spinal cord (Matsuura et al., 2008). Accordingly, a partial functional recovery has been shown after transplantation of neural progenitor cells modified to express noggin into injured mouse spinal cords (Setoguchi et al., 2004). Therefore, from these studies it is possible to propose that BMP signaling opposes to the differentiation of motor neurons. Indeed, BMP-2 plays a negative

role on the neurite outgrowth of the motor neuron cell line NSC34 (Benavente et al., 2012). This BMP-2 dependent inhibition of morphological differentiation is accompanied by increased phosphorylation of Smad1/5/8, as well as by an increase in the expression and activity of the Smad-dependent early responsive gene Id1, a negative regulator of the differentiation of neurogenic precursors (Ying et al., 2003; Viñals et al., 2004). Simultaneously, the levels of the neurogenic factor Mash1 are down-regulated by BMP-2 (Benavente et al., 2012). Together, these findings reveal that BMP signaling activation acts as a negative regulator of motor neuron differentiation.

Models of motor neuronal pathologies have also been useful to decipher a potential role of BMP signaling in these neurons. Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease characterized by the selective loss of motor neurons, leading to paralysis and death (Pasinelli and Brown, 2006). In familial cases of ALS, 20% corresponds to point mutations of superoxide dismutase-1 (SOD1; Rosen et al., 1993). Indeed, the G93A mutation of SOD1 has been widely used to generate model systems of ALS, either animals or *in vitro*, as they mimic the main clinical, pathological and cellular features of the disease (Gurney et al., 1994; Arciello et al., 2011; DuVal et al., 2014). We have analyzed BMP signaling in the context of ALS by using motor neuron-like NSC34 cells stably expressing wild-type or G93A mutated forms of human SOD1. In undifferentiated cells, phospho Smad 1/5/8 and Id1 levels are significantly higher in NSC34 cells expressing hSODG93A compared to controls, and then they are similarly down-regulated during the differentiation of both cell types (Pinto et al., 2013). Consistently, the basal phospho Smad-dependent transcription of Id1 is 2-fold higher in cells with constitutive expression of hSOD1G93A, as compared to those expressing hSOD1WT, whereas this activity is strongly diminished after 24 and 48 h of differentiation in both cell types (Pinto et al., 2013). As Smad signaling varies inversely with motor neuron differentiation, the impaired morphological differentiation observed in the ALS model may be correlated to up-regulated Smad signaling. It is appealing to note that BMP signaling inhibits the differentiation of precursor motor neuron cells, similarly to what was observed in muscle precursor cells. Comparable to the role of BMP signaling in muscle cells, it has been shown that, during mouse neural development, Smad signaling induced by BMP-2 is required to prevent premature differentiation (Di-Gregorio et al., 2007).

Hereditary Spastic Paraplegia (HSP) is a group of genetic disorders characterized by retrograde axonal degeneration of the corticospinal tract and the posterior columns in the spinal cord that result in progressive spasticity and weakness of the lower limbs (Salinas et al., 2008). Different mutated genes have been identified in HSPs (Dion et al., 2009). Atlastin, spastin, spartin and NIPA1 are among the mutated proteins related to HSP accounting for the majority of clinical cases. Remarkably, common features of these proteins in vertebrates are their localization in endosomal membrane traffic compartments and their ability to influence BMP signaling (Tsang et al., 2009; Fassier et al., 2010). For instance, morpholino-dependent knockdown of the ortholog of human atlastin (*atl1*) in developing zebrafish induced a prominent loss of larvae motility, associated with abnormal axon pathfinding and multiple aberrant branching of spinal motor neurons (Fassier et al., 2010). Interestingly, primary cultures of zebrafish spinal neurons from *atl1* morphants show a significant increase in the amount of nuclear pSmad1/5/8, suggesting that atlastin represses BMP signaling. Consistently, atl1 knockdown defects were rescued by genetic or pharmacologic inhibition of the BMP pathway (Fassier et al., 2010). As for atlastin, enhanced phosphorylation of Smad1/5 has been observed after siRNA-mediated silencing of NIPA1, spastin and spartin in mammalian cells (Tsang et al., 2009). Together, these data support the notion that different endosomal proteins related to HSP act as repressors of BMP Smad-mediated signaling. Thus, deregulations on the trafficking of BMP receptors, having as a consequence enhanced Smad signaling, may produce negative effects on motor neuron behavior or even axonal degeneration.

In spite of the aforementioned evidence, activation of BMP pathways may also exert positive roles in motor neurons (Wang et al., 2007a; Chou et al., 2013; Kelly et al., 2013). For instance, long term differentiation (72 h) of NSC34 motor neuron cells in the presence of a Smad signaling inhibitor, show a reduction in the number of cells with long axons, which is counteracted by using BMP-4. Similarly, primary motor neuron cultures allowed to initiate axon growth show that BMP enhance, whereas the Smad inhibitor delay, motor axon advancement in culture (Kelly et al., 2013). These findings are in contrast to the role of BMP as an inhibitor of motor neuron differentiation. However, the delayed effect of the inhibitor (3 days of differentiation) on the neuritogenesis of NSC34 cells, and the experimental design in primary motor neurons to assess the effect on axon advancement, suggest that BMP signaling is required later on, once motor projections have already protruded from the cell soma (Kelly et al., 2013). Thus, a unifying hypothesis is that BMP signals could play dual effects on motor neurons: an early inhibitory role on morphological differentiation, and later, a positive role on axon elongation. Interestingly, we have shown that BMPRII is up-regulated throughout NSC34 cells differentiation and becomes accumulated in somas and growth cones. Remarkably, BMP-2 treatment, that inhibits neurite outgrowth, concomitantly up-regulates BMPRII (Benavente et al., 2012). Therefore, even though BMP-2 plays inhibitory roles on neurite outgrowth, it could also provide motor neuron cells with key proteins for their subsequent differentiation. Between these proteins, the BMPRII, which becomes localized in growth cones, could be involved in local signaling because of its ability to interact with cytoskeleton modulators (see before). Indeed, the BMP receptor type II is the most abundantly expressed BMP receptor in mouse spinal motor neurons (Wang et al., 2007a). Alternatively, the effect on motor neuron cells could be dependent on members of the BMP family or other related molecular players. In the ventral horn of normal adult rat spinal cord, motor neurons express BMP-2, BMP-4, noggin, BMPRIA, BMPRIB, and BMPRII (Miyagi et al., 2012). Therefore, Smad-dependent signaling via BMPRIs, local signaling via BMPRII, BMP-2 and BMP-4, exerting individual or combined effects, as well as noggin modulating BMP availability, could be relevant for normal motor neuron physiology such as

embryonic synapse assembly, or during regeneration of neuronal connectivity.

CONCLUDING REMARKS

Besides the strong findings regarding the substantial role of Smad-dependent and independent signaling in invertebrate motor neurons, growing evidence reveals that BMPs also play important roles in the peripheral motor neuronal connectivity in vertebrate species. However, our understanding of the cellular effects of BMPs is still fragmented. Several studies suggest that the disturbance of BMP signaling is likely to be associated with a pathogenic status at the muscle fiber or the motor neurons.

Based in the literature and our studies, we propose a unifying hypothesis where BMP signal has dual effects on skeletal muscle and motor neurons. More related to the embryonic development of the neuromuscular connectivity, BMPs may have important roles in stimulating the amplification of muscle and motor neuron precursors, and simultaneously repressing precocious differentiation (**Figure 2A**). Indeed, there is convergence of neural and myogenic events within a neuromuscular synapse formation during vertebrate development (Mantilla and Sieck, 2008). Moreover, similar events could be recapitulated during axonal regeneration of peripheral nerves after injury. In fact, the recovery of contusion-induced spinal cord injury is accompanied by up-regulation of BMPRII, BMPRIA and Smad phosphorylation in spinal neurons (Matsuura et al., 2008). Likewise, Smad1 mRNA and protein levels are up-regulated in motor neurons after hypoglossal nerve injury in rats (Okuyama et al., 2007).

Afterwards, upon down-regulation of BMP signaling, the maturation of the neuromuscular synapse is reached. At this stage, BMP activation is triggered by the skeletal muscle and becomes restricted to the periphery of the postsynaptic domain, where it is modulated by the motor neuron-derived factor Agrin (**Figure 2B**). Based on these findings, it is tempting to speculate that these BMP signals could be related to axon elongation or presynaptic differentiation, as well as to NMJ formation, maturation or stability.

At present, it is still unclear how BMPs trigger different downstream signaling pathways. However, there are several possibilities including individual or mixed effects of: local concentrations of BMPs and receptors, expression of different BMPs and/or different combination of receptors expression. In this regard, it has been shown that BMP-7 specify the fate of dorsal interneurons at concentrations greater than two orders of magnitude than those required for induction of growth cone collapse (Perron and Dodd, 2011). In addition, BMPRI kinase activity that induces Smad-dependent signaling is required for cell specification but not for changes at the growth cone which require PI3K activity (Perron and Dodd, 2011). Other BMPs, including BMP-9, -4, -2, -5, 6, GDF-5 and -6 also have the ability to induce cell specification but only BMP-9, -4, -2 exhibit axon orientating activities (Perron and Dodd, 2012). Interestingly, immature mouse muscle fibers express BMP-6 whereas it is undetectable in muscle fibers or in post-synaptic domains (Wang et al., 2007a). In turn, BMP-4 is expressed in adult muscle cells and could mediate motor neuron-muscle interactions. Thus, either different levels or different BMPs expressed during the development of the muscle-motor neuron connectivity may generate different signaling events.

Further complexity arises from the expression of different receptors. For instance, it has been demonstrated a strong impact of BMP receptor utilization in conditions of BMPRII deletion (Yu et al., 2005). BMPRII expressing smooth muscle cells transduce BMP signals through BMPRII regardless of ActRIIa expression, indicating that the expression of BMPRII restrains BMP signaling via ActRIIa. BMPRII is the principal type II receptor for different classes of BMP ligands, transducing BMP-4/-2 signals through BMPRIa and also those from BMP-7/-6/-5 through either ActRIa or BMPRIa. In the absence of BMPRII, ActRIIa is the principal type II receptor for both classes of BMP ligands, transducing BMP-4/-2 signals through either ActRIa or BMPRIa and increasing BMP-7/-6/-5 signals potently through ActRIa (Yu et al., 2005). Therefore, changes in the global expression levels of BMPRII, or its local subcellular accumulation, could profoundly affect the gain or decrease of signaling activation by different BMP ligands.

Future work will provide a deeper understanding of the extracellular cues and cellular mechanisms mediated by BMPs underlying the interplay between muscle fibers and motor neurons in vertebrates. Experimental designs to establish the precise role of BMPs in muscle-motoneuronal communication must consider the evaluation of distinct cellular responses (Smad-dependent and independent), the spatial pattern of activation of specific signaling pathways in a time and concentration dependent manner, as well as if BMPs acts on cells at a distance from its source, which will imply to face these studies with morphogens criteria.

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Frizzled-9 impairs acetylcholine receptor clustering in skeletal muscle cells

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Cumulative evidence indicates that Wnt pathways play crucial and diverse roles to assemble the neuromuscular junction (NMJ), a peripheral synapse characterized by the clustering of acetylcholine receptors (AChR) on postsynaptic densities. The molecular determinants of Wnt effects at the NMJ are still to be fully elucidated. We report here that the Wnt receptor Frizzled-9 (Fzd9) is expressed in developing skeletal muscles during NMJ synaptogenesis. In cultured myotubes, gain- and loss-of-function experiments revealed that Fzd9-mediated signaling impairs the AChR-clustering activity of agrin, an organizer of postsynaptic differentiation. Overexpression of Fzd9 induced the cytosolic accumulation of β-catenin, a key regulator of Wnt signaling. Consistently, Fzd9 and β-catenin localize in the postsynaptic domain of embryonic NMJs *in vivo*. Our findings represent the first evidence pointing to a crucial role of a Fzd-mediated, β-catenin-dependent signaling on the assembly of the vertebrate NMJ.

Keywords: Frizzled receptors, Wnt proteins, neuromuscular junction, acetylcholine receptor, postsynaptic, skeletal muscle

INTRODUCTION

At the vertebrate NMJ, extracellular matrix and signaling proteins, secreted either by the nerve or the muscle, play stimulatory, and inhibitory roles to orchestrate the assembly of functional synapses (Sanes and Lichtman, 2001; Wu et al., 2010). An early hallmark of postsynaptic differentiation at the NMJ is the aggregation of several postsynaptic proteins, including the acetylcholine receptors (AChRs), in discrete domains of the sarcolemma (Sanes and Lichtman, 2001). Before innervation, a pre-pattern of AChR clusters forms on the muscle surface to guide the subsequent positioning of motor axons for NMJ assembly (Lin et al., 2001; Vock et al., 2008; Jing et al., 2009). Upon nerve-muscle contact, most pre-patterned AChR clusters are disassembled by the inhibitory effect of acetylcholine (ACh) (Lin et al., 2005; Misgeld et al., 2005; An et al., 2010), except the ones located in close apposition with the motor axon which are stabilized by the motor neuron-derived proteoglycan agrin (Lin et al., 2005; Misgeld et al., 2005). Agrin signals through the muscle-specific tyrosine kinase receptor MuSK (Valenzuela et al., 1995; Dechiara et al., 1996; Glass et al., 1996), by forming a membrane complex with the low density lipoprotein receptor-related protein 4 (Lrp4) (Weatherbee et al., 2006; Kim et al., 2008; Zhang et al., 2008). Agrin induces AChR clustering in cultured myotubes, but its *in vivo* role is likely to prevent the inhibitory role of the neurotransmitter, thus maintaining postsynaptic specializations (Misgeld et al., 2005). Therefore, the embryonic assembly of the NMJ relies on signals that play positive and inhibitory effects on AChR clustering at the postsynaptic membrane.

In the last years, strong evidence has proven a crucial role for Wnt signaling on different features related to the connectivity and function of the nervous system. Wnt ligands -of which 19 family members are found in vertebrates- signal through their cognate seven-pass transmembrane G-protein coupled Frizzled (Fzd) receptors (10 family members found in vertebrates) (Gordon and Nusse, 2006) to activate at least three different signaling pathways. In the "Wnt canonical" pathway, the glycogen synthase kinase-3β (GSK-3β) is inhibited resulting in the intracellular accumulation of the key effector β-catenin, which then translocates to the nucleus where, along with Tcf/Lef1 transcription factors, activates the expression of specific Wnt target genes (Gordon and Nusse, 2006; Kim et al., 2009). Importantly, β-catenin also plays crucial roles in cell membrane protein complexes for cell-cell adhesion (Brembeck et al., 2006). Wnts also trigger two "non-canonical" pathways: a "Wnt calcium" pathway that regulates cell fate and cell movement by increasing intracellular Ca^{2+} levels (Kuhl et al., 2000), and the "planar cell polarity" pathway which acts locally to modify the cytoskeleton through the small Rac and Rho GTPases (McEwen and Peifer, 2000). Due to the high heterogeneity of Wnt ligands, receptors and pathways, specific responses to activation of different Wnt cascades depend on specific effectors for each cell type and biological context.

Wnt pathways play pro- and anti-synaptogenic effects to regulate the formation or distribution of the vertebrate and invertebrate NMJ (Korkut and Budnik, 2009; Wu et al., 2010; Henriquez et al., 2011; Henriquez and Salinas, 2012). Early on in the formation of the vertebrate NMJ, Wnt ligands secreted by muscle

cells or the surrounding tissues have been shown to interact with MuSK to induce the aneural pre-patterning of AChR clusters on the muscle surface (Jing et al., 2009; Gordon et al., 2012). Upon NMJ synaptogenesis, Wnt ligands can play pro and inhibitory roles on postsynaptic differentiation. On the one hand, the Wnt3 ligand, which is expressed by motor neurons at the time of NMJ formation (Krylova et al., 2002), collaborates with agrin to induce AChRs clustering via a small GTPase-dependent, non-canonical Wnt signaling (Henriquez et al., 2008). On the other hand, the highly identical muscle-derived Wnt3a ligand impairs agrin-induced AChR clustering and disassemble pre-formed aggregates via a β-catenin-dependent, but TCF-independent, pathway (Wang and Luo, 2008; Wang et al., 2008). Thus, we have hypothesized that activation of different Wnt pathways could control opposite, but complementary roles on postsynaptic differentiation at nascent NMJs (Henriquez and Salinas, 2012).

In order to gain insights into the possible mechanisms employed by muscle cells to convert Wnt signals into positive or negative inputs, here we evaluated whether Fzd receptors could mediate these differential Wnt effects. Based on previous findings showing that Fzd9 expression is modulated by innervation in skeletal muscle (Magnusson et al., 2005) and due to its role in the formation of neuronal connectivity (Zhao and Pleasure, 2004; Zhao et al., 2005), we focused on the expression pattern and possible function of this Wnt receptor on postsynaptic differentiation at the NMJ.

EXPERIMENTAL PROCEDURES

ANIMALS

Swiss Webster mice were obtained from Animal Facilities at the Faculty of Biological Sciences, Universidad de Concepción. Experiments were conducted following the guidelines outlined in the Biosafety and Bioethics Manual of the National Commission of Scientific and Technological Research (CONICYT, Chilean Government). The Bioethics Committee of Universidad de Concepción (Concepción, Chile) approved all experimental procedures carried out during this study.

REVERSE TRANSCRIPTION-PCR

Total RNA was extracted from hemidiaphragms of E14.5, E19.5, and 6-week old mice, as well as from differentiated myotubes from the C2C12 cell line, using Trizol reagent (Invitrogen, Carlsbad, CA, USA), following the indications of the manufacturer. For RT-PCR, 1 μg of RNA was pre-treated with DNase I (Fermentas, Ontario, Canada) and further incubated in a buffer containing 5 μM oligo dT primer, reverse transcription buffer (0.5 M Tris-HCl, pH 8.3, 0.75 M KCl, 0.03 M $MgCl_2$), 20U RNase inhibitor (NEB, Ipswich, MA, USA) and 0.5 mM dNTPs at 37°C for 5 min. A volume to reach 160 U Stratascript reverse transcriptase (Stratagene, La Jolla, CA, USA) was added, and the mix was further incubated at 42°C for 1 h. Parallel reactions were performed in the absence of reverse transcriptase to control for the presence of genomic DNA. For amplification, a RT aliquot in 75 mM Tris buffer, pH 8.8, 20 mM (NH_4) SO_4, 0.01% Tween 20, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM primers, and 0.35 U Taq polymerase (Fermentas) was subjected to 30 cycles of PCR. The annealing temperatures were set as indicated in **Table 1**. Primers were designed to amplify specific, non-conserved regions of Fzd receptors (**Table 1**). A 233 bp fragment of mouse GAPDH mRNA was also amplified as housekeeping control.

Table 1 | List of primers used to detect the mRNA expression by RT-PCR of Frizzled 1–10 and the housekeeping control GAPDH.

Gene		Sequence	Tm (°C)	Ta (°C)	Product size (bp)
Fz1	S	CTCTTCCGCATCCGCACC	59	55	336
	AS	GCCGGACCAGATCCGGAA	60		
Fz2	S	CCCGGGCGGCCCTGGCGG	74	45	271
	AS	GTCCACTAAATAGGTGGT	48		
Fz3	S	CCACTCAAGGGACATCCA	54	50	407
	AS	AGCCAGCCATGCGAAGGC	62		
Fz4	S	TGGCTTIGTGGIGGCTCC	58	54	474
	AS	AGGAACGAGGAAGCCGGC	60		
Fz5	S	GCGCACCGGCCAAGTGCC	66	58	270
	AS	CGGCTGCAAGCGACGCTG	63		
Fz6	S	GGCAATCGCTGACCATGA	56	51	308
	AS	TGGCGGCCTGTGAAGTGC	62		
Fz7	S	CGGTGCCGGCCACCATCG	65	56	403
	AS	GTGGAGGGGGCAGGTAGC	61		
Fz8	S	CCGTGCTCTACACGGTGC	59	58	276
	AS	CTGCAGCGCCCTTGCTGG	63		
Fz9	5	GCTGGAGAAGCTGATGGT	55	50	437
	AS	CCAGAGAGGGGTCTGTCT	56		
Fz10	S	TGTGCCGGCCACCTGTGTGATTGC	66	61	246
	AS	ACGTOTTOCCAGGACTGCAGGGTC	66		
GAPDH	S	GGAGCCAAACGGGTCATCATCTC	60	55	233
	AS	GAGGGGCCATCCACAGTCTTCT	61		

The melting (Tm) and annealing (Ta) temperatures are indicated as well as the sequences and the size expected for the PCR products.

PLASMIDS

The DNA sequence coding for mouse Fzd9 was amplified from a previously cloned vector (a gentle gift of Dr. Robert Winn, University of Colorado, CO, USA). The HA tag was fused to the C-terminal end of Fzd9 by overlapping PCR, and the resulting sequence (Fzd9HA) was subsequently cloned between the XhoI and XbaI sites of the pCS2+ expression vector. A similar strategy was followed to clone mouse Wnt2, in which the full-length coding sequence was amplified from total RNA obtained from adult heart. To inhibit Fzd9 expression, we identified a potential RNA interfering sequence for mouse Fzd9 through the Dharmacon algorithm (www.dharmacon.com). A short-hairpin DNA sequence specific for Fzd9 (shFzd9) was subcloned between the BsrGI and NheI sites of the FUXH1Off-EGFP plasmid, which drives the transcription of short sequences under the H1 promoter along with expressing EGFP under the CMV promoter.

WESTERN BLOT

Mixtures of skeletal muscles from mouse hind limbs at different developmental stages (E14.5 to P0) were disrupted using a vibra cell sonicator (Sonics, Newtown, CT, USA) with five pulses at 70% power in a solution containing 0.3 M sucrose and a protease inhibitor cocktail (80 μM aprotinin, 1.5 mM pepstatin A,

2 mM leupeptin, 104 mM AEBSF, 4 mM Bestatin, 1.4 mM E-64) (Sigma, St. Louis, MO, USA). Supernatants containing the total protein extracts were obtained after centrifuging at 8000xg for 10 min at 4°C. In the case of transfected C2C12 myotubes and HEK293 cells, the samples were rinsed with PBS, harvested and homogenized in the above mentioned solution, and centrifuged for 10 min at 13,000xg and 4°C. The supernatant corresponds to the pool of cytosolic soluble proteins. The resulting pellet was resuspended in a detergent-containing buffer (0.05 M Tris-HCl, pH 7.4, 0.5% Triton X-100, 0.15 M NaCl) and centrifuged under the same conditions to obtain a membrane-enriched protein fraction. For immunoblotting, 20 μg of proteins were loaded in each lane and fractionated by PAGE-SDS, transferred to nitrocellulose membranes, and incubated with a highly specific goat anti mouse Fzd9 antibody, which does not cross-react with other related Fzd receptors (R and D Systems, Minneapolis, MN, USA). We also used goat anti human β-actin, mouse anti human β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti chicken α-tubulin (Sigma) antibodies. Primary antibodies were incubated in 5% non-fat milk blocking buffer for 12–18 h at 4°C, whereas peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA, USA) were incubated for 2 h at room temperature. The signal was developed by enhanced chemiluminiscence using the ECL Western blotting analysis system (Perkin Elmer, Waltham, MA, USA). The exposure time was adjusted to reveal the less abundant band (i.e., E19.5) in order to detect possible differences with the other samples in the gel. The intensities of the resulting bands were calculated with Image J software and correspond to the average ± s.e.m. of three independent experiments.

IMMUNOCHEMISTRY

The diaphragm muscle of E17.5 and P0 mice was removed and fixed with 4% paraformaldehyde for 1 h at 4°C and subsequently permeabilized with 0.5% Triton X-100, incubated with 0.15 M glycine, pH 7.4, for 15 min and then with 10 mg/ml $NaBH_4$ for 5 min. Muscles were blocked in 0.5% Triton X-100, 0.025% BSA, 0.2% horse serum for 12–16 h at 4°C. Primary antibodies were incubated in blocking solution for 16 h at 4°C and were then washed in PBS containing 0.1% Triton X-100. Transfected myotubes were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min and subsequently incubated with primary antibodies diluted in blocking solution (1% BSA in Tris phosphate buffer) for 12–15 h at 4°C. Antibodies used were goat anti mouse Fzd9 (R and D Systems) as well as mouse or rabbit anti human β-catenin (Santa Cruz Biotechnology). Corresponding Alexa488 and Alexa546-conjugated secondary IgGs (Invitrogen) were incubated for 2 h at room temperature, along with 2 ng/μL Alexa546- or Alexa647-conjugated αBTX (Invitrogen) to reveal AChR clusters. Nuclei were counterstained with ToPro-3 or DAPI (Invitrogen). Images were acquired with a laser confocal Nikon D-Eclipse C1 or a Zeiss LSM700 microscope at the CMA Bio-Bio facility at the Universidad de Concepción. The average fluorescence intensity of β-catenin in transfected myotubes was measured using Metamorph software. Data are expressed as the average of the relative value ± s.e.m. of three independent experiments.

C2C12 CELLS CULTURE AND TRANSFECTION

C2C12 cells were grown on glass coverslips in DMEM medium containing 10% fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin. After 24 h, myoblasts were transfected with a lipofectamine-PLUS mixture (Invitrogen) according to the instructions of the manufacturer. The DNA/lipofectamine/PLUS ratio in the mixture was 0.5 μg/1.5 μl/1 μL with 0.4 μg plasmid DNA per well. Cells were incubated with the DNA mixture and lipofectamine-PLUS in serum-free medium for 6 h before being switched to the fusion medium (DMEM containing 2% horse serum). After 5 days, fused myotubes were treated with 200 pM neural agrin (R and D Systems) for 12–18 h at 37°C. Cells were subsequently washed and fixed in 4% paraformaldehyde for 20 min at room temperature. Fzd9-transfected cells were stained with a goat anti-Fzd9 (Invitrogen) or a rat anti-HA antibody (Roche Applied Science, Mannheim, Germany). AChR clusters were stained with 2 ng/μL Alexa546-conjugated αBTX (Invitrogen), along with an Alexa488-conjugated secondary antibody (Invitrogen) for 2 h at room temperature.

IMAGE ANALYSES

Fluorescent images were acquired with a laser confocal Nikon D-Eclipse C1 microscope in *z* series of 2 μm intervals to cover the entire depth of myotubes. Stack and 2D deconvoluted images were obtained using Metamorph software. The surface of transfected myotubes (seen in the green channel) was manually traced and their area was measured using Metamorph. The area of each AChR cluster larger than 4.5 μm^2 present on transfected myotubes was automatically determined using the same software. Data are expressed as total area of AChR clusters (μm^2) per mm^2 of transfected myotube area, total average of AChR clusters ($\mu m^2/mm^2$ of myotube) and average area of AChR clusters (μm^2).

LUCIFERASE ASSAYS

For luciferase assays, the amounts of plasmid DNAs used for trasfections of HEK293 cells were: 20 ng of the Wnt reporter Top Flash, 2 ng of the control Renilla reporter, 150 ng Wnt2, 75 ng Fzd9, and 150 ng shFzd9 per well of a 24-well plate. When required, empty pCS2+ or FUXH1Off-EGFP plasmids were added in a sufficient amount to reach 0.4 μg of total plasmid DNA per well. After 48 h, cells were lysed and the activity of the Topflash and Renilla reporter genes was measured using the Dual Luciferase Report Assay System (Promega, Madison, WI, USA) according to the indications of the manufacturer. Data are expressed as the Topflash: Renilla ratio, relative to the basal background activity obtained from cells transfected with control plasmids.

STATISTICAL ANALYSES

Plots correspond to the average ± s.e.m. of three independent experiments performed by triplicate, except when indicated. Data were statistically analyzed using non-parametric *t*-test or ANOVA, as indicated in the figure legends.

RESULTS

As a first hint to examine the potential contribution of Fzd-mediated signaling on postsynaptic development at the NMJ, we

analyzed the expression pattern of the different Fzd receptors in muscle cells. We obtained total RNA extracts from 6-week old mice hemidiaphragms and from cultured myotubes of the C2C12 muscle cell line to perform RT-PCR analyses. We used specific primers (**Table 1**) to amplify internal fragments of the ten mice Fzd receptors. As shown in **Figure 1**, fragments of the expected molecular sizes were amplified from mice muscles for the ten Fzd mRNAs. A similar result was obtained from C2C12 myotubes, except for Fzd8 and Fzd10 transcripts, which were not detected. A 233 bp fragment of mouse GAPDH mRNA was amplified as housekeeping control. Negative control experiments performed in the absence of reverse transcriptase were not amplified.

Considering that most Fzd receptors are likely expressed by skeletal muscle cells, we focused on Fzd9 based on previous findings suggesting a relevant role for this Wnt receptor on nerve-muscle communication in 6-week old mice (Magnusson et al., 2005), as well as on the formation and function of central synapses (Zhao and Pleasure, 2004; Zhao et al., 2005). In order to assess a potential role for Fzd9 on the formation of the NMJ, we first analyzed the expression pattern of Fzd9 in embryonic skeletal muscle tissue (**Figure 2**). RT-PCR analyses show that the fragment amplified with specific primers for Fzd9 was diminished in E19.5 as compared to E14.5 (**Figure 2A**). Consistently, immunoblots of developing mouse skeletal muscles showed relatively high expression of Fzd9 at early stages of NMJ assembly (E14.5) and gradually decreases as development proceeds, being more than ∼4-fold less expressed in E19.5 (**Figure 2B**). Thus, the temporal expression of Fzd9 in muscle supports a potential role for this Wnt receptor on neuromuscular synaptogenesis. To analyze the distribution of Fzd9 at the developing NMJ, we performed immunohistochemical studies in whole-mounted hemidiaphragms obtained from E17.5 and P0 mice. Our findings show that Fzd9 staining was intense in discrete domains of the sarcolemma at both developmental stages (**Figure 2C**). To analyze whether the Fzd9-enriched regions corresponded to synaptic domains, we double stained with fluorescently-conjugated α-bungarotoxin (αBTX), which binds with high specificity to AChR aggregates (Sanes and Lichtman, 2001). **Figure 2C** shows that postsynaptic muscle domains contain high Fzd9 labeling, which distribute in a punctate pattern. Indeed, magnified pictures (insets in **Figure 2C**) show that this co-distribution pattern is rather incomplete, as αBTX-positive regions were not labeled with anti Fzd9 antibodies. Overall, the temporal expression and distribution of Fzd9 support a possible role for this Wnt receptor during NMJ assembly.

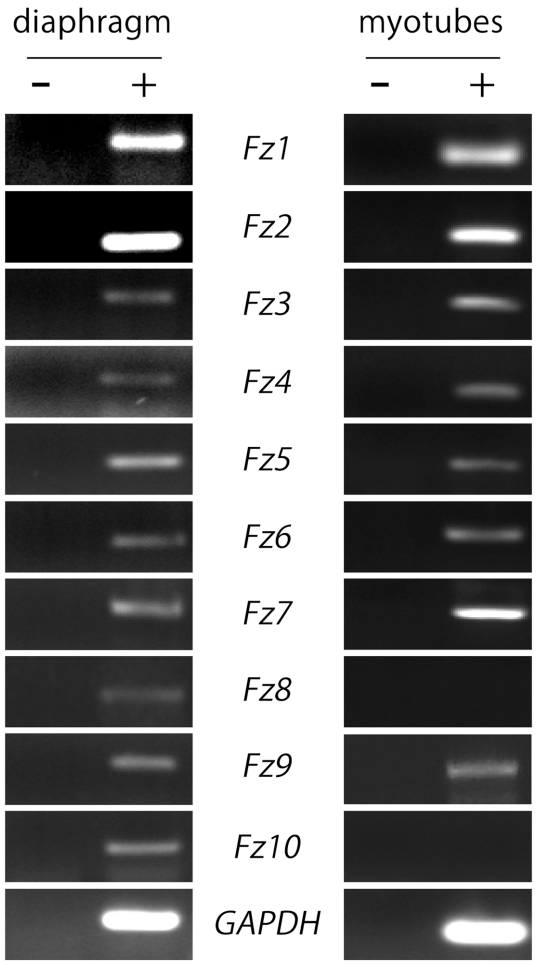

FIGURE 1 | Most *Fzd* receptors are expressed in skeletal muscle cells. Total RNAs were extracted from 6-week old mouse diaphragm and cultured myotubes and further subjected to RT-PCR to detect the mRNA expression of all ten mouse *Fzd* receptors (+). As a negative control, samples were processed in the absence of reverse transcriptase (−). *Fzd* receptors 1 to 10 are expressed in the mouse diaphragm (*left panel*). In cultured C2C12 myotubes, almost all Fzd receptors are expressed at the mRNA level, except for Fzd8 and Fzd10, which were not amplified by RT-PCR (*right panel*). GAPDH expression was used as a loading control gene. Gels are representative of at least three experiments performed by triplicate.

The possible function of Fzd9 on postsynaptic assembly at the NMJ was examined in cultures of the C2C12 muscle cell line. The expression of Fzd9 in myoblasts (day 0 of differentiation) is very low and becomes much higher as differentiation proceeds at day 3 and day 6 (**Figure 3A**). The endogenous Fzd9 protein is localized to the plasma membrane of myotubes (arrows in **Figure 3B**), where it exhibits a similar pattern as the glucose transporter Glut1. In order to perform gain-of-function experiments, we cloned the coding region of mouse Fzd9, fused to the HA tag, into the pCS2+ expression vector. In order to validate the resulting Fzd9HA plasmid, we performed control experiments. First, C2C12 myoblasts were transfected with Fzd9HA, or with the control empty plasmid, and differentiated for 5 days. We then performed a sequential subcellular fractionation to separate cytosolic- and membrane-enriched fractions. Control Western blot experiments show that α-tubulin is present only in the cytosolic fraction, whereas the sodium/vitamin C co-transporter SVCT2 (Sandoval et al., 2013) was specifically detected in the membrane fraction, thus demonstrating the efficiency of the fractionation protocol (**Figure 3C**, *left panel*). Following this approach, we observed that most Fzd9 is extracted in a membrane-enriched protein fraction after differential separation of myotube proteins, while β-actin is concentrated in the cytosolic fraction (**Figure 3C**, *right panel*). Then, as we obtained relatively low transfection efficiency in mouse muscle-derived C2C12 cells (∼10–15%), the ability of Fzd9 to transduce Wnt

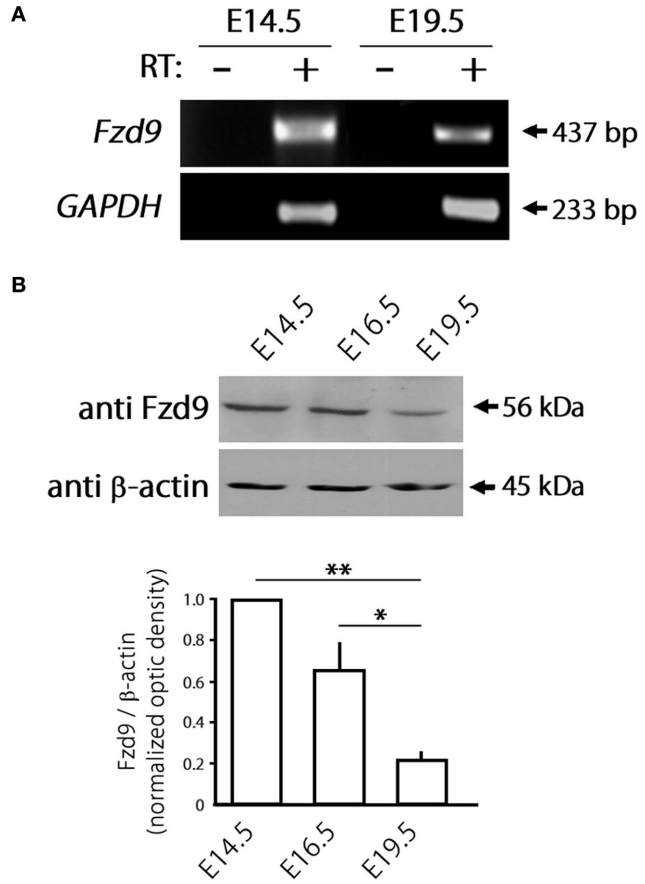

FIGURE 2 | Fzd9 is dynamically expressed in skeletal muscle tissue. **(A)** Total RNAs were extracted from E14.5 and E19.5 mouse hind limb muscles and further subjected to RT-PCR to detect the mRNA expression of *Fzd9* (+). As a negative control, samples were processed in the absence of reverse transcriptase (–). GAPDH expression was used as a loading control gene. The gel is representative of two experiments performed by triplicate. **(B)** Proteins from mouse hind limb skeletal muscles obtained at different developmental stages (E14.5, E16.5, and E19.5) were fractionated by SDS-PAGE and immunoblotted for Fzd9 and β-actin (*upper panel*). Fzd9 is expressed at the protein level in all the analyzed developmental stages since the expected 56kDa band is detected. A representative gel shows that Fzd9 band intensity displays a progressive decrease from E14.5 to E19.5. Data represent the mean ± *SD* of Fzd9/β-actin ratio from three experiments, normalized to E14.5 (*lower panel*) (*$p < 0.05$, **$p < 0.01$, ANOVA using Bonferroni's *post-hoc* analysis). **(C)** Whole-mounted diaphragms from E17.5 and P0 were stained with anti Fzd9 antibody (green) together with αBTX to reveal the postsynaptic densities (blue). Fzd9 is abundant in the synaptic domain of embryonic NMJs, where it displays a punctate expression pattern. The insets (*lower panels*) show that some αBTX-positive regions were not labeled with anti Fzd9 antibodies. Pictures are representative of at least three experiments performed by triplicate.

signaling was assayed in the human kidney-derived HEK293 cells (**Figure 4B**). In these highly transfectable (>90%) cells, Fzd9HA was able to mediate the Wnt2-dependent activation of a Wnt/β-catenin reporter gene, as it had been previously demonstrated for Fzd9 (Karasawa et al., 2002). Together, these data demonstrate the efficiency of the Fzd9HA tool to induce Fzd9 expression and function.

We then analyzed transfected C2C12 myotubes to determine if Fzd9 could modulate AChR clustering, an early hallmark of post-synaptic differentiation at the NMJ (Sanes and Lichtman, 2001). With this aim, control GFP and Fzd9HA-transfected myotubes were treated with neural agrin to induce AChR clusters, which were subsequently stained with αBTX, and quantified (Henriquez et al., 2008). Myotubes expressing Fzd9HA displayed a marked decrease of agrin-induced AChR aggregation, expressed here as total area of AChR clusters ($25.36 \times 10^3 \pm 1.3 \times 10^3 \mu m^2$ per mm^2 in control GFP-transfected cells vs. $15.00 \times 10^3 \pm 0.9 \times 10^3 \mu m^2$ per mm^2 in Fzd9-transfected myotubes, $p < 0.001$), and a significant reduction in cluster average size ($16.6 \pm 0.6 \mu m^2$ in control GFP-transfected cells vs. $14.3 \pm 0.5 \mu m^2$ in Fzd9-expressing myotubes) (**Figure 3D**). These data reveal that the overexpression of Fzd9 impairs the ability of muscle cells to aggregate AChRs.

In order to analyze the contribution of endogenous Fzd9 to agrin-mediated AChR clustering, we generated the shFzd9 plasmid, which drives the transcription of a Fzd9 RNA interference sequence under the control of the H1 promoter, plus EGFP under the control of the CMV promoter to check for transfection efficiency. Control experiments in co-transfected HEK293 cells showed that shFzd9 transfection was able to abolish the

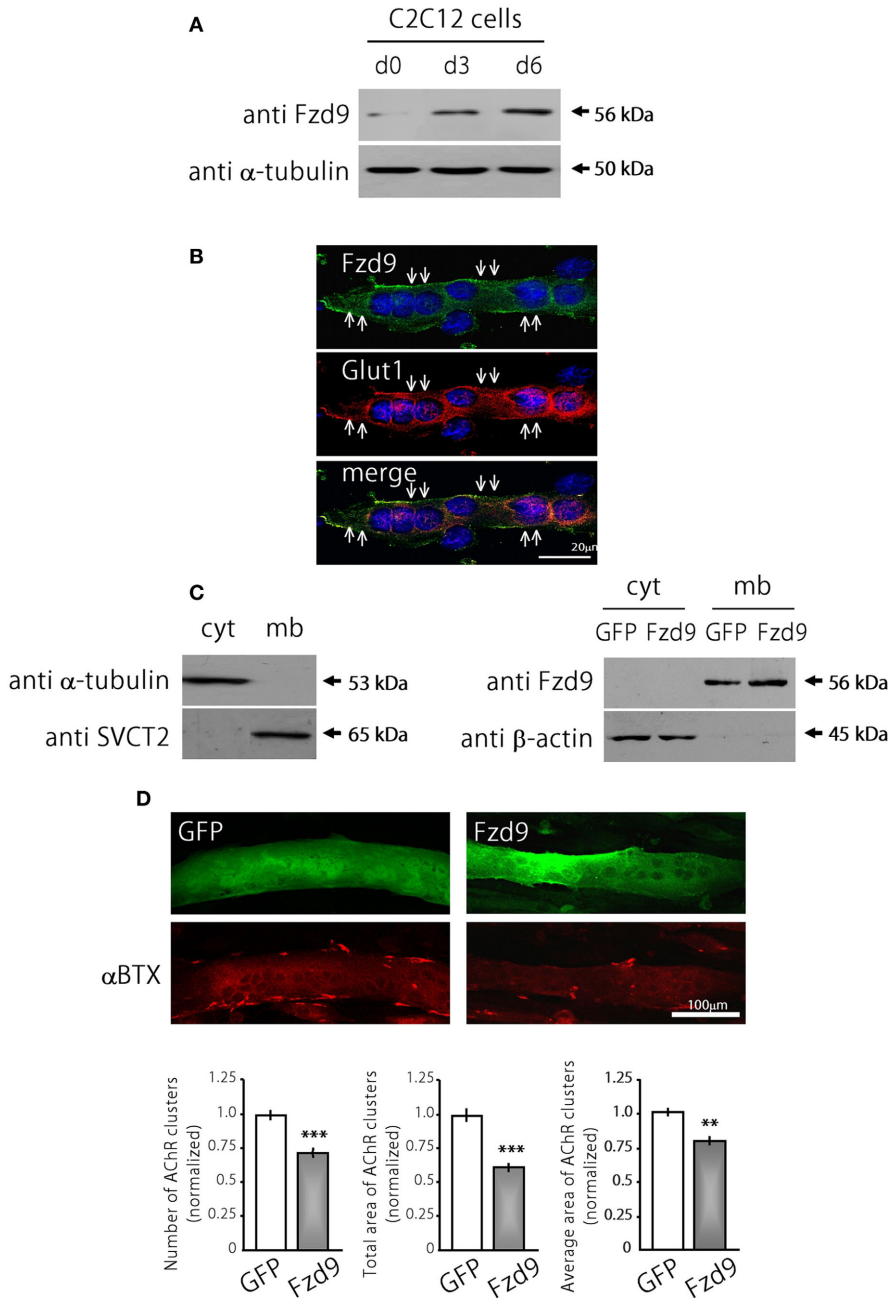

FIGURE 3 | Fzd9 impairs agrin-dependent AChR clustering in myotubes. **(A)** Fzd9 is expressed in the muscle cell line C2C12 throughout differentiation. C2C12 cells were cultured *in vitro* and differentiated for 0, 3, or 6 days (d0-d6). Total proteins were subjected to Western blot analyses. An expected 56 kDa band is gradually increased during C2C12 cells differentiation. α-tubulin expression was used as a loading control. **(B)** C2C12 myotubes differentiated for 6 days were analyzed by immunocytochemistry to detect Fzd9. Fzd9 is localized to the plasma membrane of the myotubes (green, *upper panel*), similar to Glut1, which was used as a marker of plasma membrane (red, *middle panel*). The merge image (*lower panel*) reveals the co-localization of Fzd9 and Glut1. **(C)** Differentiated C2C12 myotubes were subjected to a sequential fractionation procedure to isolate samples enriched in cytoplasm (cyt) or plasma membrane (mb) proteins. Western blot analyzes showed that α-tubulin is specifically detected in cytoplasmic fractions, whereas the vitamin C transporter SVCT2 was only present in membrane-enriched protein fractions (*left panel*). C2C12 myoblasts were transfected either with GFP or Fzd9 and differentiated. Sequential protein lysates were analyzed by Western blot. Both endogenous Fzd9 (GFP-transfected cells) or overexpressed Fzd9 (Fzd9-transfected cells) were found predominantly in the plasma membrane and were absent in the cytoplasm. As a loading control, β-actin is found only in the cytoplasm-enriched fraction (*right panel*). **(D)** Myoblasts transfected with plasmids coding for GFP (control) or Fzd9 were differentiated into myotubes and subsequently incubated with 200 pM neural agrin. αBTX staining allows the visualization of the AChRs (red). Automatized quantification of aggregates shows that Fzd9 overexpression induces a decrease in the number of AChR clusters per myotube, as well as a reduction in the total area and average size of AChR clusters, compared to controls. Data represent the mean ± s.e.m. ($n = 3$ performed by triplicate; normalized to GFP-transfected myotubes). ($^{**}p < 0.01$, $^{***}p < 0.001$ compared to GFP controls, *t*-test).

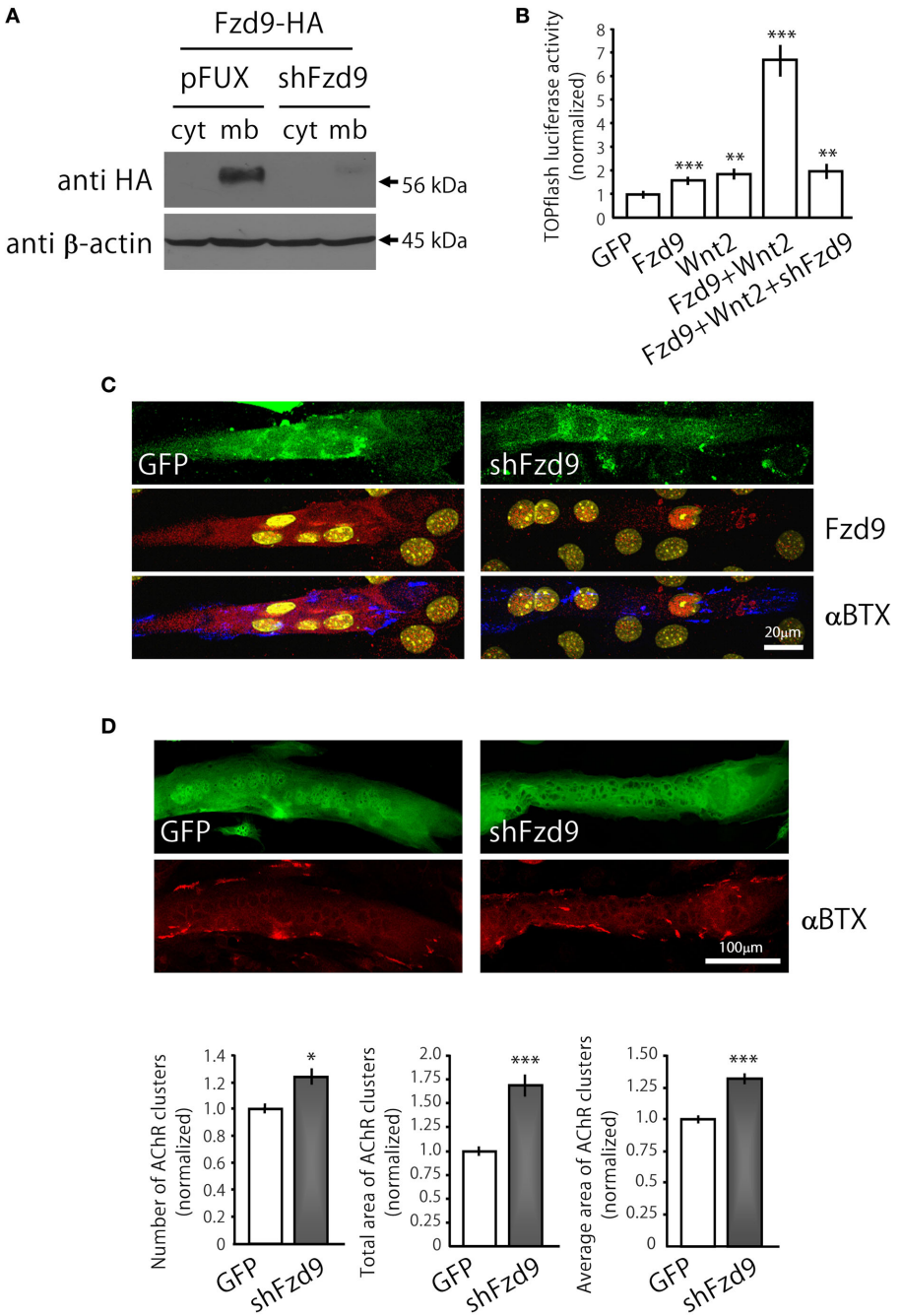

FIGURE 4 | Down-regulation of Fzd9 increases agrin-dependent AChR clustering in myotubes. (A,B) The efficiency of shFzd9 was tested by its ability to impair overexpression/function of the Fzd9 construct. **(B)** HEK293 cells were transfected with Fzd9HA together with a control shRNA (pFUX) or shFzd9, followed by protein homogenization and Western blot. Whereas a 56 kDa band corresponding to Fzd9 is detected in the membrane-enriched fraction of the control condition, Fzd9 expression is drastically silenced in cells transfected with shFzd9. **(B)** The efficiency of the shFzd9 to affect the functionality of Fzd9HA was assessed by co-transfecting Fzd9HA and Wnt2 in the presence or absence of the shFzd9 plasmid in HEK293 cells. Activation of the TOPflash luciferase reporter gene was used as a readout of activation of the canonical Wnt pathway. These experiments were performed at least three times by triplicate (**$p < 0.01$, ***$p < 0.001$, *t*-test). **(C)** Myoblasts were transfected either with GFP or shFzd9 and differentiated for 5 days. Myotubes were treated with neural agrin and further stained with an anti-Fzd9 antibody (red), along with αBTX (blue) to detect AChR clusters and DAPI (yellow) to stain nuclei. shFz9-transfected myotubes display silenced Fzd9 expression and an apparent increase in the number of AChR clusters compared to GFP-expressing myotubes. **(D)** Myoblasts transfected either with GFP or shFzd9 and grown for 5 days were treated with neural agrin and further stained with αBTX to detect AChR clusters (red). Myotubes expressing the GFP protein present in the shFzd9 plasmid show a significant increase in the number of AChR clusters, an increase in the total area of AChR clusters, as well as on the average size of AChR clusters, when compared to control myotubes that only express GFP. Data represent the mean ± s.e.m. ($n = 3$ performed by triplicate; normalized to GFP-transfected myotubes). (*$p < 0.05$, ***$p < 0.001$ compared to GFP controls, *t*-test).

expression of Fzd9HA (**Figure 4A**), and inhibited the ability of Fzd9HA to mediate the Wnt2-dependent activation of Wnt/β-catenin signaling (**Figure 4B**). In C2C12 myotubes, qualitative analyses showed that myotubes transfected with shFzd9 display reduced Fzd9 staining and increased number of AChR clusters as compared to control GFP-expressing myotubes (**Figure 4C**), suggesting that postsynaptic differentiation is up-regulated upon Fzd9 silencing. In order to quantify these results, AChR clusters were analyzed in myotubes bearing green fluorescence (**Figure 4D**). Quantification of the data shows that Fzd9 silencing by shFzd9 transfection resulted in an increase in the number and size of AChR clusters than those on the surface of control myotubes transfected with the GFP-expressing plasmid (**Figure 4D**). The total number of AChRs per myotube was increased by ∼23% in myotubes with silenced expression of Fzd9, whereas the total area of AChR clusters was significantly increased by ∼68%, compared to the control group. The size of AChR clusters was incremented by ∼32% in myotubes transfected with shFzd9. Therefore, Fzd9 inhibition enhanced the ability of agrin to induce AChR clustering. Altogether, these *in vitro* data support a specific role for Fzd9 on postsynaptic differentiation at the vertebrate NMJ.

Based on previous findings indicating that AChRs dissagregate through a β-catenin-dependent pathway (Wang and Luo, 2008; Wang et al., 2008), we next analyzed whether Fzd9 overexpression in myotubes resulted in β-catenin accumulation (**Figure 5**). Indeed, myotubes transfected with Fzd9 showed a significant 2-fold increase in cytosolic β-catenin, evaluated by

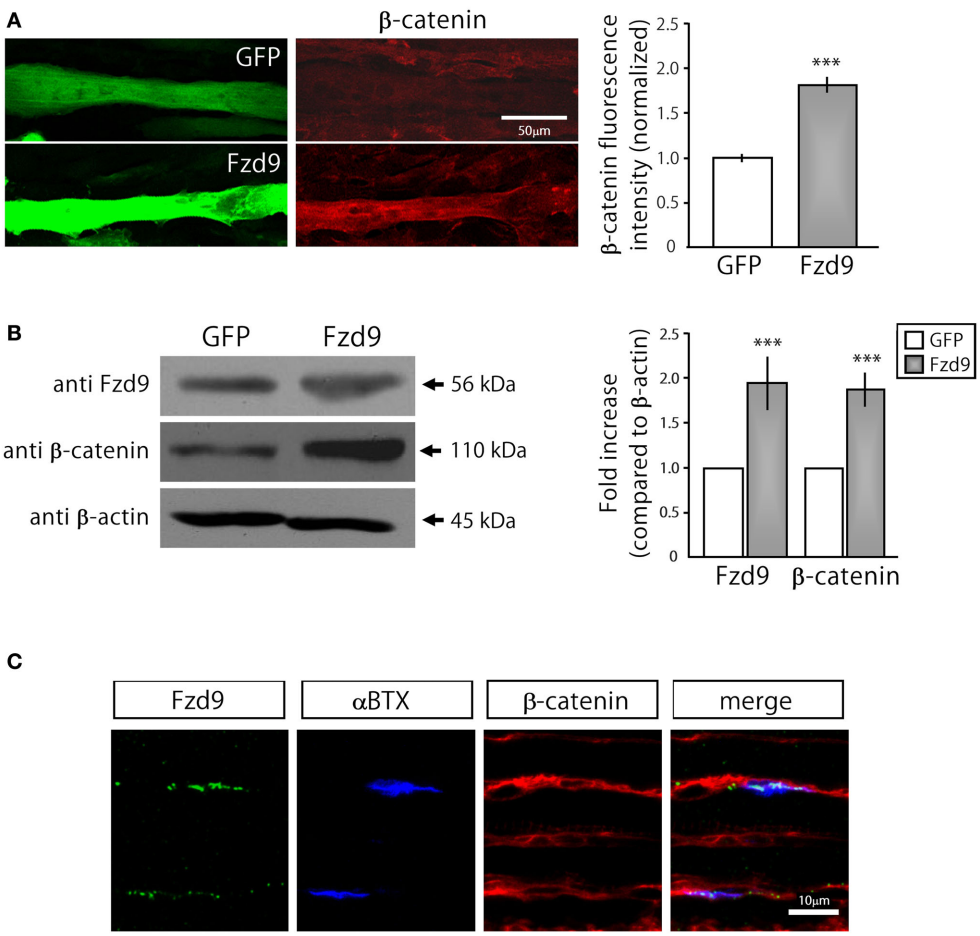

FIGURE 5 | Fzd9 enhances β-catenin accumulation in myotubes. (A) GFP and Fzd9-transfected myotubes were immunostained with an anti β-catenin antibody (red). Fluorescence intensity of transfected myotubes was quantified using Metamorph. Quantification of the data (*right panel*) shows that the expression of Fzd9 induces a significant ∼2-fold accumulation of β-catenin in the sarcoplasma, compared to control GFP-expressing myotubes. **(B)** Total protein samples from GFP- and Fzd9-transfected myotubes were separated by SDS-PAGE and immunoblotted with Fzd9 and β-catenin antibodies. Quantification of the Fzd9 or β-catenin against β-actin band intensity ratios shows that Fzd9-overexpressing myotubes display a ∼2-fold increase in β-catenin cytosolic levels, which is equivalent to the ∼2-fold increase observed for Fzd9 levels, compared to control myotubes (*right panel*). Data represent the mean ± s.e.m. ($n = 3$ performed by triplicate; normalized to control GFP cells; $^{***}p < 0.001$, *t*-test, compared to the GFP group). **(C)** Whole-mounted diaphragms of E17.5 mice were immunostained to detect Fzd9 (green) and β-catenin (red). AChR aggregates were stained with αBTX (blue). β-catenin is associated to the sarcolemma of embryonic muscle fibers, including the membrane domains where AChR clusters and Fzd9 are localized. Pictures are representative of at least three experiments performed by triplicate.

immunocytochemistry and immunoblot, when compared with control GFP-expressing myotubes (**Figures 5A,B**). When analyzed on E17.5 diaphragms by immunostaining, β-catenin is expressed all along the sarcolemma of muscle fibers, including the synaptic region, where AChRs are clustered and Fzd9 is localized (**Figure 5C**). Taken together, our present findings suggest that Fzd9 signals through a Wnt/β-catenin pathway to inhibit agrin-induced AChR clustering to shape the postsynaptic domain of the vertebrate NMJ.

DISCUSSION

Even though it has become clear that Wnt signaling plays pivotal roles during NMJ assembly, the identity of the receptors involved in these differential effects has remained elusive. In this regard, although Wnt ligands play pro- and anti-synaptogenic effects during NMJ assembly of invertebrate species, these opposite responses are both mediated by Fzd receptors (Mathew et al., 2005; Klassen and Shen, 2007). In turn, at the zebrafish NMJ, it is the agrin receptor MuSK, possibly via a non-canonical pathway, the one that transduces the signal of Wnt11r to induce the aneural pre-patterning of AChR clusters, an early event of postsynaptic differentiation at the vertebrate NMJ (Jing et al., 2009). Based on previous evidence showing differential roles for Wnt ligands on AChR clustering (Henriquez et al., 2008; Wang et al., 2008), in this study we analyzed the potential contribution of Fzd receptors on postsynaptic differentiation at the vertebrate NMJ. Our primary findings showed that most Fzd receptors are expressed by growing and/or differentiated muscle cells. We performed these analyses in 6-week old mice based on previous studies addressing a potential role for Wnt signaling in nerve-muscle connectivity at this time point (Magnusson et al., 2005). Interestingly, these studies revealed that Fzd9 mRNA is transcribed in adult skeletal muscles and it is significantly decreased upon muscle denervation, suggesting that the expression of muscle Fzd9 is positively regulated by its neuronal counterpart (Magnusson et al., 2005). Functionally, Fzd9 regulates the connectivity of central synapses. Indeed, Fzd9 is selectively expressed in cortical precursors and hippocampal neurons (Zhao and Pleasure, 2004; Zhao et al., 2005) and its deletion results in severe neuroanatomical defects that are functionally manifested with visuospatial learning and memory disabilities (Zhao et al., 2005). Consistent with these findings, the Fzd9 gene is deleted in the Williams syndrome, a developmental cognitive disorder in humans characterized by strong behavioral phenotypes (Wang et al., 1997, 1999). Based on this evidence, we decided to focus our subsequent analyses on the potential role that Fdz9 could play on NMJ synaptogenesis at relevant stages of embryonic development.

In our present studies, we show that the expression of Fzd9 in skeletal muscle varies as development proceeds, displaying relatively high expression at early NMJ development to subsequently decrease toward birth. Remarkably, a similar expression profile has been described for other muscle proteins that positively and negatively influence AChR clustering, such as the agrin receptor MuSK and Wnt3a, respectively (Valenzuela et al., 1995; Ip et al., 2000; Wang et al., 2008). Our findings also show that Fzd9 is specifically distributed in the postsynaptic domain of embryonic mice muscle fibers, as evidenced by its co-distribution with AChR-enriched areas. Together, the temporal expression of Fzd9 supports a potential role on embryonic NMJ assembly *in vivo*.

Our functional *in vitro* approach shows that Fzd9-mediated signaling inhibits the agrin-mediated clustering of AChR receptors. Such negative signals for AChR clustering are likely to be required not only at extrasynaptic regions of the developing embryonic NMJ, but also in specific domains of the innervated muscle region, where AChR aggregates become disassembled to shape the mature pretzel-like NMJ (Marques et al., 2000; Bolliger et al., 2010). Thus, our results on the expression profile of Fzd9 *in vivo* are consistent with its function in cultured myotubes. What is the mechanism by which Fzd9 inhibits AChR clustering? In this regard, neural- and muscle-derived molecules have been described to play such inhibitory roles at the vertebrate NMJ. One the one hand, the neurotransmitter ACh displays an AChR-disaggregating activity at the most abundant non-innervated ("extrasynaptic") domains of the muscle membrane at nascent NMJs (Lin et al., 2005; Misgeld et al., 2005). ACh acts through the cyclin-dependent kinase 5 (Cdk5) which phosphorylates the intermediate filament protein nestin, that becomes dissociated from the cytoskeletal network and is subsequently degraded (Fu et al., 2005; Yang et al., 2011). In addition to nestin, the local dynamics of other components of the cytoskeleton also play an important role on the formation of AChR-free areas at the NMJ. For instance, punctate areas of the postsynaptic membrane, comparable to the ones we found for Fzd9, are highly enriched in polymerized F-actin by the action of ADF/cofilin. Remarkably, these areas are subsequently converted into regions devoid of AChR aggregates as development proceeds (Lee et al., 2009). Whether the punctate pattern of Fzd9 at the developing neuromuscular synapse is related to the formation of AChR-free areas for NMJ maturation is still to be elucidated.

Experiments in cultured myotubes showed that Wnt3a, which is expressed by skeletal muscles during NMJ formation, inhibits agrin-induced AChR clustering and disperses pre-formed aggregates (Wang et al., 2008). Our present findings show that agrin-induced AChR aggregation was altered by manipulation of Fzd9 expression without the exogenous addition of any Wnt ligand. Therefore, we speculate that Fzd9 could be the functional receptor of a muscle-secreted Wnt that signals to inhibit the agrin-dependent clustering of AChRs. Since Wnt3a is likely to shape the NMJ by inhibiting AChR clustering (Wang et al., 2008), it is tempting to speculate that Fzd9 could be the receptor for this Wnt. However, since aneural muscles develop more AChR clusters than controls (Lin et al., 2001), and Fzd9 expression is regulated by innervation (Magnusson et al., 2005), it is also likely that the mechanism employed by Fzd9 to inhibit AChR clustering may somehow require the neuronal counterpart.

Wnt3a disassembles AChR clusters possibly through a β-catenin-dependent, but TCF-independent, signaling that results in the down-regulation of rapsyn (Wang et al., 2008). In addition, treatment of myotubes with lithium (Sharma and Wallace, 2003) or BIO (Henriquez et al., 2008), two GSK3β inhibitors that activate the Wnt/β-catenin pathway, inhibits agrin-induced AChR clustering. Consistent with these findings, our *in vitro* data show that Fzd9 accumulates β-catenin

in myotubes and confirm previous results showing that Fzd9 activates a Wnt/β-catenin-dependent pathway (Karasawa et al., 2002). Remarkably, we found that β-catenin is present in the synaptic domain of embryonic NMJs. Together, our present findings support the view that Fzd9 inhibits AChR clustering by activating a β-catenin dependent Wnt pathway. In this regard, the role of β-catenin in NMJ formation has remained rather controversial. Genetic ablation of β-catenin in mouse skeletal muscles, but not in motor neurons, gives rise to abnormal NMJs that are distributed in wider end-plate bands than controls, an effect that was primarily related to pre-synaptic defects in axonal branching and neurotransmission (Li et al., 2008; Wang and Luo, 2008). In turn, specific stabilization of β-catenin in muscle, but not in neuronal cells, resulted in excessive nerve branching and defasciculation, while NMJ formation and function were unaffected (Liu et al., 2012). Interestingly, β-catenin deficient mice contain bigger AChR clusters than control animals (Li et al., 2008; Wang et al., 2008), further supporting the view that β-catenin signaling in muscle cells plays a negative role on NMJ formation and growth. However, in cultured muscle cells, the blockade of β-catenin interaction with α-catenin or the silencing of β-catenin expression results in impaired agrin-dependent AChR clustering (Zhang et al., 2007). These apparent discrepancies on the role of muscle β-catenin on AChR clustering could be explained by the ability of β-catenin to activate the canonical Wnt signaling cascade but also to play a key role in cell adhesion by forming a link between cadherins and the actin cytoskeleton (Brembeck et al., 2006). Whether Fzd9 accumulation of β-catenin exerts its inhibitory effects on AChR aggregation through any of these signaling pathways is still to be determined.

Complementing previous results showing that muscle Fzd9 expression relies on innervation (Magnusson et al., 2005), our present findings suggest that the specific localization of Fzd9 in developing NMJs, and the possible subsequent activation of a β-catenin dependent signaling that disassembles AChR clusters, could depend on the establishment of specifically located postsynaptic densities *in vivo*. As a general conclusion, our present findings support the notion that a fine balance between different Wnt pathways could contribute to shape the complex postsynaptic apparatus at the vertebrate NMJ.

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Wnt signaling in the regulation of adult hippocampal neurogenesis

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In the adult brain new neurons are continuously generated mainly in two regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the hippocampal dentate gyrus. In the SGZ, radial neural stem cells (NSCs) give rise to granule cells that integrate into the hippocampal circuitry and are relevant for the plasticity of the hippocampus. Loss of neurogenesis impairs learning and memory, suggesting that this process is important for adult hippocampal function. Adult neurogenesis is tightly regulated by multiple signaling pathways, including the canonical Wnt/β-catenin pathway. This pathway plays important roles during the development of neuronal circuits and in the adult brain it modulates synaptic transmission and plasticity. Here, we review current knowledge on the regulation of adult hippocampal neurogenesis by the Wnt/β-catenin signaling cascade and the potential mechanisms involved in this regulation. Also we discuss the evidence supporting that the canonical Wnt pathway is part of the signaling mechanisms involved in the regulation of neurogenesis in different physiological conditions. Finally, some unsolved questions regarding the Wnt-mediated regulation of neurogenesis are discussed.

Keywords: neurogenesis, hippocampus, adult hippocampal progenitor (AHP), subgranular zone (SGZ), Wnt signaling pathway, β-catenin

INTRODUCTION

The adult brain is able to continuously generate new neurons, a process known as neurogenesis, which has been reported in a number of mammalian species. Adult neurogenesis occurs mainly in two specific brain regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the hippocampal dentate gyrus (Alvarez-Buylla and Garcia-Verdugo, 2002; Zhao et al., 2008) (**Figure 1A**). Through intrinsic and extrinsic factors adult neurogenesis is tightly regulated to allow the maintenance and self-renewal of the stem cell pool and the generation of fully functional neurons. Here, we review the findings specifically supporting the role of the Wnt pathway in the adult hippocampal neurogenesis, and also discuss different studies indicating that this pathway is part of the signaling mechanisms involved in the regulation of neurogenesis by different physiological conditions.

Adult neurogenesis is a multistep process. In the SVZ astrocyte-like neural stem cells (NSCs), called type B1 cells, generate type C cells that rapidly proliferate and give rise to type A neuroblasts (**Figure 1B**). These cells migrate through the rostral migratory stream to the olfactory bulb where they became interneurons (Alvarez-Buylla and Garcia-Verdugo, 2002). In the SGZ, NSCs that give rise to granule cells are present at the border between the hilus and the granule cell layer (Gage, 2000). Glial fibrillary acidic protein (GFAP)-expressing radial glia-like cells, which extend a single radial process toward the molecular layer (**Figure 1C**), are proposed to be the stem cells or undifferentiated precursors that generate dentate granule neurons (Seri et al., 2001; Kempermann et al., 2004). These progenitors (type 1 cells) that are slowly dividing or quiescent, also express nestin and the transcription factor Sox2. A morphologically distinct class of type 1 cells that has horizontal processes has also been identified (Lugert et al., 2010). The horizontal and radial progenitors have different proliferation rate and respond differently to neurogenic stimuli (Suh et al., 2007; Lugert et al., 2010), suggesting that in the SGZ there are different populations of progenitors with different properties, increasing the complexity of the cellular and molecular mechanisms underlying regulation of adult neurogenesis. Intrinsic properties of NSCs present in the SGZ, such as self-renewing and multipotency, are still a matter of debate (Bonaguidi et al., 2011; Encinas et al., 2011; Ming and Song, 2011). These cells can be isolated and cultured generating self-renewing cells that can differentiate into neurons (Palmer et al., 1997; Peltier et al., 2010).

When activated, type 1 cells give rise to fast proliferating type 2 cells or transit-amplifying progenitors that express nestin and Sox2 but not GFAP (Kempermann et al., 2004). After limited number of cell divisions, type 2 cells commit to the neuronal lineage generating type 3 cells or neuroblasts which are proliferative and express doublecortin (DCX) but not nestin (Kronenberg et al., 2003). Neuroblasts then became immature neurons that extend dendrites toward the molecular layer and project their axons through the hilus toward the CA3 region (**Figure 1C**), and during several weeks mature into functional dentate granule neurons that are integrated into the pre-existing hippocampal circuitry and are located mainly in the inner granular cell layer

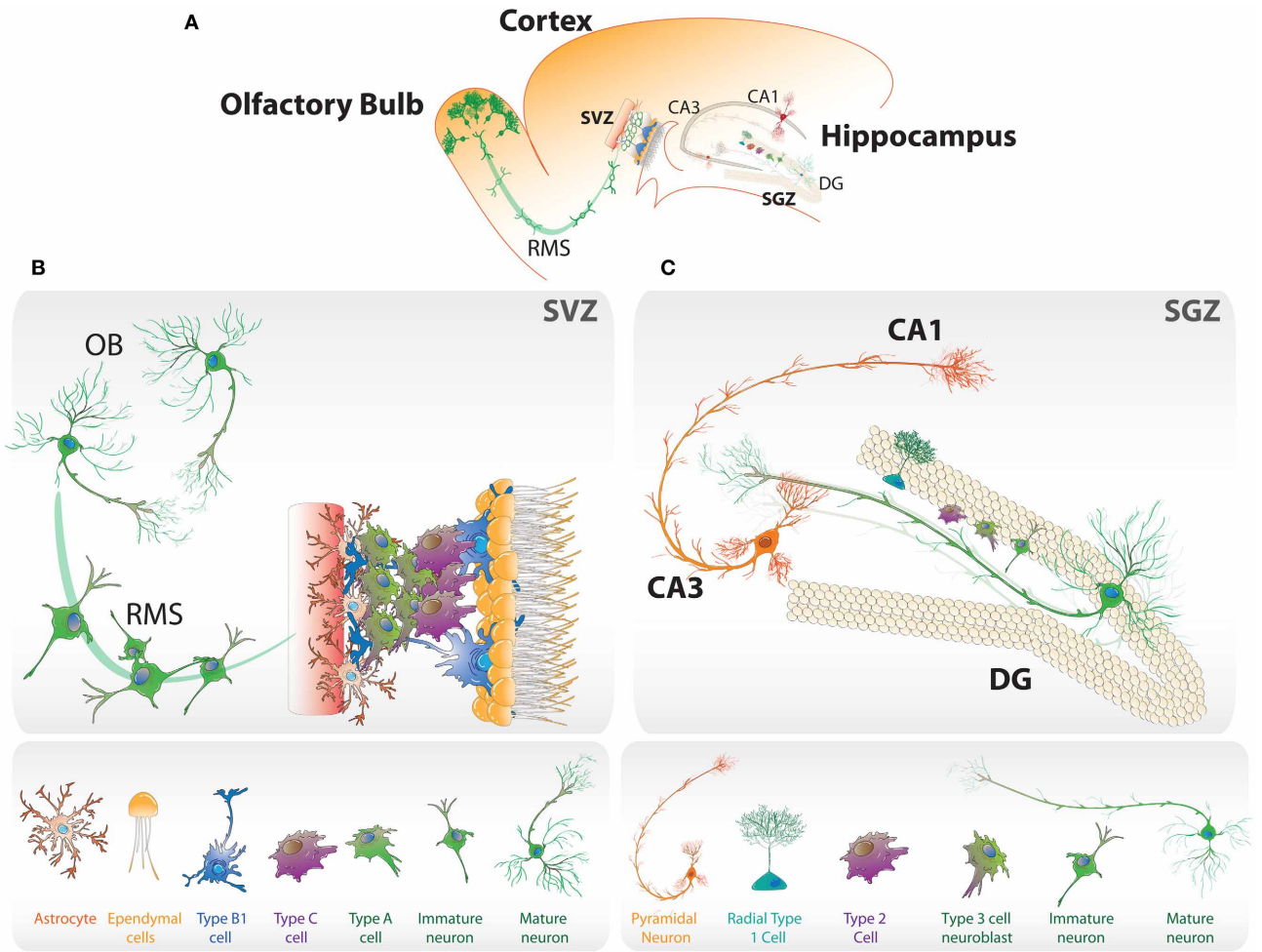

FIGURE 1 | Neurogenic niches in the adult brain. (A) Schematic representation of the neurogenic regions in the adult rodent brain: the subgranular zone (SGZ) in dentate gyrus (DG) of the hippocampus, and the subventricular zone (SVZ) in the later wall of the lateral ventricles. **(B)** SVZ niche composed of type B1 cells, that corresponds to neural stem cells, type C cells that rapidly proliferate and type A neuroblasts, which migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they mature into interneurons. **(C)** Neurogenesis in the SGZ. Radial type 1 cells give rise to type 2 cells that differentiate into type 3 neuroblasts that become immature neurons and then mature into granule neurons that migrate into the granule cell layer.

of the dentate gyrus (Van Praag et al., 2002; Zhao et al., 2006; Mathews et al., 2010). Thus, neurogenesis can be divided into sequential stages: activation of quiescent stem cells, proliferation, neuronal fate specification, maturation and integration of newborn neurons.

For the continuous generation of new neurons in the adult brain there should be a tight regulation of the sequential steps of neurogenesis. Intrinsic factors should coordinate the proper progression of neurogenesis and preserve the stem cell pool. An impaired maintenance of the quiescent pool may result in an excessive proliferation and differentiation and ultimately in the depletion of precursor cells. If commitment and differentiation of precursor cells into neurons is disturbed, there could be no generation of new neurons even in the presence of the stem cells pool. Therefore, there should be a balance between the self-renewal and maintenance of stem cells and the differentiation into neurons. Several signaling molecules regulate hippocampal neurogenesis including Wnt, Notch, sonic hedgehog (Shh), bone morphogenetic proteins (BMP), growth and neurotrophic factors and neurotransmitters (Suh et al., 2009; Schwarz et al., 2012; Faigle and Song, 2013). Also, there is a fine tuned transcriptional control involving a number of transcriptional factors and epigenetic mechanisms that will coordinate the progression of neurogenesis (Ming and Song, 2011; Hsieh, 2012; Schwarz et al., 2012). In addition, the rate of proliferation, differentiation, and survival of newborn neurons in the hippocampus can be modulated by different physiological stimuli such as hippocampal-dependent learning and neuronal activity, exposure to environmental enrichment (EE), running and stress (Kempermann et al., 1997; Gould et al., 1998; Van Praag et al., 1999; Dobrossy et al., 2003; Drapeau et al., 2007; Piatti et al., 2011; Song et al., 2012).

Increasing evidence indicate that adult neurogenesis is important for hippocampal function, being relevant for the plasticity

of the hippocampal network (Snyder et al., 2001; Mongiat and Schinder, 2011). Newborn neurons impact the hippocampal circuitry mainly while still immature, when these cells have distinct functional properties showing increased excitability and reduced GABAergic inhibition (Wang et al., 2000; Schmidt-Hieber et al., 2004; Esposito et al., 2005; Ge et al., 2007; Marin-Burgin et al., 2012). Loss of neurogenesis impairs learning and memory indicating that this process has functional implications for the adult brain (Reviewed in Deng et al., 2010; Koehl and Abrous, 2011; Marin-Burgin and Schinder, 2012).

THE Wnt/β-CATENIN SIGNALING PATHWAY IN THE REGULATION OF ADULT HIPPOCAMPAL NEUROGENESIS

Wnts compromise a large family of secreted glycoproteins that are part of the signaling molecules regulating several aspects of development such as axis formation and midbrain development (Van Amerongen and Nusse, 2009; Nusse and Varmus, 2012). The interaction of a Wnt protein with members of the Frizzled (Fz) family of seven-pass transmembrane cell-surface receptors triggers the activation of the Wnt signaling pathway (Gordon and Nusse, 2006; Wang et al., 2006; Schulte, 2010). In mammals 19 Wnt members have been found and 10 members of the Fz family have been identified. In addition, receptor-like tyrosine kinase (Ryk) and receptor tyrosine kinase-like orphan receptor (Ror2) have been identified as alternative Wnt receptors (Oishi et al., 2003; Keeble et al., 2006; Ho et al., 2012). Downstream Wnt receptors, different Wnt signaling cascades may be activated: the Wnt/β-catenin or canonical pathway that involves gene transcription, and the β-catenin-independent or non-canonical pathways that induce either an increase in intracellular calcium concentration or activation of the c-Jun-N-terminal kinase (JNK) cascade (Veeman et al., 2003; Gordon and Nusse, 2006; Angers and Moon, 2009).

In the canonical Wnt/β-catenin signaling low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) serve as co-receptors for Wnt ligands. Activation of the canonical signaling pathway activates the protein Dishevelled (Dvl) by phosphorylation, and triggers the stabilization of cytoplasmic β-catenin, that in the absence of Wnt stimulation is ubiquitinated and constantly degraded in the proteasome (Aberle et al., 1997). β-catenin enters the nucleus and binds to members of the family of T-cell factor (TCF) and lymphoid enhancer factor (Lef) and this binding activates transcription by displacing the repression of Wnt target genes (Nusse and Varmus, 2012).

During development, Wnt signaling is fundamental for the proper development of cortex and hippocampus (Li and Pleasure, 2005; Machon et al., 2007). Wnt signaling promotes self-renewal of cortical neural progenitors and the differentiation of these progenitors in a stage specific manner (Hirabayashi et al., 2004; Munji et al., 2011). During early neurogenesis the Wnt pathway promotes self-renewal and maintains neural progenitors (Chenn and Walsh, 2002; Machon et al., 2007; Wrobel et al., 2007), while it induces the differentiation of intermediate progenitors during mid and late neurogenesis (Munji et al., 2011). In addition to the key roles of the Wnt pathway during development, it has proved to be important in the adult brain, where it regulates synapse formation, neurotransmission and plasticity, and neurogenesis (Lie et al., 2005; Adachi et al., 2007; Toledo et al., 2008; Kuwabara et al., 2009; Inestrosa and Arenas, 2010). Consistent with the previous feature, in the adult brain most of the key components of the Wnt signaling including Wnts and Fz receptors are expressed (Shimogori et al., 2004; Chen et al., 2006; Chacon et al., 2008).

Different studies have shown that the Wnt/β-catenin pathway is involved in adult hippocampal neurogenesis. In the BAT-gal reporter mouse expressing a β-catenin-activated transgene with nuclear β-galactosidase under the control of TCF/Lef (Maretto et al., 2003), it was determined that the Wnt/β-catenin pathway is active in the SGZ and the dentate granule cell layer in the adult hippocampus (Lie et al., 2005). In addition, cultured adult hippocampal progenitors (AHPs) express key components of the Wnt/β-catenin signaling pathway including some Fz receptors (Lie et al., 2005; Wexler et al., 2009). Adult hippocampal astrocytes express Wnt-3, and it was shown that Wnts derived from hippocampal astrocytes stimulate Wnt/β-catenin signaling in isolated AHPs and induce the differentiation of these progenitors into neurons, since the differentiation induced by co-culture with astrocytes was reduced in the presence of the Wnt inhibitor soluble Frizzled-related protein 2 and 3 (sFRP2/3) (Lie et al., 2005). In addition to astrocytes-derived Wnts, there is an autocrine Wnt signaling activity in AHPs (Wexler et al., 2009). Interestingly, inhibition of the autocrine Wnt stimulation increases the number of neurons formed and depletes multipotent progenitors indicating that this autocrine pathway supports the proliferation and multipotency of stem cells and therefore, it may preserve the balance between NSC maintenance and differentiation (Wexler et al., 2009). Therefore, Wnts are important for both, the maintenance of the stem cell pool and the differentiation of newborn neurons.

Regulation of adult neurogenesis by Wnt signaling was also demonstrated *in vivo* by stereotactic injection of lentiviral vectors expressing Wnt-3 or a secreted mutant Wnt-1 protein that blocks the Wnt signaling (Lie et al., 2005). Wnt signaling inhibition reduced proliferation and neurogenesis in the SGZ, while activation of the Wnt signaling increased neurogenesis (Lie et al., 2005). Later on, and by using the same lentiviral approach to block Wnt signaling activation in the dentate gyrus of adult rats, it was shown that reduction of neurogenesis by Wnt inhibition impaired long-term retention of spatial memory and object recognition memory, indicating that Wnt-mediated adult hippocampal neurogenesis contributes to hippocampal function (Jessberger et al., 2009).

The importance of the Wnt pathway in neurogenesis is also supported by studies focusing on the role of a key component of the Wnt/β-catenin pathway, the enzyme glycogen synthase kinase-3β (GSK-3β). In the absence of Wnt stimulation, β-catenin is phosphorylated by GSK-3β in a multiprotein complex composed also of the scaffold protein axin and adenomatous polyposis coli (APC) (Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Kishida et al., 1998; Sakanaka et al., 1998). Phosphorylated β-catenin is recognized by β-TrCP, which is part of an E3 ubiquitin ligase complex, is ubiquitinated and subsequently degraded (Liu et al., 2002). Activation of the Wnt/β-catenin pathway inhibits the degradation pathway and induces the cytoplasmic stabilization of β-catenin and the transcription of Wnt target genes (Logan and Nusse, 2004). *In vitro* experiments show that

treatment with the GSK-3β inhibitor lithium induces the proliferation of AHPs (Wexler et al., 2008). *In vivo* treatment with lithium was also shown to stimulate proliferation and neuronal fate specification in a mouse model of Alzheimer's disease (Fiorentini et al., 2010). Besides, a decreased neurogenesis was observed in a GSK-3 knock-in mouse carrying mutations to block inhibitory phosphorylation of the kinase (Eom and Jope, 2009). In this model, it was suggested that the impaired neurogenesis was not consequence of the effects of GSK-3 in neural progenitor cells but rather by alterations in the external support for the proliferation of these cells. GSK-3β has also been involved in the disrupted in schizophrenia 1 (DISC1)-mediated regulation of adult neurogenesis. DISC-1 is a schizophrenia susceptibility gene that regulates multiple steps of neurogenesis (Duan et al., 2007; Mao et al., 2009). Interestingly, DISC1 protein inhibits GSK-3β activity, and it is required for Wnt-3a-induced proliferation of cultured AHP cells and β-catenin-dependent transcription (Mao et al., 2009). The impairment in progenitor cell proliferation caused by DISC1 knockdown could be rescued by over expression of stabilized β-catenin. *In vivo*, cell proliferation in the adult dentate gyrus could be rescued by administration of a GSK-3β inhibitor, which also suppressed schizophrenia- and depression-like behaviors caused by DISC1 loss of function (Mao et al., 2009). These findings link the DISC1-mediated regulation of neurogenesis with downstream components of the Wnt/β-catenin signaling pathway.

Recently, a negative effect on neurogenesis was determined for two Wnt inhibitors, Dickkopf 1 (Dkk1) and sFRP3 (Jang et al., 2013; Seib et al., 2013). Dkk1 binds LRP co-receptors preventing the formation of the Fz/LRP complex and the consequent activation of the Wnt signaling cascade (Clevers and Nusse, 2012); sFRPs bind to Wnts preventing its interaction with cellular receptors and the activation of the Wnt pathway (Rattner et al., 1997). Inducible deletion of Dkk1 in the adult CNS resulted in an increased self-renewal of neural progenitors and increased generation of immature neurons (Seib et al., 2013). On the other hand, sFRP3 knockdown in the dentate gyrus through a lentiviral approach increased Wnt signaling and increased neural progenitor proliferation (Jang et al., 2013). Interestingly, deletion of Dkk1 and sFRP3 knockdown resulted in an increased dendrite complexity of immature neurons indicating that Wnt signaling is important for dendritic development of newborn neurons (Jang et al., 2013; Seib et al., 2013). The negative effect of both inhibitors on neurogenesis suggests that suppression of Wnt signaling by secreted factors could be a regulatory mechanism to dynamically modulate neurogenesis under physiological and pathological stimulation as we will discuss afterward.

Also, the Wnt pathway is part of the signaling mechanisms of the orphan nuclear receptor TLX (also known as NR2E1), an important regulator of NSC maintenance and self-renewal in embryonic and adult brains (Shi et al., 2004; Li et al., 2008b) that is required for adult neurogenesis in the SVZ (Liu et al., 2008) and SGZ (Zhang et al., 2008). It was determined that TLX activates the Wnt/β-catenin pathway in adult mouse NSC to stimulate proliferation and self-renewal by activating the expression of Wnt-7a through binding to the two TLX binding sites present in the Wnt-7a gene promoter (Qu et al., 2010). In accordance, Wnt-7a expression was found down-regulated in TLX-null mice. This study also revealed that Wnt-7a is important for adult NSC proliferation *in vivo*, since a decreased proliferation was observed in the SGZ and SVZ of adult Wnt-7a knockout mice. In $TLX^{-/-}$ mice, intracranial lentiviral transduction of active β-catenin led to a considerable rescue of cell proliferation in the SVZ, suggesting that Wnt/β-catenin acts downstream of TLX to regulate NSC proliferation *in vivo* (Qu et al., 2010).

Although it is out of the scope of the present review, it is important to mention that other studies have demonstrated that neurogenesis in the SVZ is also Wnt-regulated. Retrovirus-mediated expression of a stabilized β-catenin *in vivo* promoted the proliferation of type C cells and inhibited their differentiation into neuroblasts (Adachi et al., 2007). Also in the SVZ, transduction of the β-catenin inhibitor axin by intracranial lentiviral delivery decreased cell proliferation (Qu et al., 2010), further supporting a role for Wnt/β-catenin signaling in NSC proliferation in the neurogenic areas of the adult brain.

Although the Wnt/β-catenin pathway is required for different aspects of adult hippocampal neurogenesis, excessive β-catenin signaling may impair the maturation of adult born neurons. It was determined that the impaired dendritic refinement in adult born dentate granule cells observed by primary cilia loss is a consequence of an abnormal enhancement of β-catenin signaling in newborn neurons as conditional knockout of β-catenin reversed the decrease observed in the total dendritic length in these neurons (Kumamoto et al., 2012). Moreover, expression of a constitutively active β-catenin suppresses the dendritic refinement of newborn neurons between 14 and 21 after born (Kumamoto et al., 2012). Therefore, impaired Wnt/β-catenin signaling after migration of newborn neurons may have detrimental consequences, suggesting that there should be a fine tuned activation of β-catenin signaling to allow for proper maturation and integration of newborn neurons.

Wnt/β-CATENIN PATHWAY AS PART OF THE SIGNALING MECHANISMS INVOLVED IN THE REGULATION OF NEUROGENESIS IN DIFFERENT PHYSIOLOGICAL CONDITIONS

In addition to the discussed evidence directly pointing to the involvement of the Wnt signaling pathway in the proliferation and differentiation of adult neural progenitor cells, studies have indicated this pathway as part of the mechanisms involved in the regulation of neurogenesis under some physiological conditions.

AGING

During lifespan there is a progressive reduction of hippocampal neurogenesis that have been evidenced in different species (Kuhn et al., 1996; Gould et al., 1999; Leuner et al., 2007; Olariu et al., 2007; Varela-Nallar et al., 2010b), including humans (Knoth et al., 2010). The generation of new neurons in the adult human dentate gyrus was originally evidenced in postmortem tissues from patients who were treated with the thymidine analog bromodeoxyuridine (BrdU) (Eriksson et al., 1998). Thereafter, several studies have suggested that neurogenesis in humans can be regulated by different physiological and pathological conditions (Jin et al., 2004; Li et al., 2008a; Gerber et al., 2009; Mattiesen et al., 2009; Boldrini et al., 2012). As determined in other species, it was

shown a reduction of DCX expressing cells with increasing age suggesting that there is an age-related decline in the generation of new neurons in the human hippocampus (Knoth et al., 2010).

In rodents, evidence indicate that a decline in Wnt signaling is associated to the age-dependent reduction in neurogenesis. During aging, the levels of Wnt-3 protein in hippocampal astrocytes and also the number of Wnt-3-secreting astrocytes decline (Okamoto et al., 2011). This finding is important since as previously mentioned, Wnts derived from hippocampal astrocytes stimulate Wnt/β-catenin signaling in neural progenitors and induce its neural differentiation (Lie et al., 2005). It was shown in rats that there is a progressive decrease in the expression of Wnt-3 and Wnt-3a in the dentate gyrus between 2 and 22 month, concomitantly with a decrease in the expression of NeuroD1 (Okamoto et al., 2011). NeuroD1 is a basic helix-loop-helix transcription factor important for the generation of granule cells and olfactory neurons in the embryonic and adult brain (Gao et al., 2009). This suggests that the decline in Wnt-3/Wnt-3a expression in astrocytes may cause the decreased expression of proneural genes and in consequence the decrease in neurogenesis. More recently, the Wnt inhibitor Dkk1 was also involved in the age-related decline in neurogenesis (Seib et al., 2013). As mentioned, Dkk1 is a suppressor of NSCs proliferation. The expression of Dkk1 increases with age, suggesting that suppression of Wnt signaling by this inhibitor may downregulate neurogenesis during aging (Seib et al., 2013). Whether Wnt is associated to the age-related decline in neurogenesis in humans is not known, but it was reported an association between Wnt-3 levels and cell proliferation in the human hippocampus (Gerber et al., 2009), suggesting that this pathway may also regulate neurogenesis in the human brain.

EXERCISE

Running, which is one of the physiological stimuli that strongly stimulates adult neurogenesis in the SGZ (Van Praag et al., 1999), modulates the expression of genes involved in Wnt signaling (Stranahan et al., 2010). Moreover, running was found to significantly increase the expression of Wnt-3 in astrocytes of the dentate gyrus (Okamoto et al., 2011) and to increase the population of Wnt-3 expressing cells in young and aged mice. More recently, it was shown that exercise also regulates the expression of the Wnt inhibitor sFRP3, which is a suppressor of adult hippocampal neurogenesis (Jang et al., 2013). Exercise as well as electroconvulsive stimulation (ECS) decreased the expression of sFRP3 in dentate granule neurons, and infusion of sFRP3 into the dentate gyrus abolished the ECS-induced increase of neural progenitor proliferation (Jang et al., 2013), suggesting that the reduction of sFRP3 levels is important for the activity-mediated increase in neurogenesis. In addition to the reported increase of Wnt-3 levels by running, this finding strongly implicates the Wnt pathway as a signaling mechanisms involved in the excercise-mediated increase in neurogenesis.

HYPOXIA

An association between hypoxia and neurogenesis in embryonic and adult brain has been demonstrated by different studies. Increased neurogenesis in the rodent dentate gyrus was observed in response to global ischemia (Liu et al., 1998). Also, intermittent hypobaric hypoxia regimen promoted the proliferation of endogenous neural progenitors leading to more newborn neurons in the hippocampus of adult rats (Zhu et al., 2010). Interestingly, there has been suggested an association between hypoxia and the Wnt/β-catenin pathway in embryonic stem cells (ESCs) and NSCs. Hypoxia increases β-catenin signaling in ESCs and increases the expression of Lef1 and TCF1 genes (Mazumdar et al., 2010). The hypoxia-mediated activation of the Wnt pathway is mediated by hypoxia-inducible transcription factor-1α (HIF-1α) that directly binds to the promoter of the Lef1 and TCF1 genes in cultured ESC under hypoxic conditions (Mazumdar et al., 2010). Moreover, it was determined in the BAT-gal reporter mouse that the Wnt/β-catenin signaling is active in low oxygen regions in the adult brain, including in the SGZ. This suggests an association between low oxygen and β-catenin signaling *in vivo*, which was shown to be dependent of HIF-1α (Mazumdar et al., 2010).

POTENTIAL MECHANISMS INVOLVED IN THE Wnt-MEDIATED REGULATION OF ADULT HIPPOCAMPAL NEUROGENESIS

How the Wnt/β-catenin signaling could regulate neurogenesis? The molecular mechanism may involve the transcriptional activation of NeuroD1 which depends on the Wnt/β-catenin signaling activation (Kuwabara et al., 2009). NeuroD1 gene promoter has overlapping DNA-binding site for Sox2 and TCF/Lef, then the activation of this gene implies activation of the canonical Wnt pathway and removal of Sox2 repression from the NeuroD1 gene promoter (Kuwabara et al., 2009). Prox1 is also a Wnt target gene that could be relevant for the neurogenic effect of the Wnt/β-catenin pathway (Karalay et al., 2011). Prox1 is expressed in newborn and mature granule cells and is required for the proper differentiation and survival of newborn granule cells, but not for the maintenance of granule cells after they have fully matured (Karalay et al., 2011). Interestingly, the promoter region of long interspersed element-1 (L1) retrotransposons, which was found to be actively retrotransposed during neurogenesis (Muotri et al., 2005; Coufal et al., 2009), contains dual binding sites for Sox2 and TCF/Lef (Kuwabara et al., 2009). Therefore, Wnt signaling activation could upregulate the expression of genes adjacent to the L1 loci that may be relevant for neurogenesis such as DCX (Okamoto et al., 2011).

In addition, the Wnt signaling pathway could directly or indirectly modulate neurogenesis through the regulation of glutamatergic neurotransmission in the hippocampus, since neural progenitor cells respond to neuronal activity as part of their differentiation program (Deisseroth et al., 2004). We and others have determined that Wnt ligands regulate synaptic assembly as well as synaptic plasticity and neurotransmission in the hippocampus. In cultured hippocampal neurons, Wnt-3a, Wnt-7a and Wnt-7b regulate pre-synaptic assembly increasing the number of pre-synaptic puncta (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008). In addition, Wnt-7a stimulates the clustering of the pre-synaptic receptor α7- nicotinic acetylcholine receptor (Farias et al., 2007), indicating that the Wnt signaling regulates the clustering of pre-synaptic receptors. Evidence indicate that

these ligands are able to modulate pre-synaptic differentiation by activation of the Wnt/β-catenin signaling pathway. In accordance with the structural data, electrophysiological recordings have revealed that Wnts have modulatory effects on glutamatergic neurotransmission (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Varela-Nallar et al., 2010a; Avila et al., 2010). On adult rat hippocampal slices, Wnt-7a increases neurotransmitter release in CA3-CA1 increasing the frequency of miniature excitatory post-synaptic currents (mEPSC) (Cerpa et al., 2008). Wnt-3a is also able to increase the frequency of mEPSC in cultured hippocampal neurons (Avila et al., 2010). The synaptic effects of Wnts could regulate the generation and maturation of newborn neurons.

Importantly, the release and expression of Wnt ligands is modulated by neuronal activity (Chen et al., 2006; Wayman et al., 2006; Tabatadze et al., 2012), and incubation of adult hippocampal slices with secreted Wnt inhibitors affects glutamatergic neurotransmission (Chen et al., 2006; Varela-Nallar et al., 2010a; Cerpa et al., 2011), strongly suggesting that endogenous Wnt signaling in the brain modulates hippocampal function and could modulate the generation of new neurons.

The *in vivo* relevance of the Wnt signaling in the hippocampal function is demonstrated by the effects of EE, which increases Wnt-7a/b levels in CA3 pyramidal neurons in parallel to increasing the complexity and number of large mossy fiber terminals in the CA3 region (Gogolla et al., 2009). Interestingly, inhibiting Wnt signaling through local application of the Wnt inhibitor sFRP-1 suppressed EE effects. Also, it was determined that training in the hidden platform Morris water maze task increases the levels of Wnt-7a/b in granule cells of rat dentate gyrus but not in CA3 pyramidal cells (Tabatadze et al., 2012). The increase of hippocampal Wnt-7a/b levels was still observed 30 days after training, indicating that this is a long-lasting effect that could be associated to long-term spatial memory (Tabatadze et al., 2012). Therefore, Wnt signaling pathway is regulated by neuronal activity and regulates neurotransmission. The activity-mediated Wnt signaling activation could modulate adult hippocampal neurogenesis which may contribute to Wnt-mediated increase in hippocampal plasticity.

UNSOLVED QUESTIONS AND FUTURE PERSPECTIVES

Although the evidences indicate that Wnts are part of the signaling molecules that regulate neurogenesis in physiological conditions, there are still unsolved questions remaining. In this part we will discuss two attractive issues that from our point of view should be addressed in the future.

PUTATIVE ROLE OF THE NON-CANONICAL Wnt SIGNALING CASCADES IN ADULT HIPPOCAMPAL NEUROGENESIS

One aspect that should be addressed is the potential role of non-canonical Wnt signaling cascades. There are at least two β-catenin-independent pathways: the planar cell polarity (PCP) pathway and the Ca^{2+} pathway. The PCP pathway, also known as the Wnt/JNK pathway, was originally identified in *Drosophila* where it regulates tissue polarity and cell migration (Adler, 2002; Veeman et al., 2003). This signaling pathway activates small GTPases including Rho and Rac and the protein kinase JNK, and affects cytoskeleton dynamics. It would be interesting to study whether this pathway regulates the proper polarization and migration of newborn neurons in the adult brain as it does during development. On the other hand, the activation of the Wnt/Ca^{2+} pathway triggers the increase in intracellular Ca^{2+} levels and activates the protein kinases CamKII and protein kinase C (PKC) (Veeman et al., 2003; Kohn and Moon, 2005).

We have determined that non-canonical Wnt pathways have relevant roles in the adult hippocampus. In cultured hippocampal neurons, the Wnt-5a ligand able to activate non-canonical Wnts cascades (Farias et al., 2009; Cuitino et al., 2010), plays relevant roles in synaptic structure and function. Wnt-5a increases dendritic spine morphogenesis (Varela-Nallar et al., 2010a), effect also described for Wnt-7a which increase the density and maturity of dendritic spines through a non-canonical CamKII-dependent mechanism (Ciani et al., 2011). In addition, Wnt-5a increases the clustering of the post-synaptic density protein-95 (PSD-95) (Farias et al., 2009). PSD-95 is a scaffold protein of the post-synaptic density, a multiprotein complex containing key molecules involved in the regulation of glutamate receptor targeting and trafficking and regulatory proteins relevant for neurotransmission (Li and Sheng, 2003; Han and Kim, 2008). Electrophysiological data supports the synaptic roles of Wnt-5a. Acute application of Wnt-5a increases the amplitude of field excitatory post-synaptic potentials (fEPSP) in hippocampal slices (Varela-Nallar et al., 2010a) and upregulates synaptic NMDA receptor currents facilitating induction of long-term potentiation (LTP) (Cerpa et al., 2011). Non-canonical ligands could indirectly influence the proliferation and differentiation of progenitor cells through modulating hippocampal neurotransmission, or could directly regulate synaptogenesis and connectivity of newborn neurons.

Wnt-5a also induces the recycling of functional $GABA_A$ receptors on hippocampal neurons through activation of CaMKII, and modulates inhibitory synapses (Cuitino et al., 2010). GABA is critical for the proper development and maturation of adult-born neurons (Tozuka et al., 2005; Ge et al., 2006; Jagasia et al., 2009), therefore the effect of this ligand on the inhibitory synapse may influence the development of newborn neurons.

Interestingly, it was determined that training in the hidden platform Morris water maze task increased hippocampal Wnt-5a levels (Tabatadze et al., 2012), suggesting that this ligand is also regulated by activity and may regulate neurogenesis under certain stimuli.

The effect of Wnt-5a was addressed in postnatal SVZ (Pino et al., 2011). In neural precursor cells cultured from the SVZ of mice at postnatal day 5, Wnt-5a treatment increased neurite outgrowth, effect completely different to Wnt-3a treatment that inhibited neurite development (Pino et al., 2011), suggesting that non-canonical pathway increases neurite complexity while canonical pathway suppresses dendrite maturation. Whether the same regulation takes place in the SGZ is not known.

Wnt RECEPTOR CONTEXT COULD SPECIFICALLY REGULATE THE DIFFERENT STAGES OF NEUROGENESIS

Considering that different populations of stem and progenitor cells have been identified in the SGZ, with different proliferation

rates and response to neurogenic stimuli, it should be interesting to evaluate whether these populations have different ability to respond to the signaling molecules present in the local microenvironment. Progenitor cells, as well as newborn neurons at different stages of maturity, may have different subsets of receptors and/or co-receptors for the Wnt signaling pathway, which may mimic what is observed in hippocampal neurons (Varela-Nallar et al., 2012). The expression pattern of Fz receptors during postnatal development is very different, being some of them highly expressed in adulthood and others during early postnatal development. In cultured hippocampal neurons, the distribution of Fzs is also very specific being some of them located in synaptic regions and others in the soma or growth cones of young neurons (Varela-Nallar et al., 2012). Interestingly, the distribution of Fz receptors seems to be associated to specific functions. Fz1 that is located in the synaptic region co-localizing with presynaptic proteins and with active synaptic vesicle recycling sites (Varela-Nallar et al., 2009), regulates pre-synaptic differentiation. Overexpression of Fz1 receptor increased the clustering of the active zone protein Bassoon (Varela-Nallar et al., 2009), involved in the structural organization of neurotransmitter release sites that is recruited early during synapse formation (Zhai et al., 2000). As well, Fz5 which is also present in synaptosomes and co-localizes with synaptic markers, modulates the synaptogenic effect of Wnt-7a (Sahores et al., 2010). Changes in the expression of this receptor modulates the density of synaptic sites in mature neurons (Sahores et al., 2010).

The effects of Wnts on the proliferation of progenitor cells and neuronal differentiation could be mediated by selective Wnt/receptor complexes. As mentioned, there are 19 Wnt members, 10 Fz receptors and alternative receptors and co-receptors described in mammals, therefore there is a complex scenario in which there is a wide range of possible interactions that may specifically regulate all aspects of neurogenesis. In this context, Wnt ligands constitutively expressed in the hippocampus may induce different signaling cascades in stem cells, progenitors and newborn neurons by interacting with specific sets of receptors and co-receptors, and in that way may regulate the sequential events of neurogenesis (**Figure 2**).

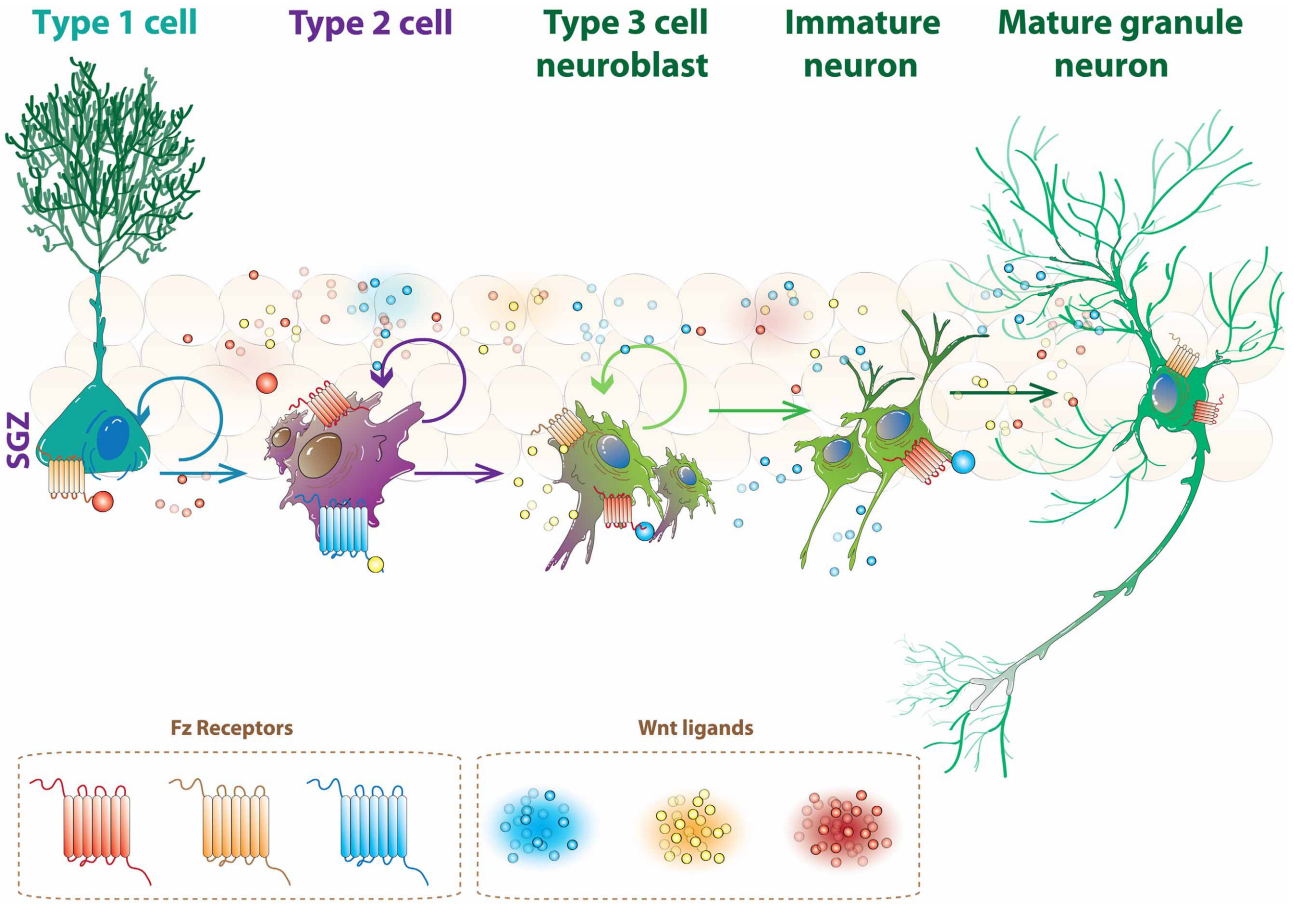

FIGURE 2 | Specific Wnt receptors could regulate the progression of neurogenesis in the hippocampus. Schematic illustration of neurogenesis in the SGZ. As represented, stem cells, progenitor cells, immature neuron and mature granule neurons are constantly present in the dentate gyrus. Wnt ligands constitutively expressed in the hippocampus could induce different signaling cascades in these cells and regulate different stages of neurogenesis by interacting with specific receptors (see text for details).

The receptor context may also be essential for specific effects of canonical and non-canonical Wnt signaling cascades. As mentioned, Wnt-5a and Wnt-3a have differential effects in neural precursor cells cultured from postnatal SVZ (Pino et al., 2011). In this neurogenic region, the dual regulation by Wnt ligands may be achieved by region-specific expression of Wnt ligands during the specification and maturation of olfactory bulb interneurons in the SVZ, rostral migratory stream and olfactory bulb. Whether the same regulation occurs during neurogenesis in the adult SGZ it is not known. In the hippocampus, all steps of neurogenesis occur at the dentate gyrus, where there are stem cells, progenitors and newborn neurons at different maturation stages in close proximity. Therefore, factors regulating each aspect of neurogenesis should be present at the dentate gyrus. It has been proposed that radial stem cells at the hippocampus have three domains that span three different anatomical layers (Fuentealba et al., 2012), which could allow specific microenvironments during neurogenesis; however, it is also plausible that signal integration of the stage-specific cues present in the niche may be given by specific Fz receptors as we have discussed.

CONCLUSIONS

Altogether, the reviewed evidences indicate that the Wnt pathway is relevant for the development of new neurons in the adult hippocampus. The canonical Wnt/β-catenin signaling pathway is important for the maintenance and self-renewal of the stem cell pool, and for progenitor cell proliferation, fate commitment and differentiation. Therefore, Wnt ligands are part of the signaling molecules in the SGZ that could regulate the progression of neurogenesis. Several Wnt ligands are constantly present in the adult hippocampus, but Wnt activity is also dynamically regulated by the expression and release of Wnt ligands and soluble Wnt inhibitors. This dynamic regulation of Wnt activity could be relevant for the regulation of neurogenesis under different physiological conditions.

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Netrin-5 is highly expressed in neurogenic regions of the adult brain

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Mammalian netrin family proteins are involved in targeting of axons, neuronal migration, and angiogenesis and act as repulsive and attractive guidance molecules. Netrin-5 is a new member of the netrin family with homology to the C345C domain of netrin-1. Unlike other netrin proteins, murine netrin-5 consists of two EGF motifs of the laminin V domain (LE) and the C345C domain, but lacks the N-terminal laminin VI domain and one of the three LE motifs. We generated a specific antibody against netrin-5 to investigate its expression pattern in the rodent adult brain. Strong netrin-5 expression was observed in the olfactory bulb (OB), rostral migrate stream (RMS), the subventricular zone (SVZ), and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, where neurogenesis occurs in the adult brain. In the SVZ and RMS, netrin-5 expression was observed in Mash1-positive transit-amplifying cells and in Doublecortin (DCX)-positive neuroblasts, but not in GFAP-positive astrocytes. In the OB, netrin-5 expression was maintained in neuroblasts, but its level was decreased in NeuN-positive mature neurons. In the hippocampal SGZ, netrin-5 was observed in Mash1-positive cells and in DCX-positive neuroblasts, but not in GFAP-positive astrocytes, suggesting that netrin-5 expression occurs from type 2a to type 3 cells. These data suggest that netrin-5 is produced by both transit-amplifying cells and neuroblasts to control neurogenesis in the adult brain.

Keywords: axon guidance, netrin, adult neurogenesis, rostral migratory stream, subventricular zone, subgranular zone

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Introduction

Netrin family proteins are diffusible axon guidance molecules. Originally netrin-1 was identified as a chemical attractant for spinal commissural axons during embryonic development (Serafini et al., 1994, 1996). Netrin-1 has a homology to the laminin B2 chain and consists of the N-terminal laminin VI domain, three EGF motifs of the laminin V domain (LE), and the C345C domain. Netrin-1 (Unc-6 in nematodes) is evolutionally well conserved in both vertebrates and invertebrates (Rajasekharan and Kennedy, 2009). Mammals express netrin-1, -3, and -4, whereas netrin-2, the ortholog of netrin-3, exists only in birds and in fish (Wang et al., 1999). The functions of netrin

Abbreviations: AD, Alzheimer's disease; DCC, deleted in colorectal carcinomas; DCX, doublecortin; DG, dentate gyrus; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; LE, EGF motifs of laminin V domain; OB, olfactory bulb; RMS, rostral migratory stream; SVZ, subventricular zone; SGZ, subgranular zone.

proteins are quite variable. In addition to regulating axonal guidance as both attractive and repulsive cues, netrin proteins promote cell survival by binding to DCC (deleted in colorectal carcinoma) and/or to the Unc5 dependent receptor for both neurons and tumor cells (Arakawa, 2004; Adams and Eichmann, 2010; Castets and Mehlen, 2010). Netrin-1 is also an angiogenic factor that acts as a guidance molecule and promotes proliferation of endothelial cells (Delloye-Bourgeois et al., 2012). Recently, it was reported to promote atherosclerosis by inhibiting the emigration of macrophages from plaques and by attenuating hypoxia-elicited inflammation at mucosal surfaces (Ramkhelawon et al., 2014).

Neurogenesis in the adult brain occurs throughout life at two major locations: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Zhao et al., 2008). In the anterior of the SVZ, neuroblasts, derived from progenitor cells, form chains and migrate along a restricted route, referred to as the rostral migrate stream (RMS). During the migration, the stabilization, and destabilization of the microtubules in the neuroblasts are regulated by DCX and stathmin1, respectively. Disruption of either DCX or stathmin1 disturbed the chain migration in the RMS (Camoletto et al., 1997; Jin et al., 2010). Subsequently neuroblasts terminate migration and localize in the olfactory bulb (OB), where they differentiate into interneurons (Doetsch et al., 1999; Alvarez-Buylla and Garcıa-Verdugo, 2002). The chains of neuroblasts migrate through a tunnel of GFAP-positive astrocytes called the "glial tube" which are derived from type B cells. During the migration, neuroblasts are guided by various molecules. Soluble netrin-1 is secreted from the mitral cells in the OB to attract DCC-positive neuroblasts (Murase and Horwitz, 2002). Inhibiting DCC by an antibody disrupted the direction of the migrating chain of cells. Another netrin-1 receptor, neogenin, is known to be expressed in both neuroblasts and GFAP-positive astrocytes in the RMS of both rodents and humans (Bradford et al., 2010). Neural stem cells in another region of the brain, the SGZ in the adult hippocampus, can produce intermediate progenitors (type 2a cells), which in turn generate neuroblasts (type 2b to type 3 cells) (Eriksson et al., 1998). Recently, it was shown that DCX-positive type 2b cells can also proliferate and expand the precursor pool (Lugert et al., 2012). After exiting the cell cycle, these cells differentiate into granule neurons. Although much is known about adult neurogenesis in the SGZ, nothing is known about the contribution of netrins and their receptors to neurogenesis.

We previously characterized new functions of FLRT2/3 as novel repulsive axon guidance molecules acting via Unc5s (Yamagishi et al., 2011). In order to identify additional axon guidance molecules, we performed BLAST analyses to search for proteins with sequences homologs to netrin-1. Here, we report an uncharacterized protein, netrin-5, found on the NCBI database, which was previously named due to its homology to other netrin family proteins. We show that netrin-5 is strongly expressed in neuroproliferative regions, namely in the SVZ and SGZ. Netrin-5 is co-expressed with Mash1, DCX, and stathmin1, which regulates microtubule stability, in neuroblasts in both the SVZ and RMS, whereas GFAP-positive cells do not co-express netrin-5. Consistent with these findings, Mash1-positive cells and DCX-positive neuroblasts in the SGZ also co-expressed netrin-5, indicating netrin-5 expression occurs from type 2a to type 3 cells. These expression patterns suggest that netrin-5 plays a role in adult neurogenesis.

Materials and Methods

Animals

Wister rats (Japan SLC Inc.) aged 2–3 months were used in most of the experiments unless otherwise stated. Two month old C57BL/6 mice (Japan SLC Inc.) were used for anti-CD31 (PECAM1) antibody staining. Glial fibrillary acidic protein (GFAP) expression was analyzed using 2 month old transgenic mice overexpressing GFP under control of the astrocyte-specific GFAP promoter (*Gfap-EGFP* mice, Mutant Mouse Regional Resource Center; Kaneko et al., 2010). All animal experiments were approved by the Committee on Animal Research at Hamamatsu University School of Medicine and performed according to the national guidelines and regulations in Japan.

Antibodies

To generate polyclonal antibodies, rabbits were immunized once and boosted 3 times with a synthetic peptide (C+FKQRAWPVRRGGQE; 353-366 aa) corresponding to the sequence of the C345C domain of mouse netrin-5a (NP_001028528.2). Antibodies were purified from serum using an antigen-specific affinity column (Operon Biotech.). The antigen sequence was chosen by the antigen prediction program (Operon Biotech). The following commercial antibodies were also used for immunofluorescence and western blotting: anti-GAPDH (1:5000, mouse monoclonal, Abcam, ab8245), anti-DCX (1:500, goat polyclonal, Santa Cruz, sc-8066), anti-stathmin1 (1:500, mouse monoclonal, GeneTex, GTX62235), anti-Mash1 (1:500, mouse monoclonal, BD, #5566045), anti-GFAP (1:1000, mouse monoclonal, Millipore, MAB3402), anti-GFP (JL-8; 1:500, mouse monoclonal, Clontech, 632381), anti-NeuN (1:100, mouse monoclonal, Millipore, MAB377), and anti-CD31 (PECAM1, 1:100, rat monoclonal, BD, #550274) antibodies.

Histological Analysis

The animals were deeply anesthetized and intracardially perfused with 4% paraformaldehyde (PFA)/PBS for 5 min. Brains were dissected, post fixed in 4% PFA/PBS for 10 min, and frozen at −80°C. Cryostat sagittal sections at 20 μm thickness were fixed in 4% PFA/PBS for 10 min. For anti-Mash1 antibody staining, 50 μm thick vibratome sections were cut. Endogenous peroxidase was quenched in 0.3% H_2O_2 in methanol for 20 min at RT. After washing with PBS, the sections were permeabilized in 0.3% Triton-X 100/PBS for 3 min. Then, the sections were incubated with blocking solution containing 3% normal goat serum (NGS) in 0.1% Triton-X 100/PBS for 1 h at RT followed by incubation of primary antibodies in 1% NGS/0.1% Triton-X 100/PBS overnight at 4°C. Either HRP-conjugated (DAKO) or Alexa Fluor dye-conjugated secondary antibodies (Life Technologies) were incubated with the sections for 30 min at RT. For anti-Mash1

antibody staining, tyramide Signal Amplification (TSA) amplification was performed according to manufacturer's instructions (PerkinElmer, NEL701001KT). The sections treated with HRP-conjugated antibody were visualized by ImmPACT DAB (DAKO) according to the manufacturer's instructions. The samples from the *Gfap-EGFP* mice were treated with anti-GFP (JL-8) antibody to amplify the GFP signal. The sections with fluorescent secondary antibodies were observed using confocal microscopy FV1000 (Olympus). Similar results were obtained from at least three independent animals.

Western Blotting

Total cell lysates from different brain regions were prepared in lysis buffer, consisting of 1% Triton X-100/TBS supplemented with a proteinase inhibitor cocktail (Roche). The samples from three rats were pooled and resolved on a SDS-PAGE gel. Then, the proteins were transferred to a PVDF membrane and the membrane was incubated sequentially with blocking solution, primary antibody, and HRP-conjugated secondary antibody. The membrane was developed with the enhanced chemiluminescence (ECL) reagent (Thermo Scientific).

Results

Netrin-5 Homology to Netrin-1

In order to search for a new axon guidance protein homologs to murine netrin-1, we performed protein BLAST analysis with the mouse netrin-1 C345C domain (472-601 aa), which is partially involved in binding to the Unc5 receptor (Kruger et al., 2004). As a result, netrin-5 isoform c (accession no. NP_001276622; 383 aa) showed the highest score with 32% identity to netrin-1. Unlike the structures of netrins-1, -3, and -4, full length netrin-5a (NP_001028528.2) consists of 452 aa comprising two laminin V-type EGF-like (LE) domains and the C345C domain, but lacking the N-terminal laminin VI domain (**Figure 1A**). A splice variant, netrin-5b, has a unique short tail at the C-terminus instead of the C345C domain. Netrin-5c is shorter than this isoform because it lacks the N-terminal sequence but contains 3 LE domains. Netrin-5s from both human and bovine are different from rodent netrin-5. Human and bovine netrin-5 has a shorter signal peptide but contains all three LE domains (**Figure 1B**). Many amino acids in both the LE1 and LE3 domains are highly conserved among these 4 species. Homology between human and mouse netrin-5 is 73%. Interestingly, most cysteine residues are highly conserved between netrin-1 and netrin-5, suggesting that the 3D structure of netrin-5 might be similar to that of netrin-1. Netrin-5 exists only in mammals, suggesting that it was evolutionarily acquired later than the other netrin family proteins.

Netrin-5 Expression in the Adult Brain

In order to explore the expression pattern of netrin-5 in adult rat brain, we generated an antibody against the C345C domain. The level of netrin-5 protein in the adult rat brain was first evaluated by immunodetection on western blotting using a rabbit polyclonal antibody against the C345C domain. Netrin-5 appeared at ∼46 kDa and is expressed in the OB, the cerebral cortex, the hippocampus, and the cerebellum in the adult brain (**Figure 2A**). In the OB, the expression level was relatively higher than that in other regions. In the hippocampus, netrin-5 showed a weak expression level. Consistent with the western blotting, immunohistochemistry on sagittal sections showed extensive expression in the OB (**Figure 2B**). Netrin-5 immunoreactivity was observed throughout the granule cell layer of the OB. In the rostral migratory stream (RMS), the immunoreactivity was strong. However, a weaker signal in the outer plexiform layer and almost no signal were observed in the anterior olfactory nucleus. In accordance with the strong immunoreactivity in the RMS of the OB, strong netrin-5 expression was also observed throughout the RMS from the anterior wall of the SVZ of the lateral ventricle (**Figure 2C**). In addition, the choroid plexus had a positive signal. Non-clustered netrin-5 positive signal was observed in both the corpus callosum and hippocampal commissure (**Figures 2C,D**). Sparse immunoreactivity was observed in both the striatum and the cerebral cortex. These cells were positive for CD31, a vascular endothelial marker (**Figure S1**). Only a subset of endothelial cells showed netrin-5-immunoreactivity. Since netrin-5 is strongly expressed in both the SVZ and RMS, it may play a role in adult neurogenesis. Therefore, we next focused on the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, the other neurogenic region in the adult brain. Although the immunoreactivity in the hippocampus was relatively low by western blotting, netrin-5 signal was specifically observed in the SGZ as expected (**Figures 2D,E**). There were few positive cells in the pyramidal cell layer of both the CA1 and CA3 regions and in the hilus region.

Netrin-5 Expression in Transit-amplifying Cells (Type C Cells) and Neuroblasts (Type A Cells), but not in Neural Stem Cells (Type B Cells), in the SVZ

In the adult SVZ, a subset of the astrocyte-like cells (type B cells) are proliferative stem cells that generate neuroblasts (type A cells) via transit-amplifying cells (type C cells). The majority of netrin-5 immunoreactive cells in the SVZ showed a morphology typical of the chain-forming neuroblasts. In order to determine the netrin-5-expressing cell types in the SVZ, we performed double-immunolabeling in a sagittal section of the rat adult brain using antibodies against netrin-5 and DCX, a microtubule binding protein widely used as a marker for neuroblasts (Zhao et al., 2008). As shown in **Figures 3A–C**, all DCX-positive cells at the SVZ co-expressed netrin-5. There were some netrin-5-positive SVZ cells that did not co-express DCX, suggesting that netrin-5 expression is initiated in DCX-negative type C cells. We also stained for the microtubule binding protein stathmin1, which is expressed in the SVZ cells, including neuroblasts (Camoletto et al., 1997; Jin et al., 2004). Double immunostaining showed complete co-localization of stathmin1 and netrin-5 (**Figures 3D–F**). Since stathmin1 expression is regulated by Mash1 (Ascl1) (Yamada et al., 2010), we then evaluated netrin-5 expression in Mash1-positive transit-amplifying cells (**Figures 3G–I**). As a result, all Mash1 positive cells were netrin-5 positive, indicating that transit-amplifying cells also express netrin-5 (**Figures 3G–I**). Next, we determined whether netrin-5 expression occurs in type B cells. Netrin-5 was not detected in GFAP-positive cells (type-B cells or other astrocytes; **Figures 3J–L**). Taken together, these results suggest that

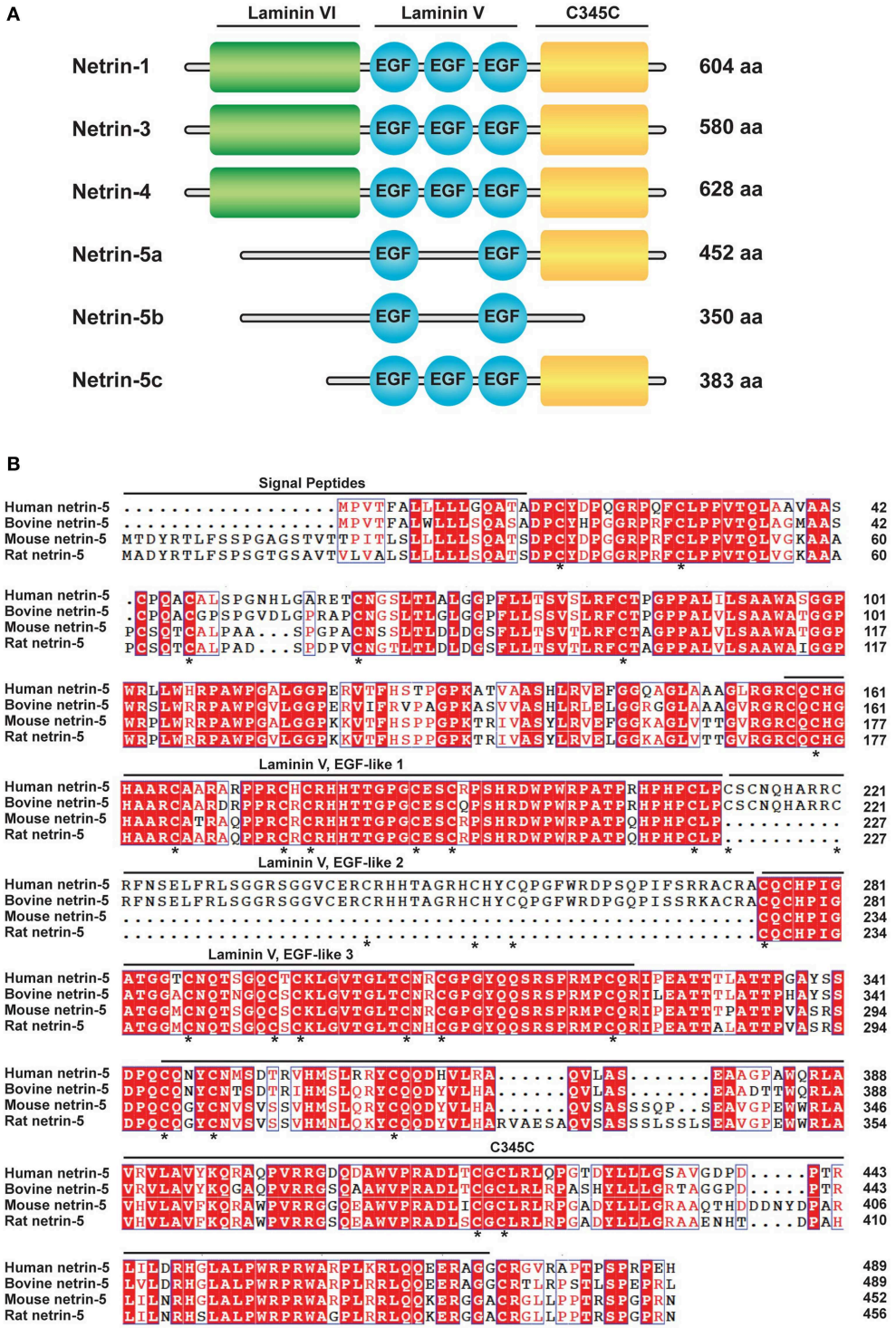

FIGURE 1 | Netrin-5, a new member of the netrin protein family. (A) Schematic drawing of the domain structure of the netrin family (*Mus musculus*; NTN1, 3, 4, and 5a-c). NTN5a and NTN5b lack the laminin VI domain and one of EGF-like motifs of the laminin V domain. NTN5b further lacks the C345C domain. NTN5c is smaller than NTN5a but contains three EGF-like domains. (**B**) Alignment of the predicted amino acid sequences of Homo sapiens NTN5 (accession number NP_665806.1), Bos taurus NTN5 (NP_001193801.1), *Mus musculus* NTN5 isoform a (NP_001028528.2), and *Rattus norvegicus* NTN5 (NP_001166997.1). Amino acid residues identical among the species are shaded in red. Similar residues are colored in red but not shaded. Putative signal peptides, EGF-like motifs of the laminin V domain, and the C345C domain are indicated. An asterisk (*) indicates conserved cysteine in netrin-1 and in netrin-5.

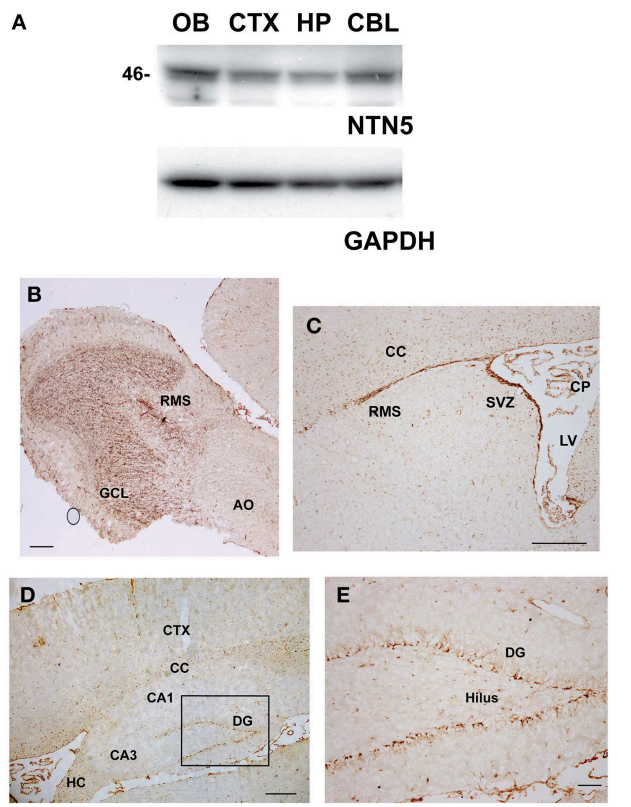

FIGURE 2 | Expression of netrin-5 in the adult brain. (A) Immunoblotting analysis revealed that anti-netrin-5 antibody recognizes a protein of ~46 kDa in the total cell lysate of the dissected adult rat brains. Netrin-5 expression was higher in the olfactory bulb and lower in the hippocampus. The same membrane was reprobed with anti-GAPDH antibody as a loading control (lower panel). **(B–E)** Immunohistochemistry of sagittal sections of adult rat brain with anti-netrin-5 antibody. Anti-netrin-5 antibody stained strongly in the GCL of the OB **(B)**, SVZ, RMS, CP **(C)**, and SGZ of the DG **(D,E)**. AO, anterior olfactory nucleus; CBL, cerebellum; CC, corpus callosum; CP, choroid plexus; CTX, cerebral cortex; DG, dentate gyrus; GCL, granule cell layer; HP, hippocampus; OB, olfactory bulb; RMS, rostral migratory stream; SGZ, subgranular zone; SVZ, subventricular zone. Bars indicate 500 μm in **(B–D)**, and 100 μm in **(E)**.

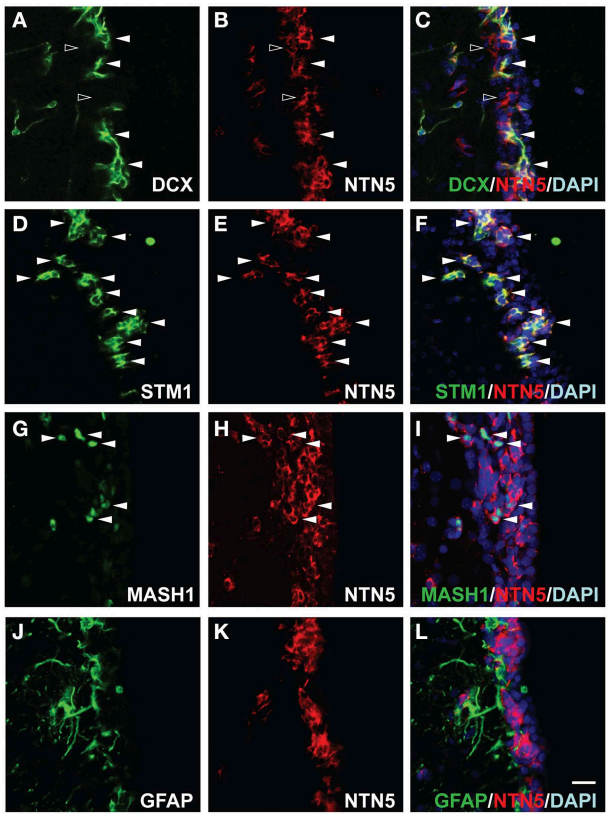

FIGURE 3 | Expression of netrin-5 in the SVZ of adult rat brain. (A–C) Immunostaining of sagittal sections of adult rat brain revealed that DCX-positive cells in the SVZ stained for netrin-5 as observed in confocal imaging (white arrowheads). Some netrin-5-positive cells were DCX-negative (black arrowheads). (**D–F**) Stathmin1 and netrin-5 showed high co-localization (white arrowheads). (**G–I**) Mash1-positive cells are also netrin-5-positive (white arrowheads). (**J–L**) Netrin-5 expression did not co-localize with GFAP. Bar indicates 50 μm.

netrin-5 is expressed in transit-amplifying cells and in neuroblasts but not in either neural stem cells or astrocytes in the SVZ.

Netrin-5 Expression in Neuroblasts in the RMS

Since the newly formed neuroblasts in the SVZ migrate toward the anterior region through the RMS, we next characterized the netrin-5-positive cells in the RMS. Consistent with the result in the SVZ, we found DCX-positive cells highly co-localized with the netrin-5 signal in the RMS (**Figures 4A–C**). In contrast to the homogeneous expression of DCX in the neuroblasts, netrin-5 was expressed at various levels in the cells. There were some netrin-5-positive cells that did not co-express DCX. On the other hand, almost all of the netrin-5-positive cells showed co-localization with stathmin1 (**Figures 4D–F**). Mash1-positive transit-amplifying cells were also positive for netrin-5 (**Figures 4G–I**), suggesting that the DCX-negative- and netrin-5-positive cells (**Figures 4A–C**) were transit-amplifying cells. The chains of rapidly migrating neuroblasts are ensheathed by a meshwork of GFAP-positive astrocytes, namely the "glial tube" (Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Kaneko et al., 2010). Although GFAP and netrin-5 immunoreactive signals are spatially intermingled, none of the GFAP-positive signal co-localized with netrin-5 (**Figures 4J–L**). Since GFAP is a cytoskeletal protein and anti-GFAP antibody staining cannot visualize the entire shape of the astrocytes, we utilized GFAP promoter-controlled EGFP transgenic mice (*Gfap-EGFP*). As expected, most of netrin-5-positive cells are GFP-negative, confirming the mutually exclusive expression of netrin-5 and GFAP (**Figures 4M–O**).

Netrin-5 Expression in both Neuroblasts and Neurons in the GCL of the OB

The migrated neuroblasts are differentiated into GABAergic neurons and integrated in the GCL of the OB (Abrous et al., 2005). Since the strong immunoreactivity of netrin-5 was observed

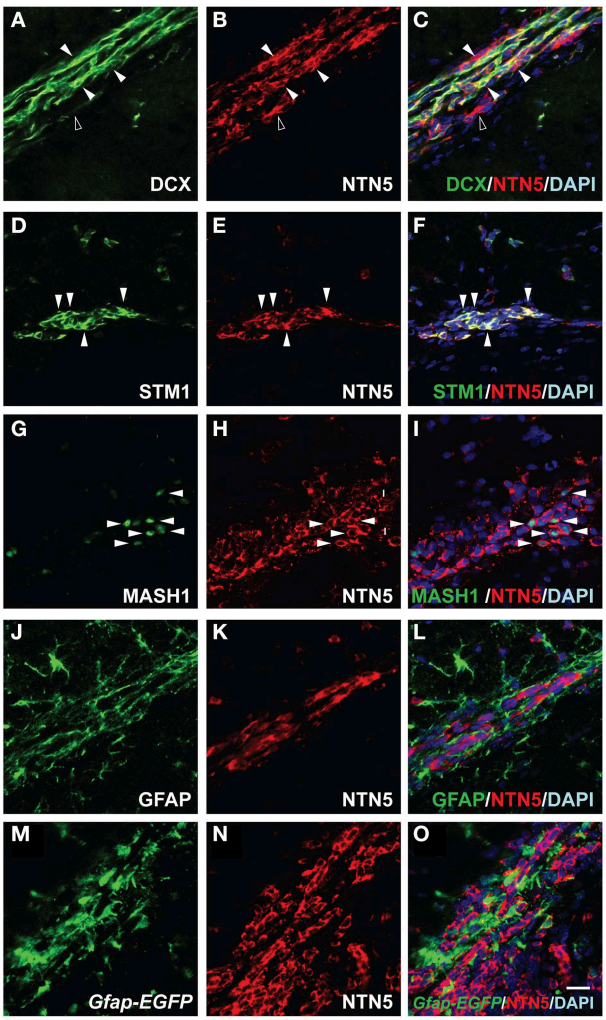

FIGURE 4 | Netrin-5 expression by neuroblasts and transit-amplifying cells but not by astrocytes within the RMS. (**A–C**) Immunostaining of sagittal sections of rat adult brain showed that most of the DCX-positive cells in the RMS stained for netrin-5 as observed in confocal imaging (white arrowheads). Some netrin-5-positive cells were DCX-negative (black arrowhead). (**D–F**) Stathmin1 and netrin-5 showed high co-localization (white arrowheads). (**G–I**) Anti-Mash1 staining revealed that Mash1-positive cells express netrin-5 (white arrowheads). (**J–L**) Anti-GFAP staining showed that netrin-5 was not expressed in astrocytes. (**M–O**) Most *Gfap-EGFP* cells did not show netrin-5 expression. (**A–L**) adult rat brain and (**M–O**) adult *Gfap-EGFP* mouse brain. Bar indicates 50 μm.

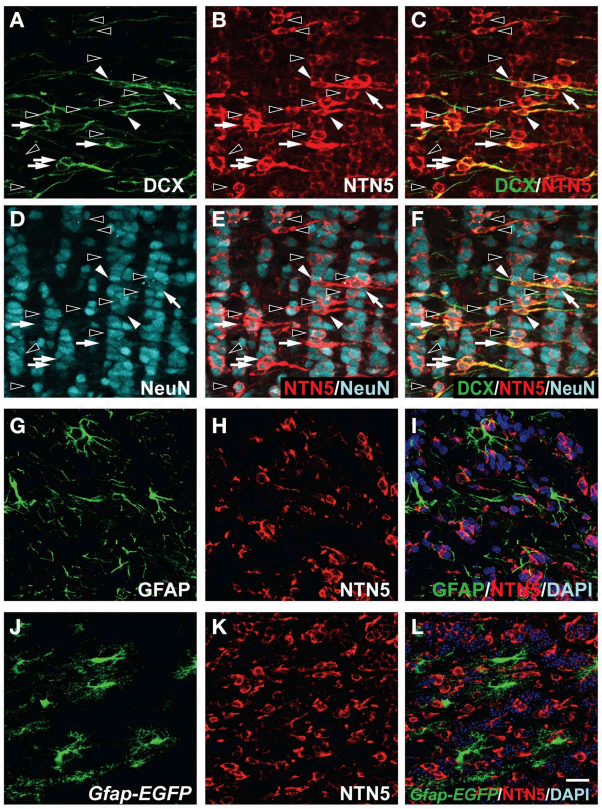

FIGURE 5 | Expression of netrin-5 in the GCL of the OB in the adult brain. (**A–F**) Triple-immunostaining of sagittal sections of adult rat brain using anti-DCX, anti-netrin-5, and anti-NeuN antibodies. Netrin-5 was highly expressed in Dcx+ neuroblasts (white arrowheads), Dcx+NeuN+ immature neurons (white arrows), and a subset of NeuN+ mature neurons (black arrowheads), and decreased but still detectable in most NeuN+ mature neurons. (**G–I**) Anti-GFAP staining showed that netrin-5 was not expressed in astrocytes. (**J–L**) *Gfap-EGFP* cells did not show netrin-5 expression. (**A–I**) adult rat brain and (**J–L**) adult *Gfap-EGFP* mouse brain. Bar indicates 50 μm.

in the GCL (**Figure 2B**), we attempted to determine the cell type in the GCL. The netrin-5 signal was observed in the cell body and in the basal part of the apical dendrites of the DCX-positive new neurons (**Figures 5A–C**). Since only about 20% of the netrin-5-positive cells in the GCL co-expressed DCX, we next analyzed whether netrin-5 expression is present in mature neurons. As expected, the DCX-negative- and netrin-5-positive cells were NeuN-positive, indicating that netrin-5 is also expressed in mature GCs (**Figures 5D–F**). The expression level of Netrin-5 is stronger in the DCX-positive cells than that in the NeuN-positive cells, suggesting that netrin-5 expression is decreased during the maturation process. Consistent with the exclusive expression of GFAP and netrin-5 in both the SVZ and RMS, we could not observe co-localization of GFAP and netrin-5 in the GCL of the OB (**Figures 5G–L**), indicating that netrin-5 is not expressed by astrocytes.

Netrin-5 Expression in Type 2a to Type 3 Cells in the SGZ of the Hippocampal Dentate Gyrus

Since DCX-positive cells showed netrin-5 immunoreactivity in the SVZ, RMS, and the GCL of the OB, we next analyzed the other major location of adult neurogenesis in rat brain, the subgranular zone (SGZ) of the hippocampal dentate gyrus (Eriksson et al., 1998). As shown in **Figures 6A–C**, all DCX-positive cells showed co-localization with the netrin-5 signal. In the same field, anti-NeuN staining revealed that most of netrin-5-positive cells contain undetectable or very low levels of NeuN (**Figures 6D–F**). This was in contrast to the OB where netrin-5 expression remained in mature neurons (**Figures 5D–F**). Most of netrin-5-positive cells also contain stathmin1 (**Figures 6G–I**),

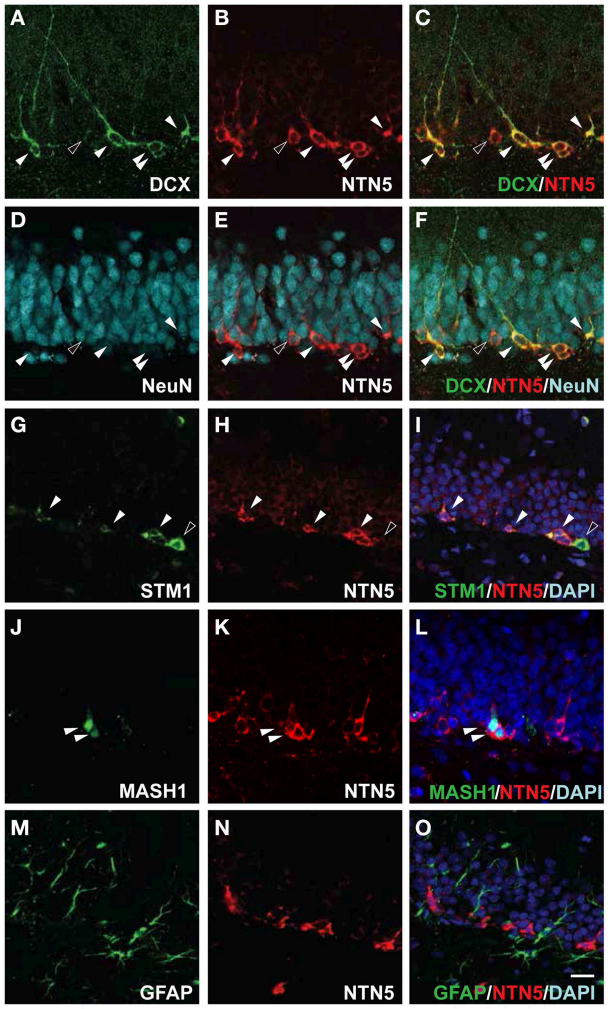

FIGURE 6 | Netrin-5 expression by type 2 a to type 3 cells but not by type 1 cells within the SGZ. (**A–F**) Triple-immunostaining of sagittal sections of adult rat brain with anti-DCX, anti-netrin-5, and anti-NeuN antibodies. DCX-positive cells in the SGZ were stained for netrin-5 as observed in confocal imaging (white arrowheads). Small populations of netrin-5-positive cells were DCX-negative, NeuN-low (black arrowhead). Please note that most of NeuN-positive cells were netrin-5 negative. (**G–I**) Stathmin1 and netrin-5 showed partial co-localization (white arrowheads). (**J–L**) Anti-Mash1 staining revealed that Mash1-positive cells express netrin-5 (white arrowheads). (**M–O**) Anti-GFAP staining showed that netrin-5 was not expressed in astrocytes. Bar indicates 50 μm.

similar to that in both the SVZ and RMS. Next, we analyzed the expression of Mash1 in netrin-5-expressing cells. Consistent with the observation in both the SVZ and RMS, netrin-5 was also observed in Mash1-positive transient-amplifying cells (**Figures 6J–L**). Finally, we analyzed netrin-5 expression in neural stem cells (type 1 cells). Double-immunostaining for both GFAP and netrin-5 revealed that the GFAP-positive signal did not co-localize with netrin-5 (**Figures 6M–O**). Consistent with this result, none of the GFAP promoter-derived GFP-positive cells merged with netrin-5-positive cells (data not shown). Taken together, these results indicate netrin-5 is expressed from type-2a through to type-3 cells during adult neurogenesis in the hippocampus.

Discussion

Here, we have characterized a new netrin family member, netrin-5, which is expressed in neuroproliferative zones and is associated with migratory pathways in the adult brain, i.e., the SVZ, RMS, OB, and SGZ. It is still unclear whether netrin-5 is a secreted or a cytosolic protein. According to the computational prediction based on the databases, netrin-5 has a putative 34 amino acids signal peptide on the N-terminus for secretion. In addition, 11 disulfide bonds and N-glycosylation sites are suggested. Given that netrin-5 is secreted, it is uncertain which receptor(s) it recognizes. Netrin-1 and -3 bind to DCC, Unc5s, DSCAM, and Neogenin (Keino-Masu et al., 1996; Wang et al., 1999; Ly et al., 2008; Lai Wing Sun et al., 2011). Netrin-1 shows higher affinity to DCC than that of netrin-3 (Wang et al., 1999). Netrin-4 does not bind to the DCC receptor but binds to various integrin family proteins, i.e., integrin α6β1, α6β4, α2β1, and α3β1 (Staquicini et al., 2009; Larrieu-Lahargue et al., 2011; Yebra et al., 2011; Hu et al., 2012). The binding of netrin-4/integrin is mediated by the unique N-terminal laminin VI domain of netrin-4, which is not conserved in netrin-1 and −3. Since netrin-5 is lacking this laminin VI domain, it may not bind to DCC, Unc5s, and integrins. On the other hand, the deletion of the laminin VI domain of netrin-1 did not affect the binding to DCC (Kruger et al., 2004). Interestingly, three binding sites on the netrin-1/DCC complex have been identified by crystal structure analysis (Finci et al., 2014; Xu et al., 2014). Among those binding sites, the laminin VI domain and LE3 domain of netrin-1 bind to DCC FN5 and FN4 domains, respectively, resulting in a continuous netrin-1/DCC assembly as proposed by Xu et al. (2014). Furthermore, Kruger et al. (2004) mapped netrin-1/Unc5c binding sites and found multiple binding sites on netrin-1, including the C345C domain. Therefore, the LE3 domain and C345C domain of netrin-5 might bind to DCC and to Unc5s, respectively. Those binding analyses should be performed in the future to identify the receptors for netrin-5.

Interestingly, netrin-5 is strongly expressed in the SVZ, RMS, and SGZ of the DG in the hippocampus where neurogenesis occurs in the adult brain (**Figures 2B–D**). Netrin-5 is also expressed in the choroid plexus, where netrin-1 is also expressed (Lein et al., 2007), and in both the corpus callosum and the hippocampal commissure. In the RMS, a major population of netrin-5-positive cells express Mash1, DCX, and stathmin1, namely in transit-amplifying cells and in neuroblasts, but it is not expressed in the GFAP-positive astrocytes, suggesting the involvement of netrin-5 in neurogenesis. Consistently, netrin-5 expression is decreased during neuronal maturation in both the GCL and the DG. On the other hand, NeuN-positive neurons also express weak levels of netrin-5 in the GCL, suggesting that netrin-5 might have a function during the maturation process, such as dendritogenesis and synaptogenesis in GABAergic neurons.

The netrin-1 receptors, DCC and neogenin, are also expressed in neuroblasts in both the RMS and SVZ (Murase and Horwitz, 2002; Bradford et al., 2010). In contrast to netrin-5, Netrin-1 is

not detected in the RMS but is located on the outer border of the most ventral end of the descending limb of the RMS and in mitral cells in the OB (Bradford et al., 2010). Inhibition of DCC function by a blocking antibody disturbs the direction of neuroblast migration and reduces the speed of migration (Murase and Horwitz, 2002). Therefore, we hypothesize that this DCC-dependent migration may be also mediated by netrin-5 acting either in an autocrine or paracrine manner. DCC is also known as a dependent receptor, meaning that DCC-positive cells undergo apoptosis without their ligand. Since netrin-1 expression is limited in the anterior region of the RMS, another ligand is needed to maintain the survival of the neuroblasts. We hypothesize that netrin-5 binds to DCC to prevent cell death. In order to clarify the molecular function of netrin-5 as a guidance molecule, *in vitro* analysis, such as binding assays to define receptors, a turning assay, and a growth cone collapse assay are necessary. In addition, loss and/or gain of function studies using animal models will be needed to better understand the *in vivo* functions of netrin-5.

Netrin-1 is well characterized as a repulsive guidance cue during angiogenesis via the Unc5B receptor, which is expressed in arterial endothelial cells, sprouting capillaries, and tip cells (Lu et al., 2004). UncB activation by Netrin-1 causes retraction of the filopodia of the endothelial tip cells and inhibits the neovessel sprouting processes. Since netrin-5 expression was observed in a subpopulation of CD31-positive endothelial cells, netrin-5 might have a role in guiding angiogenesis as a repulsive guidance cue acting either in an autocrine or paracrine manner via Unc5B.

A number of studies revealed that cognition and adult neurogenesis are highly correlated in rodents (Mu and Gage, 2011). Indeed, many mouse models of Alzheimer's disease (AD), such as Presenilin-1 with both M146V and ΔExon9 mutations in knock-in mice and transgenic mice with the Swedish APP mutation, showed decreased adult neurogenesis (Wang et al., 2004; Zhang et al., 2007; Choi et al., 2008). Even in humans, substantial neurogenesis occurs in the hippocampus of the adult brain (Spalding et al., 2013). Although it is still controversial whether the proliferative ratio of neural stem cells is altered, neuronal maturation is impaired in the DG of AD patients (Li et al., 2008). Recently, it has been reported that new neurons are generated in the adult human striatum, which is impaired in patients with Huntington's disease (Ernst et al., 2014). Therefore, it would be of interest to investigate netrin-5 expression in the neurogenic regions in the human brain especially in patients with neurodegenerative disorders.

Although it is widely accepted that new neurons are continuously generated and incorporated into the functional neural network of the adult brain, the neurogenic cues, and the soluble factors involved in the proper transportation of neuroblasts to their appropriate location are still unclear. Our finding of the expression of netrin-5 in neurogenic regions may provide some hints toward understanding the fundamental processes (proliferation, migration, and differentiation) in adult neurogenesis.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2015.00146/abstract

Figure S1 | Netrin-5 is expressed in vascular endothelial cells in both the cerebral cortex and the striatum. (**A–F**) Immunostaining of sagittal sections of adult rat brain with anti-CD31 and anti-netrin-5 antibodies in the cerebral cortex (**A–C**) and in the striatum (**D–F**). Please note that netrin-5 is expressed in a small population of vascular endothelial cells. White and black arrowheads indicate co-localization and non-co-localization, respectively. Bar indicates 50 μm **(A–F)**.

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SVCT2 vitamin C transporter expression in progenitor cells of the postnatal neurogenic niche

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Known as a critical antioxidant, recent studies suggest that vitamin C plays an important role in stem cell generation, proliferation and differentiation. Vitamin C also enhances neural differentiation during cerebral development, a function that has not been studied in brain precursor cells. We observed that the rat neurogenic niche is structurally organized at day 15 of postnatal development, and proliferation and neural differentiation increase at day 21. In the human brain, a similar subventricular niche was observed at 1-month of postnatal development. Using immunohistochemistry, sodium-vitamin C cotransporter 2 (SVCT2) expression was detected in the subventricular zone (SVZ) and rostral migratory stream (RMS). Low co-distribution of SVCT2 and βIII-tubulin in neuroblasts or type-A cells was detected, and minimal co-localization of SVCT2 and GFAP in type-B or precursor cells was observed. Similar results were obtained in the human neurogenic niche. However, BrdU-positive cells also expressed SVCT2, suggesting a role of vitamin C in neural progenitor proliferation. Primary neurospheres prepared from rat brain and the P19 teratocarcinoma cell line, which forms neurospheres *in vitro*, were used to analyze the effect of vitamin C in neural stem cells. Both cell types expressed functional SVCT2 *in vitro*, and ascorbic acid (AA) induced their neural differentiation, increased βIII-tubulin and SVCT2 expression, and amplified vitamin C uptake.

Keywords: SVCT2, vitamin C, brain, niche, stem cells, progenitor, ependymal cells

INTRODUCTION

Active neurogenesis occurs within the anterior wall of the lateral ventricle in the adult mammalian brain (Lois and Alvarez-Buylla, 1993; Doetsch et al., 1999). Neurogenic precursors have also been found in the human brain, specifically located in the periventricular region before 18 months of age (Johansson et al., 1999; Nunes et al., 2003; Sanai et al., 2011; Bergmann et al., 2012). The formation of new neurons, which are βIII-tubulin-positive, occurs in restricted, organized compartments termed neurogenic niches (Doetsch et al., 1997, 1999; Alvarez-Buylla and Garcia-Verdugo, 2002; Conover and Notti, 2008; Mirzadeh et al., 2008; Nualart et al., 2012). The neuroblasts formed in this region migrate tangentially in chains throughout the rostral migratory stream (RMS), where the presence of neurogenic progenitors and astrocytes has also been described (Doetsch and Alvarez-Buylla, 1996). The neuroblasts present in the RMS reach the olfactory bulb, where they differentiate into interneurons (Lois et al., 1996; Alvarez-Buylla and Garcia-Verdugo, 2002; Lledo et al., 2008). Ultrastructural, immunohistochemistry, and proliferation analyses of the cytoarchitecture of the neurogenic niche (Doetsch et al., 1997) have revealed the presence of four cell types. B-type cells or astrocytes (GFAP- and nestin-positive) are preferentially located in the subventricular zone (SVZ) and are precursor cells. C-type cells are intermediate transient neuronal cells (nIPC) that proliferate rapidly (Eisch and Mandyam, 2007; Ihrie and Alvarez-Buylla, 2008) and differentiate into neuroblasts or type-A cells (Doetsch et al., 1999; Tramontin et al., 2003; Chojnacki et al., 2009; Kriegstein and Alvarez-Buylla, 2009; Nualart et al., 2012). E-type cells, which are cube-shaped and multiciliated, are ependymocytes. B-type cells are found in the ependymal layer, projecting cilium to the ventricular lumen, similar to what has been described in the radial glia (Tramontin et al., 2003; Spassky et al., 2005); they also have a close relationship with blood vessels (Mirzadeh et al., 2008). Precursor cells reactive to GFAP have been identified in the SVZ of the human brain (Roy et al., 2000; Gibbons and Dragunow, 2010), and some are in direct contact with the cerebrospinal fluid (CSF) (Sanai et al., 2004, 2011; Quinones-Hinojosa et al., 2006).

Vitamin C, which is present in high concentrations in the CSF (Spector and Lorenzo, 1974; Kratzing et al., 1985), may be important in postnatal neural differentiation. An important role for vitamin C in embryonic cerebral development and in the differentiation of dopaminergic and serotoninergic neurons has been described (Lee et al., 2000; Yan et al., 2001). Embryonic precursors supplemented with vitamin C show an increase in neural and glial markers (Lee et al., 2003). Recently, Esteban et al. (2010) found that vitamin C favored the generation of induced pluripotent stem cells (iPS) (Esteban et al., 2010). Furthermore, cells grown *in vitro* in the presence of vitamin C expressed two histone demethylases, Jhdm1a and Jhdm1b (Wang et al., 2011), which are required for iPS cell production. Together, these results suggest that vitamin C is able to positively regulate stem cell generation and proliferation.

The intracellular incorporation of ascorbic acid (AA) by neurons is carried out by SVCT2, the sodium and AA co-transporter (Daruwala et al., 1999; Castro et al., 2001; Hediger, 2002; Harrison and May, 2009; Nualart et al., 2012). This protein is formed by 12 transmembrane domains, with a molecular mass of ∼75 KDa (García et al., 2005). In the CNS, SVCT2 is expressed primarily in neurons of the cerebral cortex, hippocampus, and hypothalamus (Tsukaguchi et al., 1999; García et al., 2005); its expression has also been described in microglia (Mun et al., 2006) and tanycytes of the hypothalamus (García et al., 2005). In addition, functional SVCT2 was observed in cultures of embryonic rat cortical neurons (Castro et al., 2001; Astuya et al., 2005). Recently, SVCT2 mRNA expression was detected in radial glial cells of the fetal rat brain (Caprile et al., 2009). Moreover, SVCT2 knockout mice die at birth due to respiratory defects and cerebral hemorrhaging; low levels of AA in various tissues were also noted in SVCT2-null mice (Sotiriou et al., 2002). These data suggest that SVCT2 and vitamin C are important for normal nervous system development and neuronal maturation. The neurogenic niche stem cells are in contact with the CSF, which has high a concentration of vitamin C. Therefore, vitamin C may be a factor involved in stem cell differentiation; however, studies regarding the expression and distribution of the vitamin C transporter, SVCT2, in neural stem cells of the postnatal brain neurogenic niche and the effect of vitamin C on neuronal differentiation of stem cells from the periventricular areas of the brain have not been performed.

In this study, the expression of SVCT2 at the initial stages of differentiation of the ventricular neurogenic niche was analyzed in the rat brain. In addition, the distribution of SVCT2 in the human ventricular wall at 1 month postnatal development was assessed. Using P19 cells (an *in vitro* progenitor cell line with active proliferation) and primary neurospheres isolated from rat brain, SVCT2 expression and the effects of vitamin C on neural differentiation were determined.

MATERIALS AND METHODS

ANIMALS

Adult Sprague–Dawley rats and animals at 15–21 days postnatal development were used throughout the experiments. Animals were maintained in a 12 h light/dark cycle with food and water *ad libitum*. The handling of the animals was performed in agreement with the "Manual de Normas de Bioseguridad" (Comición Nacional de Ciencia y Tecnología, CONICYT), and the procedures were described in the publication entitled, "*Guide for Care and Use of Laboratory Animals*" (National Academy of Science, 2011; http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf). One month postnatal human brain tissue samples were obtained from archived samples previously fixed in 4% paraformaldehyde from the Department of Pathological Anatomy at Concepcion University. The samples were obtained in accordance with the accepted standards of the ethics committee on the use of human specimens and after informed consent was obtained from all patients.

IMMUNOHISTOCHEMISTRY AND CONFOCAL MICROSCOPY

Rat brain tissue samples were fixed in formalin at 10% v/v or in Bouin solution and embedded in paraffin after which 7-μm saggital sections were obtained. For the immunohistochemical analysis, the deparaffinized samples were incubated for 15 min in absolute methanol with 3% v/v H_2O_2. The sections were incubated with the following primary antibodies diluted in Tris-phosphate buffer and 1% bovine serum albumin: anti-PCNA (1:100 DAKO, Carpinteria, CA, USA); anti-Nestin (1:25 Amersham Pharmacia Bitech., Pittsburgh, PA, USA); anti-βIII-tubulin (1:500, Promega, Madison, WI, USA); anti-GFAP (1:200, DAKO); anti-PSA-NCAM (1:25 Hybridoma Bank, Iowa. IA, USA); anti-S100A (1-200, DAKO); and anti-SVCT2 (G19; 1:50, Santa Cruz Biotechnology, Sta. Cruz, CA, USA). The samples were then incubated with the appropriate secondary antibody conjugated to horse radish peroxidase (HRP), including HRP-conjugated goat anti-IgG, HRP-conjugated rat anti-IgG, and HRP-conjugated rabbit anti-IgG (ImmunoPure; PIERCE Biotechnology, Rockford, IL, USA). The enzymatic activity of the peroxidase was revealed with diaminobenzidine and H_2O_2. To perform inmunofluorescence analysis, secondary antibodies conjugated to different fluorophores, including Cy2-conjugated goat anti-IgG, Cy2- or Cy3-conjugated rat anti-IgG; and Cy3- or Cy5-conjugated rabbit anti-IgG (Jackson Immuno Research, Pennsylvania, USA), were used. The images (512 × 512 × 8 bits or 1024 × 1024 × 8 bits) were obtained using a confocal microscope.

In situ HYBRIDIZATION

A PCR product of 620 bp, which was obtained from pcDNA3-hSVCT2 that was subcloned into pCR-4-Blunt-TOPO (Clontech, Palo Alto, CA, USA), was used to generate sense and antisense digoxigenin-labeled riboprobes. RNA probes were labeled with digoxigenin-UTP by *in vitro* transcription with SP6 or T7 RNA polymerase following the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). *In situ* hybridization was performed on brain sections mounted on poly-L-lysine-coated glass slides. The sections were baked at 60°C for 1 h, deparaffinized in xylene, and rehydrated in graded ethanol. Following proteinase K treatment (5 min at 37°C in PBS, 1 mg/ml), the tissue sections were fixed with 4% paraformaldehyde at 4°C for 5 min, washed in cold PBS, and then acetylated with 0.1 M triethanolamine-HCl (pH 8.0) and 0.25% acetic anhydride at room temperature for 10 min. After a brief wash, the sections were incubated in pre-hybridization solution (Boehringer Mannheim) at 37°C for 30 min, and then 25 μl of hybridization mix [50% formamide, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 × Denhart's solution, 10% polyethylene glycol 8000, 10 mM DL-dithiothreitol (DTT), 500 mg yeast tRNA/ml, 50 mg/ml heparin, 5.0 mg/ml DNA carrier, and 1:20–1:100 dilutions of riboprobe) were added to each slide. The slides were covered with glass coverslips and placed in a humidified chamber at 42°C overnight. The slides were washed at 37°C for 30 min each in 2 × SSC, 1 × SSC, and 0.3 × SSC. Visualization of digoxigenin was performed by incubation with a monoclonal antibody coupled to alkaline phosphatase (anti-digoxigenin alkaline phosphatase Fab fragments diluted 1:500; Boehringer Mannheim) at room temperature for 2 h. Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) were used as substrates for the alkaline phosphatase. Controls

included use of the sense riboprobe and omission of the probe.

In vivo BrdU LABELING

Five intraperitonial injections of BrdU at a concentration of 40 mg/kg were administered to animals at 19 days postnatal development. Samples were obtained at 21 days postnatal development and fixed in Bouin, and the sections were obtained as previously described. Briefly, after denaturation of the DNA with 2N HCl for 30 min at 37°C, the sections were incubated with 5% BSA for 30 min and anti-BrdU (1:1000, Amersham Pharmacia Biotech) overnight. Detection was made by incubation with rat peroxidase-labeled anti-IgG or rat Cy3-labeled anti-IgG.

CELL CULTURE

The embryo-derived teratocarcinoma cell line, P19 (McBurney, 1993), was cultured in Minimum Essential Medium Eagle (MEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS), penicillin, and streptomycin. The neurospheres were generated in pretri dishes (10 cm in diameter) seeded with 1×10^6 cells maintained in culture for 4 days in (1) MEM (control), (2) MEM supplemented with 200 or 400 μM AA or (3) cultured in neurobasal-B27 (Gibco-BRL).

For neurosphere preparation, adult Sprague–Dawley rats were sacrificed by cervical dislocation ($n = 6$ per preparation), and their brains were removed. The lateral walls of the lateral ventricle were dissected and collected in DMEM-F12 (GIBCO). The cells were dissociated using a Pasteur pipette in NeuroCult NS-A (Stem Cells Technologies Inc), and cells were collected by centrifugation at 100 × g for 5 min. The cell pellets were resuspended in NeuroCult NS-A, supplemented with 20 ng/ml EGF, 10 ng/ml bFGF, 2 μg/ml heparine and proliferation supplement (Stem Cells Technologies Inc). The cells were cultured at 40,000 cells per cm^2 on uncoated dishes in the same medium. To analyze the effects of vitamin C, neurospheres were cultured for 7 days and were treated with 200 or 400 μM AA the last 4 days. The same concentration of vitamin C was added to the cultured cells each day.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Total RNA was isolated using Trizol (Invitrogen, Rockville, MD, USA). For RT-PCR, 1 μg of RNA was incubated in 10 μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 20 U RNase inhibitor, 1 mM dNTPs, 2.5 μM of oligo d(T) primers, and 50 units of MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA) for 10 min at 23°C followed by 30 min at 42°C and 5 min at 94°C. Parallel reactions were performed in the absence of reverse transcriptase to control for the presence of contaminant DNA. For amplification, a cDNA aliquot in a volume of 12.5 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.6 mM $MgCl_2$, 0.4 mM dNTPs, 0.04 units of *Taq* DNA polymerase (Gibco-BRL) and 0.4 mM primers was incubated at 95°C for 5 min, 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s for 35 cycles with a final extension at 72°C for 7 min. PCR products were separated by 1.2–1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. The following set of primers was used to analyze the expression of SVCT2: sense: 5′TGTTTCAGGCCAGTGCTTT 3′ and antisense: 5′GAAAGGATGGACGGCATACA 3′ (expected product of 457 bp). Additionally, the following sets of primers were used to analyze the expression of nestin and βIII-tubulin: nestin sense, 5′GGAGTCTCGCTTAGAGGTGC 3′ and antisense, 5′ CAGCAGAGTCCTGTATGTAGCC 3′ (expected product 103 bp) and βIII-tubulin sense, 5′ TTTATCTTCGGTCAGAGTGG 3′ and antisense, 5′ GAGCAGCAGTAGAAGTATGT 3′ (expected product 1500 bp).

WESTERN BLOT ANALYSIS

Membrane proteins from P19 cells or neurospheres were obtained by homogenizing the cells in 0.3 mM sucrose, 3 mM DTT, 1 mM EDTA, 1.0 mg/ml PMSF, 1 mg/ml pepstatin A, 2 mg/ml leupeptin, and 2 mg/ml aprotinin. Total membranes were collected by high-speed centrifugation. For immunoblotting, 50 mg of membrane protein was loaded in each lane and separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, transferred to PVDF membranes (0.45 μm pore, Amersham Pharmacia Biotech., Piscataway, NJ, USA) and probed against an anti-SVCT2 (1:100) antibody or a preabsorbed antibody (1:500) (García et al., 2005). The secondary antibody was rabbit anti-goat IgG coupled to peroxidase (1:5000). The reaction was developed with enhanced chemiluminescence according to the manufacturer's instructions (Amersham Corporation, Arlington Heights, IL).

VITAMIN C UPTAKE STUDIES

P19 cells were carefully selected under the microscope to ensure that only plates showing uniformly growing cells were used at 200,000 cells/well. Additionally, neurospheres isolated from rat brain were also used for vitamin C uptake. The cells were incubated in buffer containing 15 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgCl2 at room temperature for 30 min. Uptake assays were performed in 500 ml of incubation buffer containing 0.1–0.4 mCi of 1-^{14}C-AA (Dupont NEN, Boston, MA, USA, specific activity 8.2 mCi/mmol) to a final concentration of 100 mM. Data represent means ± SD of three experiments with each analysis performed in duplicate. To analyze the effect of AA on SVCT2 expression and function, P19 cells were pre-incubated with 400 mM AA for 4 days. Initial velocity transport assays were performed after incubation with 100 mM AA with or without 135 μM sodium chloride (replaced by sodium choline) or 20 μM cytochalasin B.

STATISTICAL ANALYSIS

For inhibition experiments, statistical comparisons between two or more groups of data were carried out using analysis of variance (ANOVA, followed by Bonferroni post-test). $P < 0.05$ was considered to be statistically significant. The statistical software, GraphPad Instat 3.0 (GraphPad Software, Inc., La Jolla, CA 92037 USA), was used for data analysis.

RESULTS

POSTNATAL NEUROGENIC NICHE DIFFERENTIATION

The ventricular neurogenic niche of postnatal brains at 15 days showed a similar cellular structure to that described in the adult

brain (Doetsch et al., 1997; Alvarez-Buylla and Garcia-Verdugo, 2002; Alvarez-Buylla and Lim, 2004). The ependymal cells were differentiated in the ventricular wall, and the SVZ was formed by different cell types (**Figure 1**). The migration of cells to the olfactory bulb, the RMS, was also assessed (**Figures 1A**, insets 1–2 and **B**). At 15 days, a positive reaction with anti-PCNA in the SVZ was observed (**Figures 1A**, inset 1, **C,G**); the ependymal cells were also labeled with anti-S100A (**Figures 1D**,**H**). Type-B precursor cells (GFAP-positive) were also detected in the SVZ (**Figures 1E,I**). βIII-tubulin expression was observed in neuroblasts (**Figures 1F**,**J**,**K**, arrows) surrounded by B-type cells reactive to GFAP (**Figures 1L**,**M**). GFAP-positive cells presented radial morphology, and their processes extended to the subventricular region (**Figures 1L**,**M**).

Mitotically active cells were next detected by *in vivo* BrdU administration. All the BrdU-positive cells were observed in the subventricular region (**Figure 1N**), a result that was confirmed by analysis with anti-S100, an ependymal cell marker (**Figures 1O**,**P**). Approximately, ten percentage of the BrdU-positive cells were also GFAP-positive (**Figures 1Q–S**). BrdU-positive cells were also detected at the RMS (**Figure 1 N,Q**).

EXPRESSION OF SVCT2 IN THE NEUROGENIC NICHE AND RMS

Using *in situ* hybridization, SVCT2 mRNA expression was observed in the anterior ventricular zone and RMS (**Figure 2A**). SVCT2 mRNA expression was also located in other areas of the brain, including neurons of the hippocampus (**Figure 2B**), cerebral cortex (**Figure 2D**), and the cerebellum (**Figure 2E**). As a negative control, the sense probe was employed; no reaction was observed in the different cerebral areas analyzed (**Figure 2C**). Furthermore, a positive reaction to astrocytes was not observed; however, ependymal cells presented an intense reaction in different regions of the ventricular wall (data not shown).

García et al. (2005) had demonstrated that SVCT2 mRNA expression did not directly correlate with its protein levels. Specifically, neurons presenting an intense *in situ* hybridization signal for SVCT2 showed a weak immunoreactivity with anti-SVCT2. Thus, SVCT2 protein expression was determined using immunofluorescence and confocal microscopy in the neurogenic niche, and intense immunoreactivity in the anterior ventricular area and RMS was detected (**Figure 3A**, insets). The positive signal was observed in the ependymal cells and different cells present in the SVZ (**Figure 3A**, inset and arrows). To determine whether SVCT2 was associated with proliferative cells, co-localization

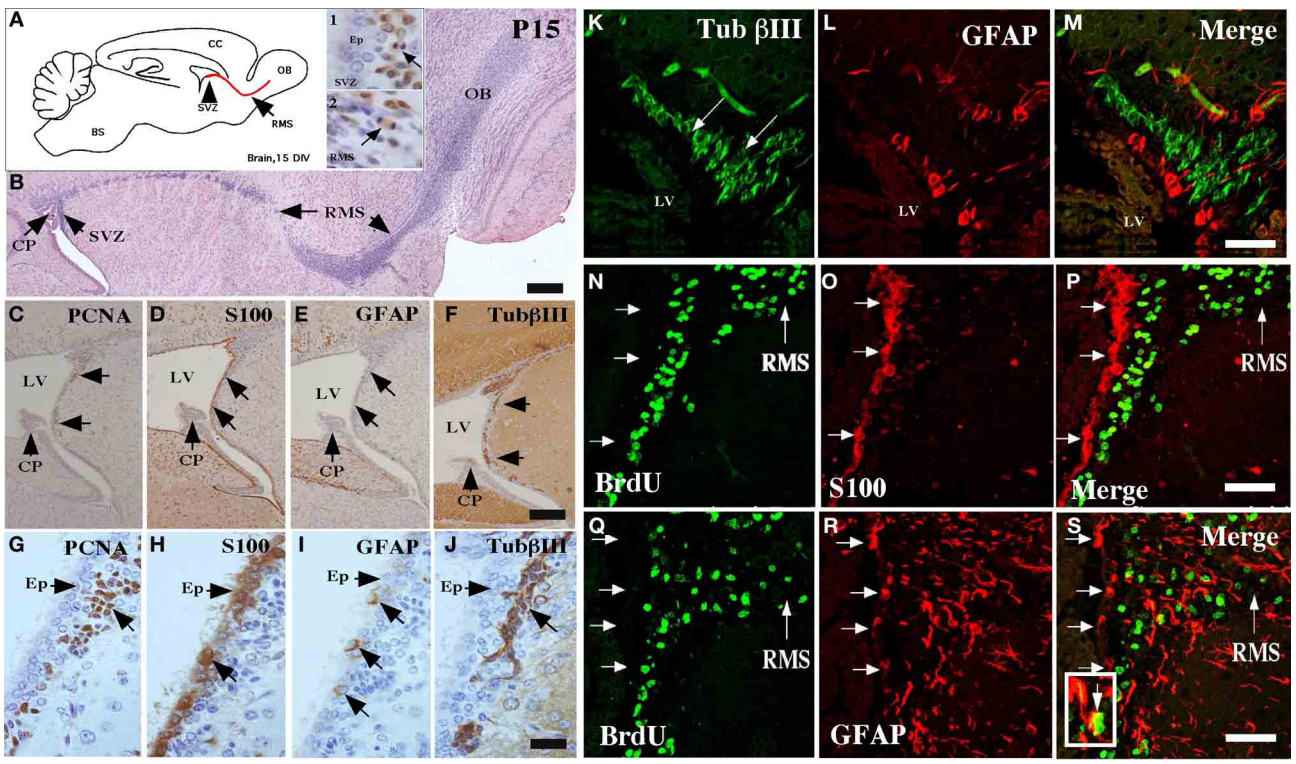

FIGURE 1 | Characterization of the neurogenic niche at 15 days postnatal development. (A) Scheme of a sagittal section of rat brain, highlighting the anterior ventricular zone, the neurogenic niche of the subventricular zone (SVZ), and the rostral migratory stream (RMS). Insets 1 and 2: Immunohistochemical analysis with anti-PCNA (1:100) in the SVZ and the RMS, respectively. **(B)** Sagittal cut of rat brain stained with hematoxylin and eosin showing the RMS (arrows). **(C–J)** Neurogenic niche of the brain at 15 days postnatal development analyzed with anti-PCNA, anti-S100a, anti-GFAP, and βIII-tubulin. **(K–S)** Immunofluorescence and confocal microscopy analyses using antibodies to identify glial and neural cells in different states of proliferation. **(K–M)** Brain tissue analyzed with anti-βIII-tubulin and GFAP. **(N–P)** Co-localization of S100a-positive ependymal cells and BrdU-positive cells. **(Q–S)** Co-localization of GFAP-positive glial cells and BrdU-positive cells. The images are representative of three independent experiments. CP, choroid plexus; LV, lateral ventricle; OB, olfactory bulb. Scale bars in **(B)**, 250 μm; **(C–J)**, 100 μm; **(K–S)**, 50 μm.

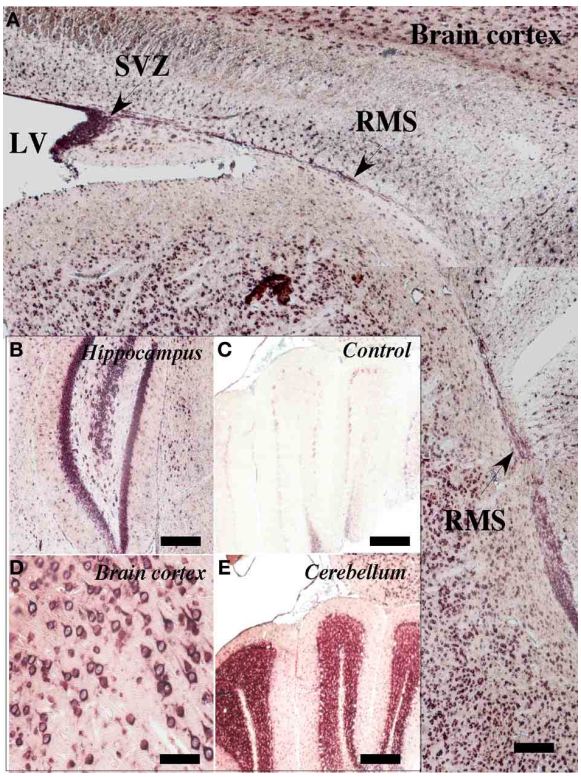

FIGURE 2 | Expression of SVCT2 mRNA in the neurogenic niche and RMS. *in situ* hybridization analysis of a sagittal section of rat brain at 21 days postnatal development. **(A)** A positive reaction was observed in the anterior wall of the lateral ventricle as well as in the ventricular and subventricular zones. A positive reaction was also observed in the RMS (arrows). **(B,D,E)** Positive *in situ* hybridization in different regions of the CNS: hippocampus **(B)**, cerebral cortex **(D)**, cerebellum **(E)**. **(C)** Negative control using the sense probe. LV, lateral ventricle; SVZ, subventricular zone; RMS, rostral migratory stream. Scale bar, 100 μm.

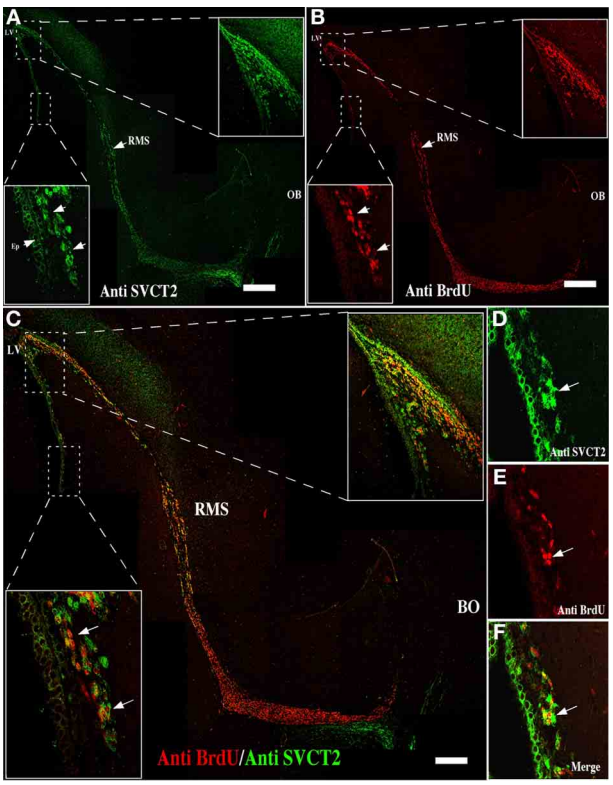

FIGURE 3 | Mitotically active cells express SVCT2. Immunofluorescence and confocal microscopy analyses of the neurogenic niche and RMS at 21 days postnatal development, using antibodies to identify proliferative cells and the ascorbic acid transporter, SVCT2. **(A)** Sagittal section of the rat brain analyzed with anti-SVCT2. **(B)** Sagittal section of rat brain analyzed with anti-BrdU, 5 days after incorporation of BrdU *in vivo*. **(C–F)** Co-localization of SVCT2- and BrdU-positive cells in the neurogenic and RMS areas. The images are representative of three independent experiments. LV, Lateral ventricle; RMS, rostral migratory stream. Scale bars in **(A–C)**, 100 μm. Arrows point positive cells for SVCT2 and BrdU.

analysis was undertaken with BrdU-positive cells (**Figure 3B**, insets). Most of the BrdU-positive cells were also SVCT2-positive (**Figure 3C**, insets), and a high percentage of these cells were localized in the SVZ (**Figures 3C**, insets and **D–F**). The number of BrdU/SVCT2-positive cells was next quantified in two rat brains; of 494 BrdU-positive cells in the neurogenic niche, 95.1% were also SVCT2-positive.

To determine the cell types within the neurogenic niche that express SVCT2, immunofluorescence analysis with triple labeling was undertaken (**Figure 4**). At 21-days postnatal development, SVCT2 did not co-localize with βIII-tubulin (**Figure 4A**) or with GFAP (**Figure 5B**). High power imaging in different regions of the neurogenic niche and in the RMS confirmed these observations (**Figures 4C–N**). SVCT2 reactions were observed in ependymal cells and cells located in the SVZ, again without co-localizing with GFAP (**Figures 4D**,**H**) and with low co-localization with βIII-tubulin (**Figures 4E**,**I**). A similar result was found in the RMS (**Figures 4K–N**), suggesting that SVCT2 is present in highly proliferative intermediate progenitors (C type cell or nIPC).

Analysis of the ventricular wall of a human brain at 1 month postnatal development was next performed. The presence of βIII-tubulin-positive neuroblasts in the SVZ was observed (**Figure 5**). These cells had varying morphologies; some were spherical and closely located to ependymal cells while others were more elongated and positioned tangentially to the ependymal layer (**Figures 5A–C**, arrows). Analysis of GFAP identified the subventricular astrocyte band that was located immediately below the ependymal cells (**Figure 5D**). Some cells had processes inserted among the ependymal cells, directly contacting the CSF (**Figures 5E**,**F**, inset and arrows). SVCT2 expression was identified in the ependymal cells and in the SVZ. Whereas the immunoreaction of the ependymal cells was intense throughout the whole cell, the reaction to SVCT2 was concentrated in specific areas of the cells in the SVZ (**Figures 5G**,**J**). The signal for SVCT2 rarely co-localized with GFAP-positive or βIII-tubulin-positive cells (**Figures 5I**,**L**).

VITAMIN C INCREASES SVCT2 AND βIII-TUBULIN EXPRESSION

Because various factors can enhance neural differentiation (Dhara and Stice, 2008), the effect of vitamin C on neural differentiation was determined using P19 cells. P19 cells form neurospheres

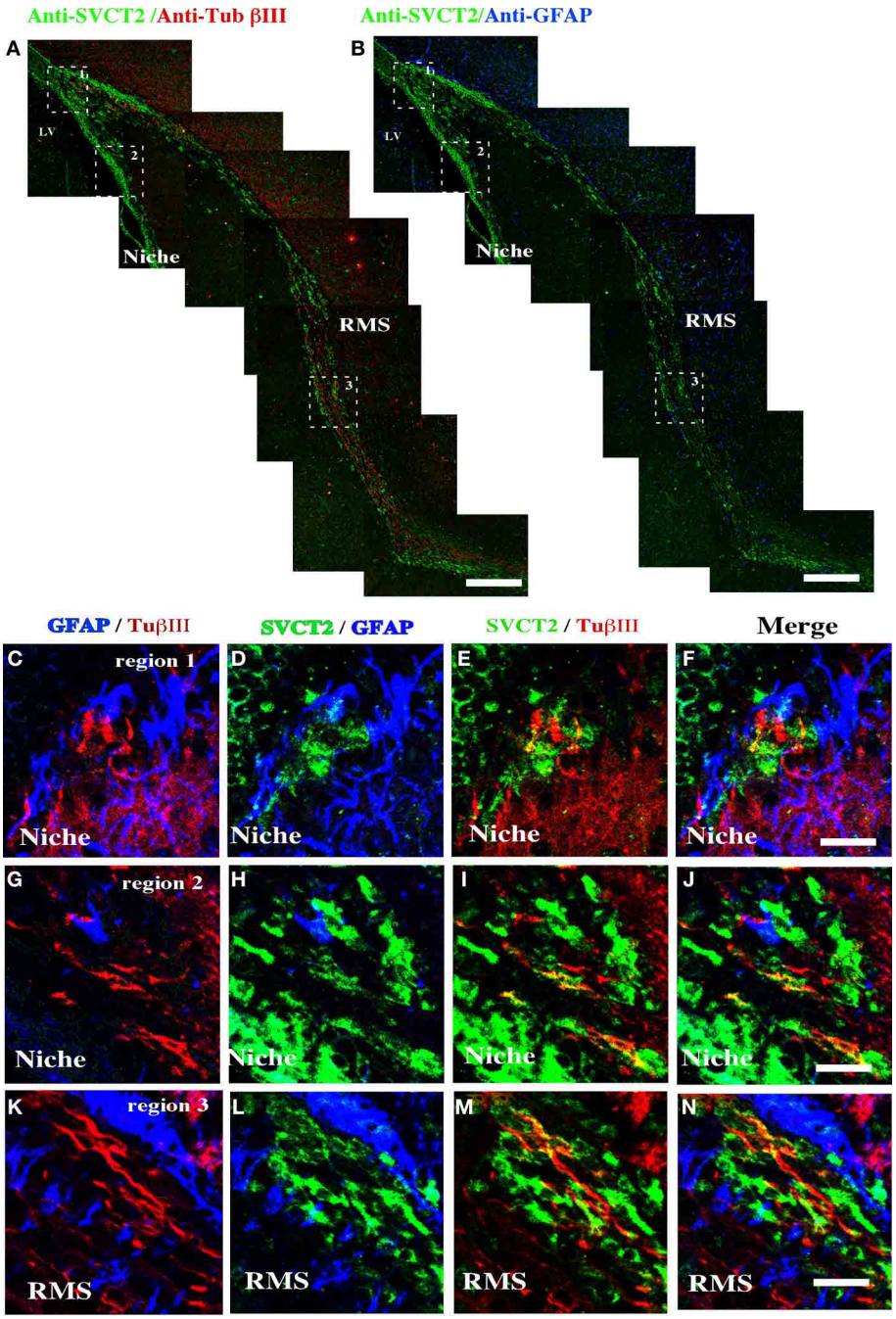

FIGURE 4 | Distribution of SVCT2, GFAP, and βIII-tubulin in the cells of the ventricular area and the RMS. Immunofluorescence and confocal microscopy analyses of the ventricular neurogenic niche after 21 days postnatal development using antibodies to identify glial cells, neurons and SVCT2. **(A)** Sagittal section of rat brain analyzed with anti-SVCT2 and anti-βIII-tubulin. **(B)** Sagittal section of rat brain analyzed with anti-SVCT2 and anti-GFAP. **(C–P)** Co-localization of glial and neural cells with anti-SVCT2 (green), anti-GFAP (blue), and anti-βIII-tubulin in the dorsal **(C–F)** and ventral neurogenic niche **(G–J)**. **(K–N)** Co-localization of glial and neural cells in the RMS. The images are representative of three independent experiments. Ep, ependymal cells. LV, lateral ventricle. Scale bars in **(A–B)**, 100 μm; **(C–N)**, 20 μm.

in vitro, which are immunoreactive to nestin, SVCT2 and βIII-tubulin (**Figures 6A–C**). The expression of these markers was also confirmed using RT-PCR (**Figure 6D**) and Western blot analyses (**Figure 6E**). Moreover, functional studies revealed that AA is incorporated at a velocity of 100 pmoles × 10^6 cells/min in the P19 cells, an uptake that remained constant for up to two min of transport (**Figure 6F**). In the absence of sodium, AA uptake was inhibited by 70% (**Figure 6G**). To confirm that AA is not being

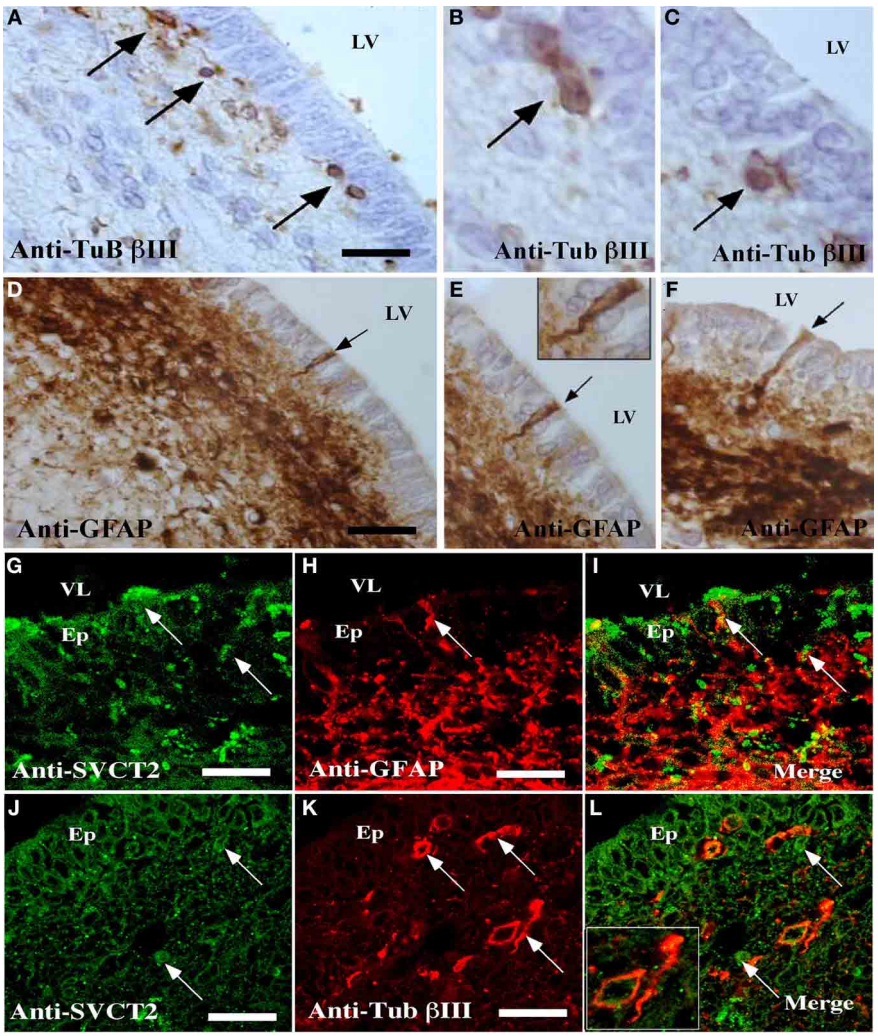

FIGURE 5 | Detection of cells reactive to GFAP, βIII-tubulin, and SVCT2 in the ventricular wall of the postnatal human brain. (A–F) Immunohistochemical analysis using antibodies to identify glial and neural cells. **(A–C)** Sagittal section of the ventricular wall of the human brain at 1 month postnatal development analyzed with antibodies specific for βIII-tubulin **(A–C)** or anti-GFAP **(D–F)**. **(G–L)** Immunofluorescence and confocal microscopy analyses of the human ventricular neurogenic niche, using antibodies to identify glial and neural cells and the SVCT2. Sagittal section of the ventricular wall analyzed with anti-GFAP and anti-SVCT2 **(G–I)** or anti-βIII-tubulin and anti-SVCT2 **(J–L)**. The images are representative of three independent experiments. Ep, ependymal cells; LV, lateral ventricle. Scale bars in **(A–K)**, 20μm. Arrows point positive cells for the respective antibodies.

transported by GLUTs, AA transport was determined in the presence of cytochalasin B, an inhibitor of GLUT transporters that incorporates DHA. The presence of this molecule did not inhibit AA transport (**Figure 6G**). Consequently, these results indicate that P19 cells express functional SVCT2.

To evaluate the effect of vitamin C on neurosphere formation by P19 cells, the culture were maintained with MEM (**Figure 6H,K,O**) or supplemented with 200 or 400 μM AA for four days (**Figures 6I,L,P**). As a positive control, the neurospheres were cultured in neurobasal/B27 medium to stimulate neural differentiation (**Figures 6J,M,N,Q**). After culture in MEM alone, the neurospheres had a lower number of βIII-tubulin-positive cells, even when the majority of the cells in the neurosphere were slightly positive for SVCT2 (**Figure 6K**). After treating the neurospheres with 400 μM AA vitamin C, an increase in neural differentiation was detected as observed by the presence of intensely βIII-tubulin-positive cells (**Figure 6I**). These cells were also highly immunoreactive to SVCT2 (**Figures 6L,P**). A similar response was observed with neurospheres cultured with neurobasal/B27 medium (**Figures 6J,M,Q**). Western blot analysis confirmed that AA as well as neurobasal/B27 medium increased the levels of βIII-tubulin by two-fold (**Figures 6R,S**). Finally, AA-treated P19 cells increased AA incorporation by approximately six-fold as compared to the control (**Figure 6T**).

Similar results were observed using primary neurospheres isolated from the lateral wall of the lateral ventricle of adult rats supplemented with 200 μM AA or 400 μM (data not shown) for four days. After 7 days *in vitro* (**Figure 7A**), the neurospheres cells showed positive immunostaining for anti-GFAP and anti-nestin (**Figures 7B,C**); however, low immunoreaction was observed with

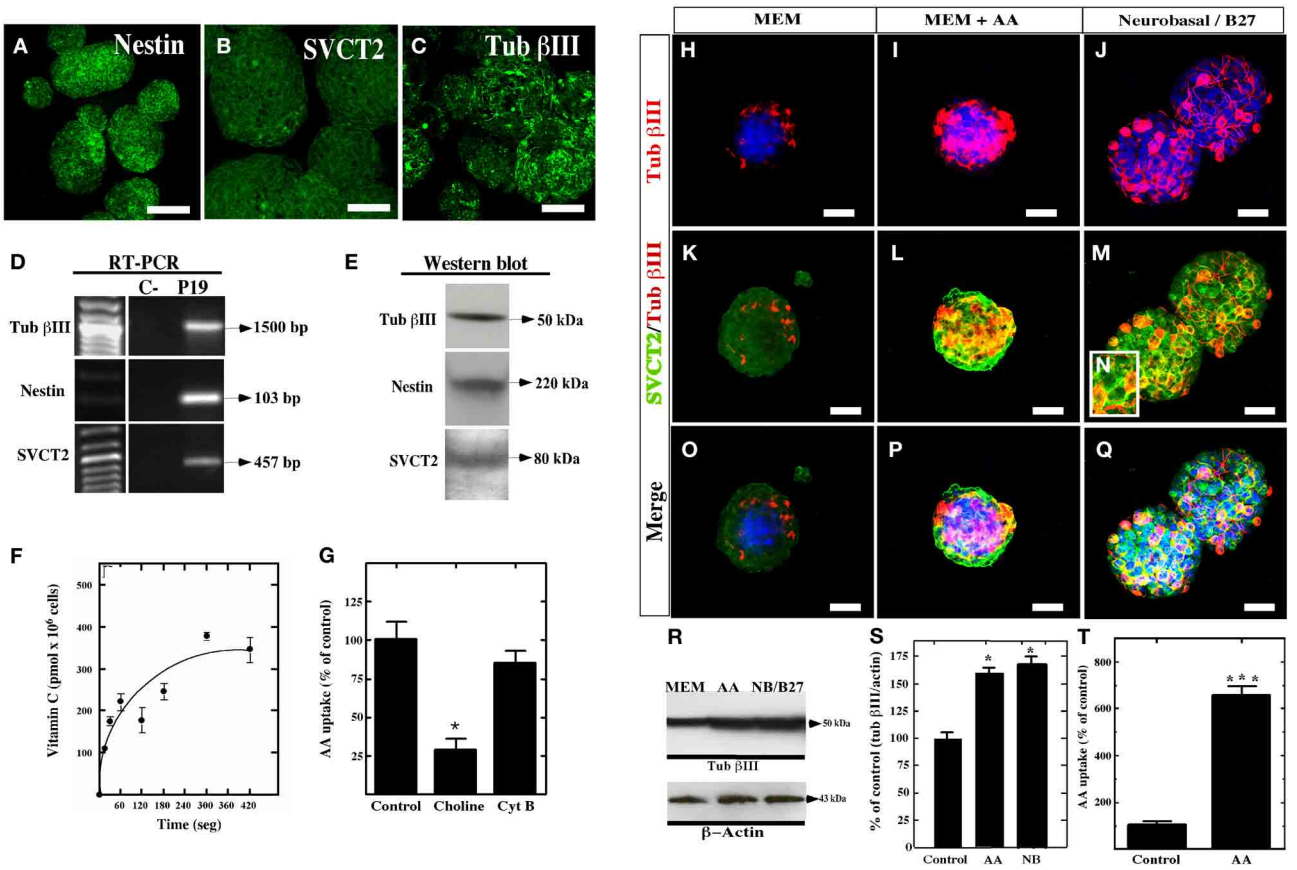

FIGURE 6 | Expression and function of SVCT2 in P19 cells. (A–C) Culture of P19 cells for 4 days *in vitro* in MEM medium. The expression of nestin **(A)**, SVCT2 **(B)**, and βIII-tubulin **(C)** was analyzed in the resultant neurospheres. **(D)** RT-PCR analysis using specific primers for βIII-tubulin, nestin, and SVCT2. **(E) 3** Western blot analysis using antibodies that recognize βIII-tubulin, nestin, and SVCT2. **(F)** Functional analysis of the uptake of 100 μM AA over 7 min. **(G)** Uptake of 100 μM AA in the presence/absence of sodium ions or cytochalasin **(B)**. **(H–Q)** Analysis with anti-SVCT2 and anti-βIII-tubulin antibodies in P19 cell cultures at 4 days *in vitro* using different culture conditions. Topro was used to identify the nucleus of the cells (blue). **(H,K,O)** Cells cultured in MEM medium. **(I,L,P)** Cells cultured in MEM medium supplemented with 400 μM vitamin C. **(J,M,Q)** Cells cultured in neurobasal/B27. **(R)** Western blot analysis for βIII-tubulin in P19 cells cultured in the presence of AA or Neurobasal/B27. **(S)** Quantitative analysis of the Western blot analysis. **(T)** Uptake of 100 μM AA in P19 cells cultured in MEM supplemented with 400 μM of vitamin C for 4 days *in vitro*. The data represent the average and standard deviation of three experiments analyzed by One-Way ANOVA with the Bonferroni post-test. $*p < 0.05$, $***p < 0.005$. Scale bars in **(A–C)**, 50 μm, **(H–Q)**, 30 μm.

anti-βIII-tubulin (**Figure 7D**). In addition, uptake of AA was inhibited by choline and quercetin (**Figure 7E**). When the cells were treated with AA, decreased anti-GFAP immunoreaction was observed (**Figures 7F,G**); however, the reaction for βIII-tubulin increased (**Figures 7H,I,L,M**). Additionally, an increased immunoreaction for SVCT2 was observed by immunohistochemistry and Western blot analyses (**Figures 7J,K,L,N**).

DISCUSSION

Different studies suggest that intracellular vitamin C may regulate the activation of diverse protein kinases, and thereby regulate transcription factor activity and the expression of pro-neural genes (Bowie and O'Neill, 2000; Carcamo et al., 2002; Park et al., 2005; Frebel and Wiese, 2006; Mimori et al., 2007). Vitamin C also helps to maintain iPS cells *in vitro*, which favored their proliferation (Esteban et al., 2010). Neural tissue has been shown to attain AA concentrations that rank among the highest of mammalian tissues (Horning et al., 1975; Kratzing et al., 1982; Milby et al., 1982). In fact, vitamin C levels are particularly high in fetal rat brain, doubling from the 15th to the 20th day of gestation.

To determine the postnatal period during which the neurogenic niche is formed, a detailed immunochemical characterization of the first 3 weeks of postnatal development was conducted. Cellular distribution during this period was compared to the cellular distribution of the neurogenic niche and the RMS of the adult brain. Ependymal differentiation was observed beginning at day 7 postnatal development (data not shown), which was similar previous studies (Silva-Alvarez et al., 2005; Spassky et al., 2005). Between days 7 and 21 of development, an intense immunoreactivity to GLUT1 (Silva-Alvarez et al., 2005) and the S100 protein was observed (**Figure 8A**). However, the radial glia was not present (Hartfuss et al., 2001). Thus, the first cell in the neurogenic niche to differentiate itself was the ependymal

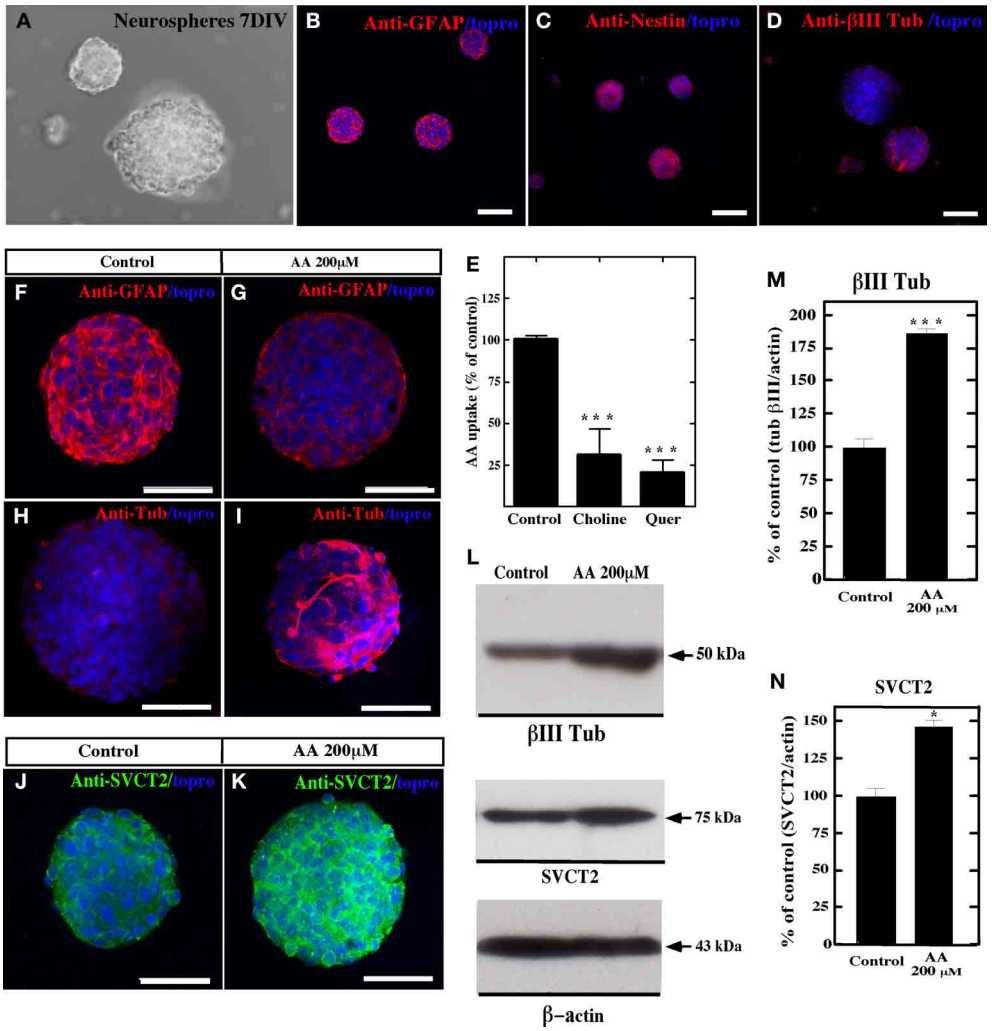

FIGURE 7 | Vitamin C treatment increases neural differentiation in neurospheres. *In vitro* culture of neurospheres at 7 days using different culture conditions, and analysis with anti-GFAP, anti-nestin, anti-SVCT2 and anti-βIII-tubulin. Topro was used to identify the nucleus of the cells (blue). **(A–D)** Neurospheres cultured in NeuroCult NS-A medium showed immunoreaction for anti-GFAP and anti-nestin. Low immunoreaction for anti-βIII-tubulin was observed. **(E)** Uptake of 100 μM AA (10 min) and effect of choline and quercetin in cultured neurospheres. (**F–K)** Neurospheres cultured in NeuroCult NS-A medium supplemented with 200 μM vitamin C **(G,I,K)** or control neurospheres without supplementation **(F,H,J)**. Increased βIII-tubulin-positive cells were detected in AA-supplemented neurospheres **(I)**. **(L)** Western blot analysis for βIII-tubulin and SVCT2 expression in neurospheres cultured in the presence of AA or control medium. (**M,N)** Quantitative analysis of the Western blot. The data represent the average and standard deviation of three experiments analyzed by One-Way ANOVA with the Bonferroni post-test. $^{*}p < 0.05$, $^{***}p < 0.005$. Scale bars in **(A–D)**, 120 μm; **(F–K)**, 50 μm.

cell. Ependymal differentiation could be directly related to the subsequent formation of the neurogenic niche. Noggin secretion by the ependyma stimulates the neurogenic process, acting as an antagonist of bone morphogenic proteins 2 and 4 (BMP2 and BMP4, respectively), factors that stimulate gliogenesis (Lim et al., 2000; Sabo et al., 2009).

GFAP-positive cells were only identified in the SVZ at day 15, indicating the presence of type-B precursor cells (Doetsch et al., 1999; Sanai et al., 2011). The distribution of these cells was similar to what has been described previously for the adult brain (Doetsch et al., 1999; Ma et al., 2005, 2009; Nualart et al., 2012). In addition to the type-B cells, neuroblasts that form the RMS were also identified. Furthermore, the RMS was composed preferentially of cells from the neural lineage reactive to βIII-tubulin and GFAP (Alvarez-Buylla and Lim, 2004; Merkle et al., 2007). The formation of the RMS is additional evidence for concluding that the neurogenic niche forms at day 15. Using *in vivo* BrdU labeling, proliferative type-B cells increased notably at day 21 (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1997; Kee et al., 2002) and were in close contact with the ependymal layer. These cells had a long process that contacted the blood vessels forming a specialized vascular niche (Voigt, 1989; Leprince and Chanas-Sacre, 2001; Alvarez-Buylla and Garcia-Verdugo, 2002; Noctor et al., 2002; Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). Therefore, factors arising from the CSF and blood vessels could regulate the differentiation

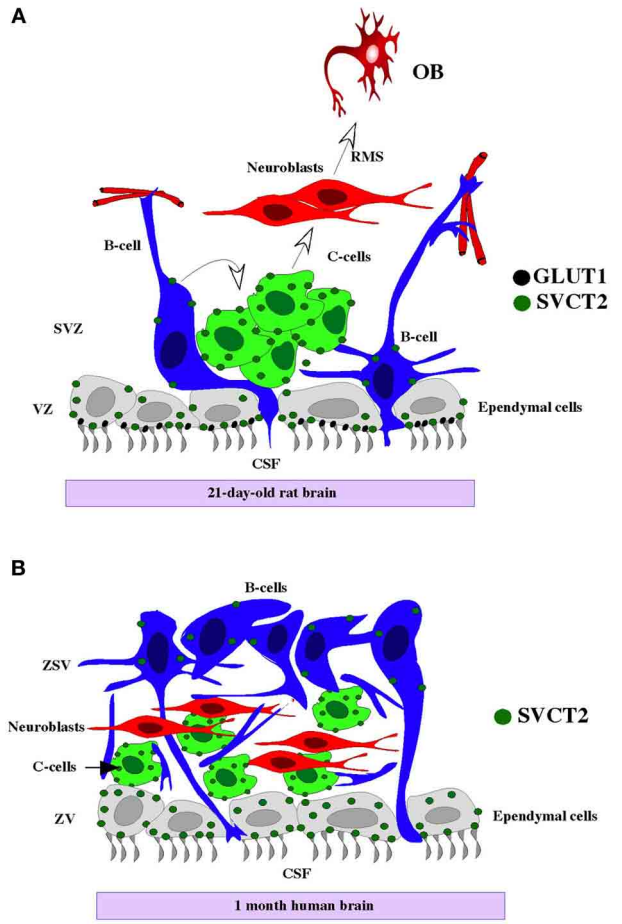

FIGURE 8 | Cytoarchitecture of the human and rat neurogenic niche and SVCT2 expression. Rat **(A)** and human neurogenic niche **(B)**, showing type-B GFAP-positive cells (blue cells) in the ependymal, contacting the CSF (grey cells) and subventricular neuroblasts (red cells). SVCT2 is expressed principally in the ependyma and in GFAP-negative and βIII-tubulin-negative type C subventricular progenitor cells (green cell). OB, olfactory bulb; CSF, cerebrospinal fluid; RMS, rostral migratory stream; VZ, ventricular zone; SVZ, subventricular zone.

of the neurogenic niche (Shen et al., 2004; Mirzadeh et al., 2008).

Previous studies have observed SVCT2 expression in cerebral cortex, hypothalamus, hippocampus, thalamus and cerebellar neurons (Tsukaguchi et al., 1999; Castro et al., 2001; Astuya et al., 2005; García et al., 2005; Caprile et al., 2009; Nualart et al., 2012). This transporter is fundamental for the neural development of CNS, which was confirmed with the use of SVCT2 knock-out animals (Sotiriou et al., 2002). Qiu et al. (2007) demonstrated that neural cultures from SVCT2 knock-out animals had lower rates of neurite growth and reduced neural activity (Qiu et al., 2007). SVCT2 expression was also reported in hypothalamic tanycytes, ependymal cells, marginal astrocytes and microglia (García et al., 2005; Mun et al., 2006; Nualart et al., 2012).

Vitamin C may induce neural and glial differentiation in embryonic cortical precursors and mesencephalic precursor cells (Lee et al., 2000, 2003; Yan et al., 2001). AA concentrations in the CSF may reach up to 500 and 200–400 μM in regions of the cerebral parenchyma (Spector and Lorenzo, 1974). The presence of the vitamin C transporter, SVCT2, in the ventricular neurogenic area after 15 days postnatal development was analyzed in the present study. The expression of SVCT2 in ventricular and subventricular areas has only been described in the embryonic rat brain (Caprile et al., 2009). However, the expression of SVCT2 in postnatal neurogenic areas remains unknown. Using immunohistochemical analysis, the present study was the first to identify SVCT2 expression in postnatal rat brain and in the human neurogenic niche at 1 month postnatal development (**Figures 8A**,**B**). These results were confirmed by *in situ* hybridization, observing positive hybridization in the SVZ and RMS. Double and triple-labeling techniques revealed low co-localization of SVCT2 (rat and human) with GFAP and βIII-tubulin, indicating that type-B cells and neuroblasts would have a low capacity to transport vitamin C. Alternatively, co-localization of SVCT2 with BrdU-positive cells, in particular in the SVZ, suggests that type-C cells with high mitotic activity (Doetsch et al., 1999; Alvarez-Buylla and Garcia-Verdugo, 2002; Kriegstein and Alvarez-Buylla, 2009) preferentially express this transporter. SVCT2 also co-localized with BrdU in the RMS, suggesting the presence of progenitors or similar cells (Doetsch and Alvarez-Buylla, 1996).

The transport of vitamin C to type-C cells of the SVZ can be explained by diffusion from the CSF as vitamin C is present at concentrations up to 10-fold higher than that observed in blood (Tsukaguchi et al., 1999; García et al., 2005). Finally, as observed in the present study, SVCT2 was widely expressed in the postnatal neurogenic area and RMS, suggesting that mitotically active type-C progenitors preferentially express the transporter. Unfortunately, it was not possible to co-localize SVCT2 with markers of type C cells due to technical problems associated with the use of picric acid within the Bouin solution that was used as a fixative for identifying SVCT2.

Precursor cells have the ability to form neurospheres (Reynolds and Weiss, 1992, 1996; Doetsch et al., 1999; Nunes et al., 2003; Chojnacki et al., 2009). In this work, primary neurospheres from rat brain and P19-derived neurospheres expressed nestin, GFAP and βIII-tubulin, and functional SVCT2. Supplementation of 4 days *in vitro* P19-derived neurospheres with 400 μM vitamin C increased their expression of βIII-tubulin, which has also been observed in embryonic precursor cells (Yan et al., 2001). In addition, a six-fold increase in the incorporation of AA was observed in P19 cells cultured with vitamin C, which was similar to the results obtained after culturing cells in Neurobasal/B27 medium, a condition that induces neural differentiation (Hemmati et al., 2003; Beier et al., 2007). Finally, we used primary neurospheres isolated from rat brain to define the effect of 200 or 400 μM vitamin C; 200 μM vitamin C produced a similar differentiation effect to that observed in P19 cells treated with 400 μM vitamin C. The differential response to vitamin C concentration may be due to the level of SVCT2 expression and/or its functional activity in the cells. Because vitamin C uptake by cells is dependent on the affinity of the transporter as well as its concentration in the cellular membrane, we think that the functional activity of SVCT2 is increased in neurospheres. Additionally, increased

SVCT2 expression was observed in primary neurospheres after AA treatment. In conclusion, we suggest that vitamin C and SVCT2 may be important in inducing neurogenesis in postnatal stages.

The specific mechanisms through which vitamin C and SVCT2 induce neural differentiation remain unknown although some evidence suggests the involvement of gene expression related to neurogenesis and maturation (Lee et al., 2003; Shin et al., 2004; Yu et al., 2004). SVCT2 may be regulated in differentiation through its phosphorylation sites dependent on protein kinase C (PKC) and protein kinase A (PKA) (Daruwala et al., 1999; Rajan et al., 1999; Tsukaguchi et al., 1999; Wang et al., 1999, 2000) since its phosphorylation is essential for translocation to the cellular membrane and subsequent AA uptake (Wu et al., 2007). The level of SVCT2 in the cellular membrane is also increased by prostaglandin E through the activation of the EP4 and PK prostaglandin receptor (Wu et al., 2007).

In this study, differentiation of the postnatal neurogenic niche at days 15 and 21 was analyzed by determining the expression pattern of the AA transporter, SVCT2 (**Figure 8**). SVCT2 was expressed in the highly proliferative cells of the neurogenic niche. Additionally, primary neurospheres prepared from rat brain and the P19 teratocarcinoma cell lines also expressed SVCT2, which is involved in AA uptake. Vitamin C-induced neural differentiation increased βIII-tubulin and SVCT2 expression.

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Chronic hypoxia induces the activation of the Wnt/β-catenin signaling pathway and stimulates hippocampal neurogenesis in wild-type and APPswe-PS1ΔE9 transgenic mice *in vivo*

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Hypoxia modulates proliferation and differentiation of cultured embryonic and adult stem cells, an effect that includes β-catenin, a key component of the canonical Wnt signaling pathway. Here we studied the effect of mild hypoxia on the activity of the Wnt/β-catenin signaling pathway in the hippocampus of adult mice *in vivo*. The hypoxia-inducible transcription factor-1α (HIF-1α) was analyzed as a molecular control of the physiological hypoxic response. Exposure to chronic hypoxia (10% oxygen for 6–72 h) stimulated the activation of the Wnt/β-catenin signaling pathway. Because the Wnt/β-catenin pathway is a positive modulator of adult neurogenesis, we evaluated whether chronic hypoxia was able to stimulate neurogenesis in the subgranular zone (SGZ) of the hippocampal dentate gyrus. Results indicate that hypoxia increased cell proliferation and neurogenesis in adult wild-type mice as determined by Ki67 staining, Bromodeoxyuridine (BrdU) incorporation and double labeling with doublecortin (DCX). Chronic hypoxia also induced neurogenesis in a double transgenic APPswe-PS1ΔE9 mouse model of Alzheimer's disease (AD), which shows decreased levels of neurogenesis in the SGZ. Our results show for the first time that exposure to hypoxia *in vivo* can induce the activation of the Wnt/β-catenin signaling cascade in the hippocampus, suggesting that mild hypoxia may have a therapeutic value in neurodegenerative disorders associated with altered Wnt signaling in the brain and also in pathological conditions in which hippocampal neurogenesis is impaired.

Keywords: hypoxia, HIF-1α, hippocampus, Wnt signaling pathway, β-catenin, neurogenesis, Alzheimer's disease

INTRODUCTION

Neurogenesis in the adult brain is mainly restricted to the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the hippocampal dentate gyrus (Alvarez-Buylla and Garcia-Verdugo, 2002; Zhao et al., 2008a). In the SGZ, neural stem cells (NSCs) give rise to neuroblasts that mature into functional dentate granule neurons that are integrated into the preexisting hippocampal circuitry (van Praag et al., 2002; Zhao et al., 2006; Mathews et al., 2010). Increasing evidence indicates that neurogenesis is relevant for hippocampal functions, such as spatial learning, object recognition and memory (Reviewed in Deng et al., 2010; Koehl and Abrous, 2011; Marin-Burgin and Schinder, 2012).

Neurogenesis is modulated by different physiological stimuli such as running, exposure to environmental enrichment, learning and stress (Kempermann et al., 1997; Gould et al., 1998; van Praag et al., 1999; Dobrossy et al., 2003; Drapeau et al., 2007; Piatti et al., 2011; Song et al., 2012). Several signaling molecules have been determined to be essential for the maintenance, self-renewal and proliferation of NSCs and for the differentiation into fully functional neurons (Suh et al., 2009; Schwarz et al., 2012; Faigle and Song, 2013; Varela-Nallar and Inestrosa, 2013). The possibility to activate endogenous NSCs and stimulate the generation of new neurons in the adult brain could have therapeutic potential in pathological conditions in which neurogenesis is altered, such as mood disorders, schizophrenia and neurodegenerative diseases (Kaneko and Sawamoto, 2009; Winner et al., 2011; Petrik et al., 2012). It has been shown that exposure to low oxygen concentrations (or hypoxia) can stimulate the proliferation and differentiation of cultured embryonic and adult NSCs (Vieira et al., 2011). This effect has been associated with β-catenin (Mazumdar et al., 2010; Cui et al., 2011), a key component of the Wnt/β-catenin signaling pathway.

The Wnt/β-catenin cascade is initiated by the binding of a Wnt ligand to its receptor, Frizzled, and co-receptors, such as the low-density lipoprotein receptor-related protein 5 (LRP5) and

LRP6 (Cadigan and Liu, 2006; Gordon and Nusse, 2006), which triggers the phosphorylation of the protein Dishevelled (Dvl), and inhibits the degradation of β-catenin, which in the absence of Wnt stimulation is phosphorylated in a multiprotein complex, ubiquitinated and degraded by the proteasome (Aberle et al., 1997; Liu et al., 2002). The stabilization of β-catenin results in its translocation into the nucleus where it binds to members of the T-cell factor (TCF) and lymphoid enhancer factor (Lef) family and activates the transcription of Wnt target genes (Logan and Nusse, 2004). The Wnt/β-catenin signaling pathway regulates several aspects of central nervous system development and also plays fundamental roles in the adult nervous system (Salinas and Zou, 2008; Inestrosa and Arenas, 2010) where it regulates synaptic assembly and plasticity (Ahmad-Annuar et al., 2006; Cerpa et al., 2008) and adult neurogenesis (Lie et al., 2005; Kuwabara et al., 2009; Karalay et al., 2011; Varela-Nallar and Inestrosa, 2013).

It was previously found that hypoxia increases β-catenin signaling in cultured neonatal hippocampal NSCs (Cui et al., 2011) and embryonic stem cells (ESCs) (Mazumdar et al., 2010). Under hypoxic conditions, the hypoxia-inducible transcription factor-1α (HIF-1α) directly binds to the promoters of the Lef1 and TCF1 genes (Mazumdar et al., 2010), therefore regulating the transcriptional activity of β-catenin. Moreover, it was determined that Wnt/β-catenin signaling is active in low oxygen regions in the adult brain, including the SGZ, suggesting an association between low oxygen and β-catenin signaling *in vivo* (Mazumdar et al., 2010). However, it has not been determined whether hypoxia modulates the activation of the Wnt/β-catenin signaling cascade in the hippocampus. Here, we assessed whether chronic exposure to hypoxia stimulates the activation of the Wnt/β-catenin signaling pathway, specifically in the hippocampus of adult mice, and also we studied whether this hypoxic condition could stimulate SGZ neurogenesis in adult wild-type mice as well as in a double transgenic mouse model of Alzheimer's disease (AD).

MATERIALS AND METHODS

ANIMALS AND TREATMENTS

APPswe/PSEN1ΔE9 mice, which express the Swedish mutation of APP (K595N/M596L) and PS1 with the deletion of exon 9 (APP-PS1 mice stock #004462), were obtained from The Jackson Laboratory (Bar Harbor, Maine). All procedures involving experimentation on animal subjects were approved by the Bioethical Committee of the P. Catholic University of Chile. All animals had access to water and food *ad libitum*, in a 12:12 h light/dark cycle.

BROMODEOXYURIDINE (BrdU) ADMINISTRATION

A single dose of Bromodeoxyuridine (BrdU) (Sigma-Aldrich, St Louis, MO, USA) was injected i.p. at 100 mg kg^{-1}.

HYPOXIC EXPOSURE

Animals were exposed to hypoxia (10% O_2 at normal barometric pressure) by placement of a mice cage in a plexiglass chamber for 6–72 h. The hypoxic environment in the chamber was achieved by inflow of N_2 gas. The hypoxic level was controlled by an oxygen controller (Pro-Ox model 110, BioSpherix, USA). Mice had free access to water and food *ad libitum* during the hypoxic exposure. Control animals were kept at normoxic condition (21% O_2).

PERFUSION AND POSTFIXATION

Animals were anesthetized (100 g ketamine + 10 g xylazine in 10 μl saline/g), and then transcardially perfused with saline, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The brain was removed and placed in a vial with 4% PFA in PBS for 24 h at room temperature, dehydrated in 30% sucrose, and kept at 4°C until analysis.

TISSUE SECTIONING

Each mouse brain was sectioned on a cryostat in 12 sets of serial coronal sections of 40 μm thickness (Leica Microsystems, Wetzlar, Germany) and collected in ice-cold-PBS in multiwall dishes (Encinas and Enikolopov, 2008). Each set contained a representative sample of the whole hippocampus (Abbott et al., 2013).

IMMUNOFLUORESCENCE

Immunodetection of BrdU and neuronal markers in tissue sections was carried out as previously described (Abbott et al., 2013). Primary antibodies used were: rat anti-BrdU (Abcam), rabbit anti-Doublecortin (Cell Signaling Technology Inc., Beverly, MA, USA), monoclonal anti-NeuN (Millipore, Billerica, MA, USA) and rabbit anti-Ki67 (Abcam). As secondary antibodies, Alexa (Molecular Probes) and DyLight (Abcam) conjugated antibodies were used. BrdU and Ki67 positive cells were counted using a fluorescence microscope (Olympus BX51, Tokyo, Japan) as described (Abbott et al., 2013). Double-labeled sections were analyzed by confocal laser microscopy (Olympus FV 1000). Image analysis and *z*-projections were made with ImageJ software (NIH, USA).

IMMUNOBLOTTING

The hippocampus and cortex of treated and control mice were dissected on ice and either immediately processed or frozen at −150°C. Immunoblotting was performed as previously described (Varela-Nallar et al., 2009). Primary antibodies used were: mouse anti-Dvl3, mouse anti-β-catenin, mouse anti-c-myc, mouse anti-cyclin D1 and rabbit anti-β-tubulin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-rabbit anti-HIF-1α (Novus Biologicals, Littleton, CO, USA).

STATISTICAL ANALYSIS

Statistical analysis was performed using Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance of differences was assessed using the non-paired Student's *t*-test or ANOVA, and non-normally distributed data was analyzed using the Mann-Whitney test or Kruskal Wallis. $P < 0.05$ was considered significant.

RESULTS

CHRONIC HYPOXIA INDUCES THE ACTIVATION OF THE Wnt/β-CATENIN SIGNALING PATHWAY IN THE HIPPOCAMPUS OF ADULT MICE

An association between low-oxygen and the transcriptional activity of β-catenin has been previously reported (Mazumdar et al., 2010); however, it is not known whether it also involves the activation of the Wnt/β-catenin signaling cascade. We aimed to

determine whether hypoxia exposure could stimulate the activation of the Wnt signaling pathway in the hippocampus of adult mice *in vivo*. For this purpose, 2-month-old mice were placed in hypoxic chambers with 10% oxygen for 0 (normoxic control), 6, 24 or 72 h. After treatment animals were immediately sacrificed, the brain was removed and the hippocampus dissected and analyzed by immunoblot (**Figure 1A**). Hypoxia induced a significant increase in HIF-1α for all exposure times compared to the normoxic control (**Figure 1B**), indicating that the hypoxic procedure used stimulated a hypoxic response in the hippocampus.

To investigate the effect of hypoxia on the Wnt/β-catenin signaling pathway, we evaluated the stabilization of β-catenin and observed a significant increase in its levels with all hypoxic treatments compared to control animals (**Figure 1C**), suggesting that the canonical Wnt pathway was activated. Importantly, hypoxia induced a mobility shift of Dvl3 (**Figure 1A**, arrow heads), suggesting that the phosphorylation of Dvl3 was induced, which is normally triggered by the activation of the Wnt pathway due to the binding of a Wnt ligand to Frizzled receptors and to co-receptors (Gao and Chen, 2010). The highest effect on β-catenin levels and Dvl3 phosphorylation was observed after a 24 h exposure to hypoxia, however a clear effect was already observed after 6 h. An increase in c-myc and cyclin D1 levels (**Figure 1D**), two well-known Wnt target genes (Mann et al., 1999; Hodar et al., 2010), was also observed in response to hypoxia treatment. Altogether, these results suggest that chronic hypoxia induces the activation of the Wnt/β-catenin signaling cascade in the adult hippocampus.

CHRONIC HYPOXIA INCREASES NEUROGENESIS IN THE SUBGRANULAR ZONE (SGZ) OF ADULT MICE

Next, we evaluated the effect of hypoxia on neurogenesis *in vivo*. First, proliferation was evaluated by immunostaining for the mitotic marker Ki67 (Kee et al., 2002) in the hippocampus of 2-month-old mice exposed to hypoxia for 6, 24 and 72 h. The strongest effect was seen after 24 h of treatment (**Figure 2A**), which induced a significant increase in total number of $Ki67^+$ cells in the SGZ compared to the normoxic control (**Figure 2B**). These results indicate that chronic hypoxia increases proliferation of hippocampal neural progenitor cells *in vivo*.

To evaluate the differentiation of newborn cells into neurons, mice received a single i.p. injection of 100 mg kg^{-1} BrdU and were

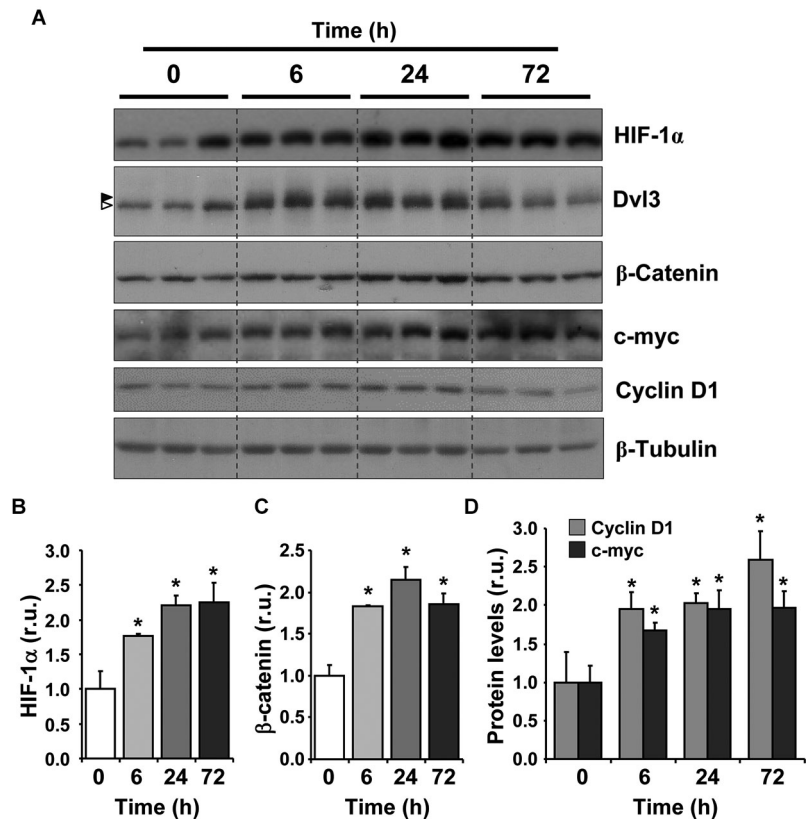

FIGURE 1 | Hypoxia induces the activation of Wnt/β-catenin signaling in adult mice. (A) Immunoblot of total protein extracts from the hippocampus of 2-month-old mice exposed to hypoxia (10% O_2) for 6, 24 and 72 h. Time 0 corresponds to control animals that were maintained at normoxic conditions (21% O_2). Immunoblots of three different animals are shown in the control condition and in each time of exposure to hypoxia. In Dvl3 immunoblot, arrowheads on the left indicate dephosphorylated (white) and phosphorylated and shifted (black) Dvl3. **(B–D)** Densitometric analysis expressed in relative units (r.u.) of HIF-1α **(B)**, β-catenin **(C)**, cyclin D1 and c-myc **(D)** levels normalized to β-tubulin levels and compared to control mice that were not exposed to hypoxia. Bars represent mean ± S.E ($n = 3$ mice). * $p < 0.05$.

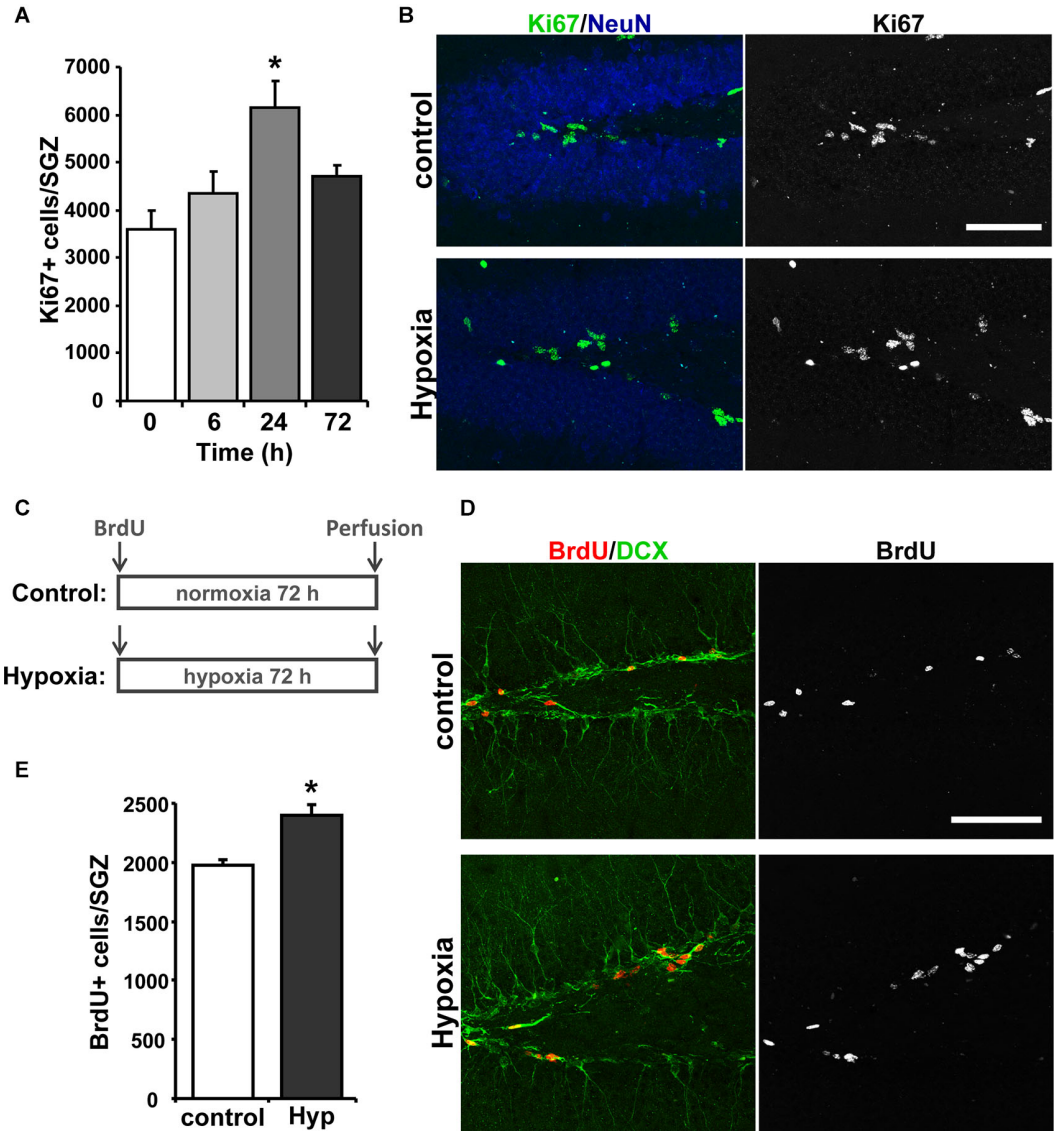

FIGURE 2 | Hypoxia induces neurogenesis in the hippocampus of adult mice. (A) Quantification of total number of Ki67 positive (Ki67+) cells in the SGZ of control mice and mice exposed to 6, 24 and 72 h of hypoxia. **(B)** Representative immunofluorescence staining of Ki67 in the hippocampus of control mice and mice exposed to hypoxia for 24 h. Scale bar: 50 μm. **(C)** Schematic representation of the treatment protocol. Control and hypoxia mice received an i.p. injection of 100 mg kg^{-1} BrdU and were then exposed for 72 h to normoxia or hypoxia, respectively, after which were then immediately transcardially perfused. **(D)** Representative double labeling of BrdU and DCX in the hippocampus of control mice and mice exposed to hypoxia for 72 h. Scale bar: 50 μm. **(E)** Quantification of total number of BrdU+ cells in the SGZ of control mice and mice exposed to hypoxia for 72 h. Bars represent mean ± S.E ($n \geq 3$ mice). * $p < 0.05$.

exposed to hypoxia or maintained at normoxic conditions for 72 h (**Figure 2C**), and immunoreactivity for BrdU and the immature neuronal marker doublecortin (DCX) in the hippocampus was investigated (**Figure 2D**). An increase in the total number of BrdU positive ($BrdU^+$) cells was observed in the SGZ of mice exposed to hypoxia (**Figure 2E**), indicating an increase in cell proliferation. The percentage of the $BrdU^+$ cells that were also positive for DCX^+ was not significantly changed (% $BrdU^+/DCX^+$: control: 69.42 ± 5.20; hypoxia: 70.68 ± 2.91), indicating that the differentiation of $BrdU^+$ cells into DCX^+-neuroblasts was not affected. However, since there is a significant increase of $BrdU^+$ cells that differentiate into DCX^+ cells in mice exposed to hypoxia, these results indicate that chronic hypoxia induces neurogenesis in the hippocampus of adult mice.

HYPOXIA INDUCES NEUROGENESIS IN THE SUBGRANULAR ZONE (SGZ) OF DOUBLE TRANSGENIC APPswe/PS1ΔE9 MICE

Considering the effects on neurogenesis observed in the SGZ of adult wild-type mice, the effect of hypoxia was evaluated in the double transgenic APPswe/PS1ΔE9 mouse model of AD, which

shows reduced levels of neurogenesis (Hu et al., 2010; Abbott et al., 2013). For this experiment, 9-month-old APPswe/PS1ΔE9 mice were exposed to 72 h hypoxia, and the effect on cell proliferation was studied by Ki67 immunoreactivity (**Figure 3A**). A strong increase in total number of $Ki67^+$ cells was observed in APPswe/PS1ΔE9 exposed to hypoxia compared to age-matched control APPswe/PS1ΔE9 mice that were not exposed to low oxygen conditions (**Figure 3B**).

To assess neurogenesis, mice received a single i.p. injection of 100 mg kg^{-1} BrdU before exposure to hypoxia for 72 h. As a control, age-matched wild-type and APPswe/PS1ΔE9 mice received the BrdU injection but were not exposed to hypoxia and were sacrificed 72 h after BrdU administration. As expected, the total number of $BrdU^+$ cells was lower in 9-month-old wild-type (**Figures 4A, B**) than in 2-month-old wild-type mice (**Figure 2D**), because of the age-dependent decline in hippocampal neurogenesis (Kuhn et al., 1996; Gould et al., 1999; Leuner et al., 2007; Snyder and Cameron, 2012). In addition, as previously reported (Abbott et al., 2013), a decreased number of $BrdU^+$ cells was observed in APPswe/PS1ΔE9 compared to wild-type mice, which was significantly increased after hypoxia (**Figures 4A, B**). The differentiation of newborn cells into DCX+ neuroblasts and immature neurons, evaluated by double labeling of BrdU and DCX (**Figure 4C**), was decreased in APPswe/PS1ΔE9 mice compared to age-matched wild-type mice (% $BrdU^+/DCX^+$: wild-type: 68.64 ± 2.656; APPswe/PS1ΔE9: 41.05 ± 6.094), and it was strongly increased in transgenic mice exposed to hypoxia (72.46 ± 4.493). In fact, when analyzing the total number of $BrdU^+/DCX^+$ cells in the hippocampus (**Figure 4D**), we observed a significant increase in APPswe/PS1ΔE9 mice exposed to hypoxia compared to control APPswe/PS1ΔE9 mice maintained in normoxic conditions (**Figure 4D**). Altogether, these results indicate that hypoxia stimulates neurogenesis in APPswe/PS1ΔE9 mice.

DISCUSSION

In the present study, we have determined that *in vivo* exposure to mild hypoxia increases the activation of the Wnt/β-catenin signaling pathway in the hippocampus of adult mice and stimulates cell proliferation and neurogenesis in the SGZ of adult wild-type mice and in the double transgenic APPswe/PS1ΔE9 mouse model of AD.

Hypoxia normally occurs during embryonic stages and it is fundamental for proper neurogenesis during development (Zhu et al., 2005a; Zhang et al., 2011). Moreover, in the adult hippocampus, there are normally low oxygen regions in the SGZ (Mazumdar et al., 2010), indicating a hypoxic microenvironment in the neurogenic niche. Low oxygen stimulates the proliferation and differentiation of embryonic NSC *in vitro* (Studer et al., 2000; Zhao et al., 2008b), and intermittent hypobaric hypoxia increases cell proliferation and neurogenesis in the SVZ and SGZ of adult rats (Zhu et al., 2005b, 2010). HIF-1α has been shown to be critical for the hypoxia-induced proliferation of NSCs *in vitro* and *in vivo* (Zhao et al., 2008b; Mazumdar et al., 2010). The HIF-1α-mediated effect on NSCs involves β-catenin-dependent transcription since HIF-1α increases the expression of Lef1 and TCF1 (Mazumdar et al., 2010), the nuclear partners of β-catenin for the activation of Wnt target genes (Logan and Nusse, 2004). Here we determined that concomitantly with the increase in the levels of HIF-1α, exposure to 10% O_2 stimulated Dvl3 phosphorylation, β-catenin stabilization and the transcription of Wnt target genes in the hippocampus of adult mice. These results indicate that hypoxia not only regulates transcriptional activation of β-catenin, but also induces the activation of the Wnt/β-catenin signaling cascade *in vivo*, which has not been previously reported. The mechanism involved may comprise the increased transcription of Wnt ligands and/or Frizzled receptors. In mammals, 19 Wnt ligands and 10 Frizzled receptors have been identified, many of them being present in the adult brain (Shimogori et al., 2004; Chen et al., 2006; Chacon et al., 2008). Also, hypoxia may regulate

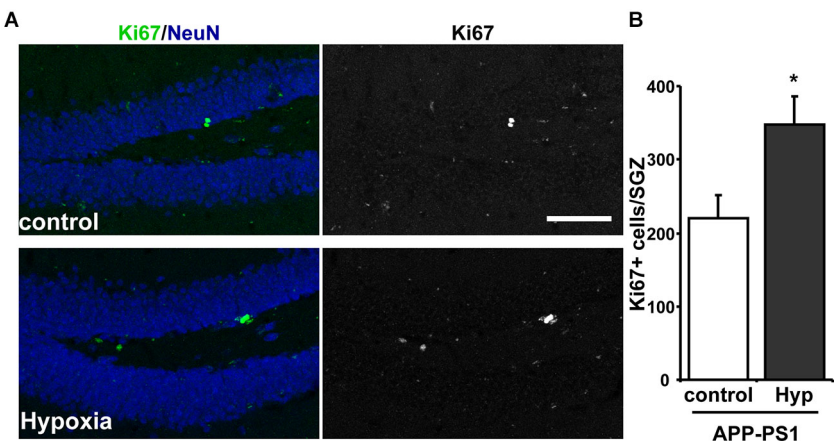

FIGURE 3 | Increased proliferation in the SGZ of APPswe-PS1ΔE9 mice exposed to hypoxia. (A) Representative immunofluorescence staining of Ki67 in the hippocampus of control APPswe-PS1ΔE9 mice and APPswe-PS1ΔE9 mice exposed to hypoxia for 72 h. Scale bar: 50 μm. **(B)** Quantification of total number of Ki67 positive (Ki67+) cells in the SGZ of APPswe-PS1ΔE9 mice maintained in control conditions or exposed to hypoxia for 72 h. Bars represent mean ± S.E (n = 3 mice). * $p < 0.05$.

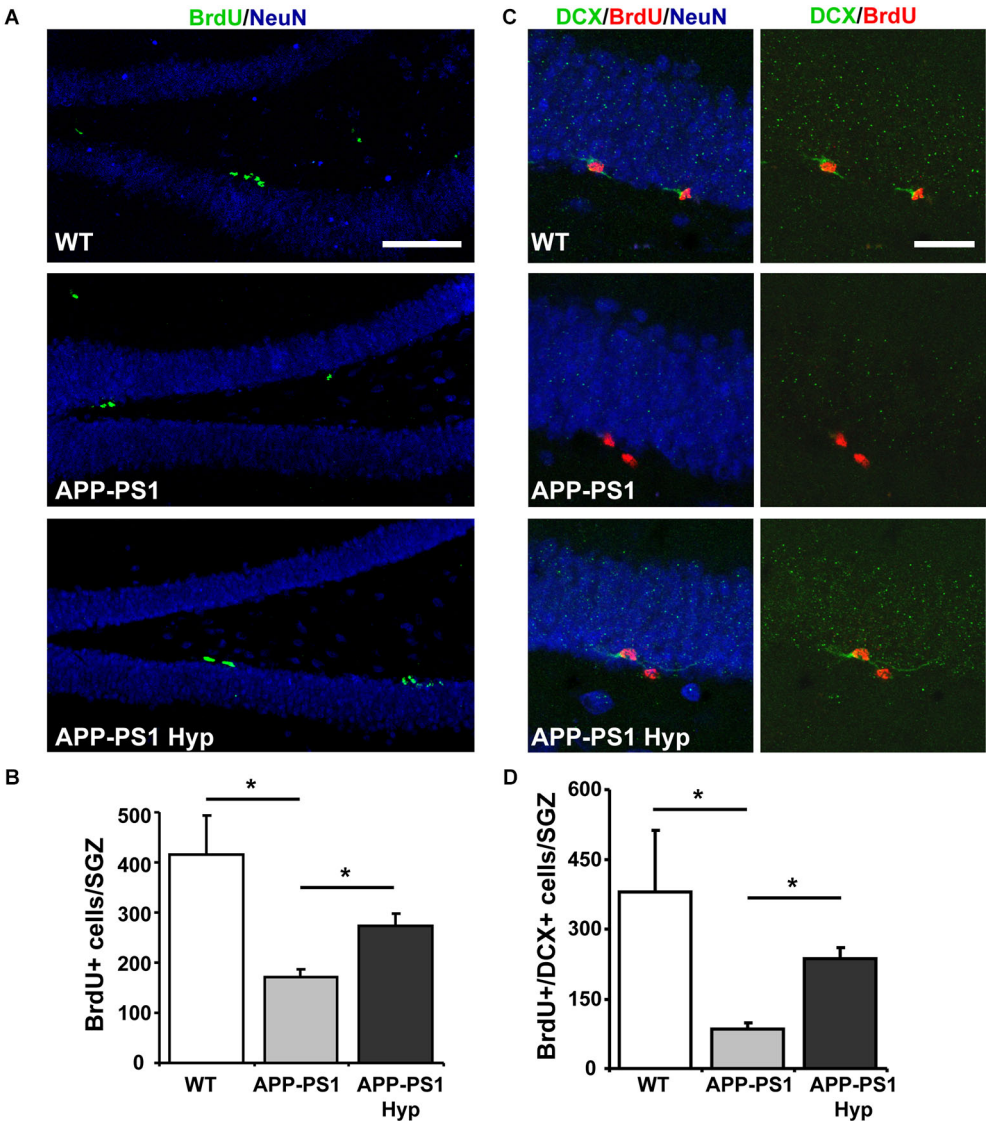

FIGURE 4 | Hypoxia induces neurogenesis in the hippocampus of APPswe-PS1ΔE9 mice. (A) Representative double labeling of BrdU and the mature neuronal marker NeuN in the hippocampus of 9-month-old wild-type mice, APPswe-PS1ΔE9 and APPswe-PS1ΔE9 exposed to hypoxia for 72 h. Scale bar: 50 μm. **(B)** Total number of $BrdU^+$ cells in the SGZ of all experimental groups. **(C)** Representative double labeling of BrdU and DCX in the same animals. Scale bar: 20 μm. **(D)** Total number of $BrdU^+$ cells also positive for DCX ($BrdU^+/DCX^+$) in the hippocampus of all experimental groups. Bars represent mean ± S.E ($n \geq 3$ mice). * $p < 0.05$.

the levels of secreted inhibitors of the Wnt signaling pathway such as Dickkopf 1 (Dkk1) and soluble Frizzled-related protein 3 (sFRP3), both recently described as negative regulators of adult hippocampal neurogenesis that can be regulated under certain physiological conditions (Jang et al., 2013; Seib et al., 2013). Whether or not exposure to hypoxic conditions regulates the expression of Wnt signaling components will have to be explored further.

We also determined that exposure to chronic hypoxia induced cell proliferation in the SGZ of adult mice as determined by BrdU incorporation and Ki67 staining. Importantly, the neuronal differentiation of newborn cells was not changed, indicating that hypoxia-induced proliferation results in increased newborn neurons. The hypoxia-induced proliferation was also observed in the SGZ of a double transgenic mouse model of AD. AD is a neurodegenerative disease characterized by progressive deterioration of cognitive abilities. Two neuropathological hallmarks of AD are the extracellular senile plaques mainly composed of amyloid-β (Aβ) peptide and intracellular neurofibrillary tangles formed by hyperphosphorylated tau protein (Castellani et al., 2010; Ballard et al., 2011; Mandelkow and Mandelkow, 2012). The double transgenic APPswe-PS1ΔE9 mice at the age used in the present study show most histopathological markers of AD (Inestrosa et al., 2011), and show decreased levels of neurogenesis

as previously reported (Abbott et al., 2013) and as observed here. Hypoxia strongly stimulated proliferation and neuronal differentiation in AD mice, indicating that hypoxia could stimulate this process in the diseased brain.

The possibility to stimulate neurogenesis in the adult brain may offer an exciting alternative for brain repair. Considering the described roles of neurogenesis in learning and memory (Deng et al., 2010; Koehl and Abrous, 2011; Marin-Burgin and Schinder, 2012), the hypoxia-induced activation of progenitor cells in the adult hippocampus may help to ameliorate the cognitive decline associated to neurodegenerative diseases. Not only is the effect of hypoxia on neurogenesis of therapeutic interest. Our findings indicating that mild hypoxia induces the activation of the Wnt signaling pathway in the adult brain may also have therapeutic benefits. The dysfunction of the Wnt/β-catenin signaling pathway has been linked to neurodegenerative disorders such as schizophrenia, autism and AD (Moon et al., 2004; Lovestone et al., 2007; Inestrosa et al., 2012). Several studies have shown that Wnt signaling components are altered in AD (De Ferrari and Inestrosa, 2000; Caricasole et al., 2004; Ghanevati and Miller, 2005; De Ferrari et al., 2007; Toledo and Inestrosa, 2010), and that Wnt signaling activation has neuroprotective properties against the toxicity of Aβ peptide (De Ferrari et al., 2003; Alvarez et al., 2004; Chacon et al., 2008). Therefore, the hypoxia-induced activation of the Wnt/β-catenin signaling pathway may be relevant for the treatment of AD and other pathologies associated with impaired Wnt signaling.

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Implication of fibroblast growth factors in epileptogenesis-associated circuit rearrangements

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The transformation of a normal brain in epileptic (epileptogenesis) is associated with extensive morpho-functional alterations, including cell death, axonal and dendritic plasticity, neurogenesis, and others. Neurotrophic factors (NTFs) appear to be very strongly implicated in these phenomena. In this review, we focus on the involvement of fibroblast growth factor (FGF) family members. Available data demonstrate that the FGFs are highly involved in the generation of the morpho-functional alterations in brain circuitries associated with epileptogenesis. For example, data on FGF2, the most studied member, suggest that it may be implicated both in seizure susceptibility and in seizure-induced plasticity, exerting different, and apparently contrasting effects: favoring acute seizures but reducing seizure-induced cell death. Even if many FGF members are still unexplored and very limited information is available on the FGF receptors, a complex and fascinating picture is emerging: multiple FGFs producing synergic or antagonistic effects one with another (and/or with other NTFs) on biological parameters that, in turn, facilitate or oppose transformation of the normal tissue in epileptic. In principle, identifying key elements in these phenomena may lead to effective therapies, but reaching this goal will require confronting a huge complexity. One first step could be to generate a "neurotrophicome" listing the FGFs (and all other NTFs) that are active during epileptogenesis. This should include identification of the extent to which each NTF is active (concentrations at the site of action); how it is active (local representation of receptor subtypes); when in the natural history of disease this occurs; how the NTF at hand will possibly interact with other NTFs. This is extraordinarily challenging, but holds the promise of a better understanding of epileptogenesis and, at large, of brain function.

Keywords: fibroblast growth factors, epilepsy, cell death, neurogenesis, synaptogenesis

INTRODUCTION

Acquired epileptic syndromes are characterized by the spontaneous appearance of seizures in a previously healthy brain. Many acquired epilepsies have an identifiable cause, such as a head trauma, an episode of status epilepticus (SE), a stroke, or a brain infection (Pitkänen and Sutula, 2002). It is thought that these damaging insults set in motion a cascade of neurobiological alterations that, in time, will lead to the occurrence of spontaneous seizures and to the diagnosis of epilepsy. This phenomenon is termed "epileptogenesis".

Conventional "antiepileptic" agents exert only symptomatic effects on seizures but do not interfere with epileptogenic processes (Temkin, 2001). Moreover, a third of the people with epilepsy do not get adequate seizure control with the current medications (Schmidt and Sillanpää, 2012). Thus, there is an urgent need for more effective (and better tolerated) treatments to control drug-resistant seizures, as well as for innovative therapies to prevent, stop or reverse the development of epilepsy in at-risk individuals (Galanopoulou et al., 2012).

In principle, understanding the molecular mechanisms underlying the neurobiological alterations occurring during epileptogenesis and finding ways to manipulate them should allow development of effective agents. Although the epileptogenic process remains incompletely understood, recent molecular studies began to elucidate the mechanisms that regulate some components of the circuitry reorganizations (including cell death, axonal and dendritic plasticity, neurogenesis and functional alterations in ion channel and synaptic properties) that occur during epileptogenesis and likely contribute to the development of hyperexcitability and spontaneous seizures (Pitkänen and Lukasiuk, 2011). Microarray-based gene expression studies indicate that products of genes regulated during epileptogenesis belong to a variety of functional classes, including signal transduction, transcription regulation, neurogenesis and immune response proteins (Pitkänen and Lukasiuk, 2009).

Which of these many molecular changes should be a target for intervention? Neurotrophic factors (NTFs) appear to be very strong candidates, because an extensive literature demonstrates

their involvement in each of the above-mentioned cellular alterations associated with epileptogenesis (Simonato et al., 2006; Simonato and Zucchini, 2010): not only their trophic effects suggest an involvement in cell death, neurogenesis and axonal sprouting, but they also exert functional effects at the synaptic level, with distinct modulatory actions at excitatory and inhibitory synapses (Schinder and Poo, 2000). Furthermore, NTFs are greatly involved in brain development, and epileptogenesis is thought to recapitulate several aspects of developmental processes (Kim et al., 2010; Simonato and Zucchini, 2010; Ueda et al., 2011).

Identification of the specific roles played by NTF families or even single NTFs in the morpho-functional changes associated with epileptogenesis is very difficult. It seems likely that multiple NTFs are involved in the process at distinct phases and with distinct roles (Simonato et al., 2006). Nonetheless, a few specific molecules have gained particular interest and attention, like the brain-derived neurotrophic factor (BDNF) and members of the fibroblast growth factor (FGF) family, especially FGF2 (Simonato et al., 2006). In fact, the combined supplementation of BDNF and FGF2 in the epileptogenic area during the latency period between an epileptogenic insult and the first spontaneous seizure has been reported to produce a dramatic attenuation of the adaptive morpho-functional changes occurring in the tissue (namely cell loss, aberrant neurogenesis, sprouting of the mossy fibers—i.e., of hippocampal granule cell axons—and neuroinflammation), ultimately leading to reduced frequency and severity of spontaneous seizures, i.e., a disease-modifying and maybe truly anti-epileptogenic effect (Paradiso et al., 2009; Bovolenta et al., 2010; Paradiso et al., 2011; Simonato et al., 2013).

These very promising results prompt further investigation of the role played in epileptogenesis by neurotrophins like BDNF and FGFs like FGF2. Here, we will focus on the involvement of FGF family members in the morpho-functional alterations associated with epilepsy. We will first summarize the biological features of this class of NTF and then describe existing evidence supporting their role in epileptic disorders and specifically in epileptogenesis.

THE FGFs

The human FGFs contain 150–300 amino acids and have a conserved core of 120 amino acids with 30–60% identity. The family encompassed 18 members. FGF15 has not been identified in humans and FGF19 has not been identified in mice and rats, thus it has been hypothesized that they are the products of orthologous genes. Four previously listed members, now termed FGF homologous factors (FHF1-4), have been removed from the original list of 23 (Goldfarb et al., 2007) because they exert purely intracrine effects. FHF1, FHF2, FHF3 and FHF4, which in the old nomenclature correspond respectively to FGF12, FGF13, FGF11 and FGF14, are not secreted extracellularly and act intracellularly in an FGF receptor-independent manner. They interact with intracellular domains of voltage gated sodium channels and with a neuronal mitogen-activated protein kinase (MAPK) scaffold protein, islet-brain-2. FGF homologous factor (FHFs) are thought to be mainly active in postnatal life, and their only known role is in the regulation of excitability by association with sodium channels (Itoh, 2010).

The currently classified 18 members of the mammalian FGF family can be functionally sub-divided into canonical FGFs and hormone-like FGFs (hFGFs) based on their paracrine or endocrine actions (Itoh and Ornitz, 2011). Both sub-groups mediate biological responses in an FGF receptor-dependent manner, but hFGF can act over long distances like endocrine hormones. Most FGFs are secreted proteins with cleavable N-terminal secretion signal sequences. In this respect, FGF1 and FGF2 are atypical, because they do not have these N-terminal sequences, but they may nonetheless be released from damaged cells or via exocytotic mechanism(s) independent from the endoplasmic reticulum-Golgi pathway.

Almost all FGF effects are mediated by binding to cell surface tyrosine kinase receptors (FGFRs). Heparin/heparan sulfate acts as a cofactor for the binding of FGFs to FGFRs. FGF1, FGF2 and FGF3 can also directly translocate to the nucleus and act in an intracrine manner (Itoh and Ornitz, 2011). Four genes, *FGFR1-FGFR4*, have been identified in humans and mice that encode for high-affinity FGFRs (FGFR1 through FGFR4). These genes display substantial sequence homology and are similar in their general structure: all are tyrosine kinase receptors with one membrane-spanning domain and an extracellular ligand-binding domain with three immunoglobulin-like motifs (Ig I, II and III). The Ig-like domain III is an essential determinant of ligand binding. *FGFR1-FGFR3* encode two main versions of this domain (termed IIIb and IIIc) generating, by alternative splicing, a total of six FGFR proteins (FGFR 1b, 1c, 2b, 2c, 3b, 3c), whereas *FGFR4* generates a single protein (FGFR4). FGF binding to FGFRs induces dimerization, receptor trans-phosphorylation and activation of four key downstream signaling pathways: RAS-RAF-MAPK, PI3K-AKT, STAT and PLCγ (Beenken and Mohammadi, 2009; Turner and Grose, 2010).

Heparan sulfate interacts with heparan binding sites in the FGFR Ig II domain and in the FGF molecule, favoring protein-protein contacts at the dimer interface and thereby sustaining dimerization. Dimerization enables tyrosine trans-phosphorylation of the intracellular kinase domains and generates docking sites for the recruitment and phosphorylation of downstream signaling substrates, ultimately leading to alterations in expression of specific genes and to the biological effects. The acid box-containing linker between Ig domains I and II may serve as auto-inhibitory control on heparan sulfate-dependent receptor dimerization (Kalinina et al., 2012). The endocrine hFGFs (FGF15/19, FGF21 and FGF23) bind to FGFRs and heparin/heparan sulfate with very low affinity (Itoh, 2010). FGF23 activates FGFR1c, which forms a complex with α-Klotho, a single-pass transmembrane protein of 1,000 amino acids with a short cytoplasmic domain predominantly expressed in the kidney, parathyroid glands, and epithelial cells of choroid plexuses in the brain. Similarly, FGF15/19 can bind to FGFR4 and β-Klotho, a protein structurally and functionally similar to α-Klotho that is predominantly expressed in the liver, pancreas and adipose tissue.

FGFs IN EPILEPSY

The production and release of many members of the FGF family have been reported to be altered (increased in most cases)

in epilepsy: these include FGF1, −2 and −5 (Gómez-Pinilla et al., 1995; Riva et al., 1995; Cuevas and Giménez-Gallego, 1996; Simonato et al., 1998; Bregola et al., 2000), FGF7 and −22 (Terauchi et al., 2010; Lee et al., 2012) FGF8 and −17 (McCabe et al., 2011; Zanni et al., 2011), as well as FHF4 (Hu et al., 2011) and α-Klotho (Park et al., 2009). These observations prompted investigations on possible functional roles played by the FGFs in epileptic models and human syndromes. At present, data are still rather fragmentary and incomplete. **Figure 1** provides a schematic representation of some of the best-characterized effects of FGFs on the morpho-functional changes associated with epileptogenesis in the hippocampus. The member on which available information is most robust and convincing is FGF2 (Zucchini et al., 2005, 2008), that will be therefore discussed separately and in greater detail.

Research on FGFs involvement in epilepsy has been conducted mainly in three types of models. First, acute seizure models in which convulsions are produced in normal animals. These include maximal electroshock (MES) and administration of convulsant agents. Second, kindling: a model in which the repeated administration to a discrete limbic brain area of an initially subconvulsive electrical stimulation induces seizures that progressively intensify in duration and severity, from focal to secondarily generalized. Kindling can be evoked by stimulating different areas, including the amygdala, hippocampus, piriform cortex. Third, chemically (pilocarpine or kainate) or electrically (self-sustained SE) evoked SE: these are models in which induction of an epileptogenic insult SE is followed by a latency period during which the animals are apparently well and then by spontaneous recurrent seizures (SRSs), i.e., epilepsy. This situation

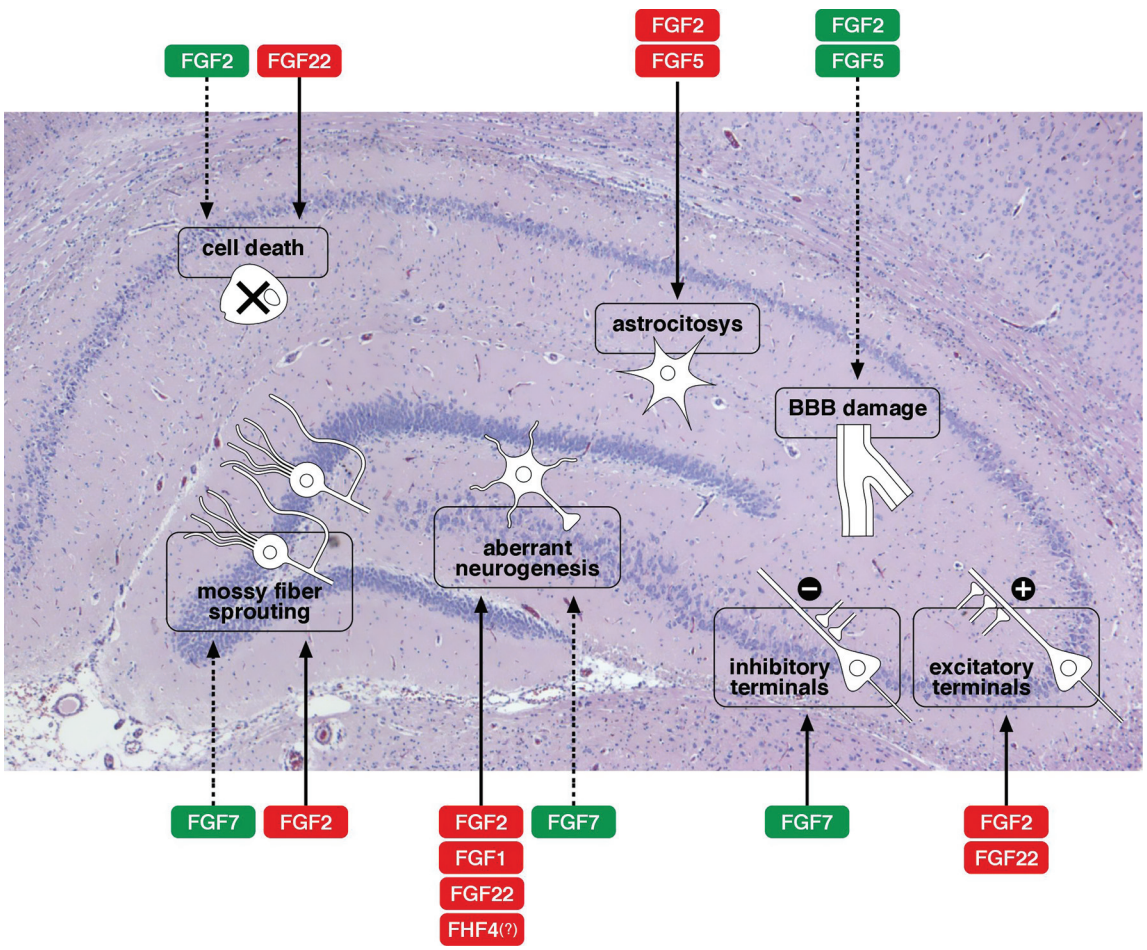

FIGURE 1 | Effects of FGFs on morpho-functional alterations associated with epileptogenesis in the hippocampus. A schematic representation of some of the best-characterized effects of FGFs on the morpho-functional changes associated with epileptogenesis in the hippocampus: cell death, astrocytosis, blood-brain barrier (BBB) damage, alterations in synaptogenesis (density of excitatory or inhibitory terminals), axonal sprouting (like sprouting of the mossy fibers), aberrant neurogenesis with newborn neurons in the dentate gyrus hilus. Solid arrows indicate facilitation, dotted arrows inhibition of a specific event. The name of the FGF members is framed in red to indicate a putatively negative implication in epilepsy (favoring epileptogenesis), in green to indicate a putatively positive implication (contrasting epileptogenesis). Note that these are only tentative indications, because evidence regarding the FGFs is often fragmentary and because the patho-physiological consequences of each alteration are sometimes uncertain.

most closely mimics the one occurring in humans with acquired epilepsies.

FGF1

FGF1 (also known as acidic FGF, aFGF) has been reported to be implicated in rodent models of epilepsy but, to date, it has not been studied in the human disease (Naegele, 2009). MES in rodents can produce epigenetic modifications of DNA leading to increased neurogenesis. This phenomenon appears to be FGF1-dependent and related to *Gadd45b*, a gene involved in DNA repair and DNA 5-methylcytosine excision (Ma et al., 2009): Gadd45b is responsible for demethylating regulatory promoter regions in the genes encoding for FGF1 and BDNF. However, the role in epilepsy of neurogenesis (and, based on these studies, of FGF1) is still uncertain. Many consider it as a maladaptive plastic change (Parent, 2007; Jung et al., 2009; Naegele, 2009; Hattiangady and Shetty, 2010), implicating a pro-epileptic role for FGF1. In contrast with this view, however, systemic administration of the recombinant human FGF1 has been reported to exert anticonvulsant effects in kainate-induced convulsions and mortality (Cuevas and Giménez-Gallego, 1996).

FGF5

Increased expression of FGF5 has been found shortly after acute seizures, in association with an increased transcription of *FGF2* and *FGFR1* (Gómez-Pinilla et al., 1995). In contrast with FGF2, whose constitutive expression is mainly in astrocytes (see below), FGF5 is mostly found in neurons. Similar to FGF2 knock-out mice, however, decreased expression levels of the intermediate filament component glial fibrillary acidic protein (GFAP) has been reported in FGF5 deficient mice, even if the density of astrocytes remains unchanged (Reuss et al., 2003). FGF5 has been hypothesized to favor astrocyte proliferation, based on the observation that FGF5 and its high-affinity receptor FGFR1 IIIc are overexpressed in astrocytic brain tumors (Allerstorfer et al., 2008). Whether this effect also occurs in epileptogenesis and favors epilepsy-associated astrocytosis is still unknown. Astrocytosis contribute to induce many pathological events associated with epileptogenesis, including increased inflammation and neuronal hyperexcitability (Vezzani et al., 2011; Kim et al., 2012).

In functional contrast with these putative effects on astrocytosis, FGF5 may contribute to the stabilization of the blood—brain barrier (BBB). A reduction of GFAP protein levels has been observed in the perivascular astroglial endfeet of FGF5 (and FGF2) deficient mice (Reuss et al., 2003), resulting in increased BBB permeability, an event associated with epileptogenesis. Therefore, FGF5 appears to exert contrasting effects in epilepsy development: on one hand, it may protect the BBB; on the other hand, it may favor reactive astrocytosis.

FGF7 AND FGF22

FGF22 and FGF7 have been reported to promote the organization of excitatory and inhibitory presynaptic terminals, respectively, as target-derived presynaptic organizers. These factors are target-derived molecules that promote differentiation of neuritic segments into presynaptic nerve terminals. FGF22 and FGF7 are expressed in the CA3 pyramidal neurons of the rodent and human (at least FGF22) hippocampus (Umemori et al., 2004; Katoh and Katoh, 2005; Terauchi et al., 2010; Lee et al., 2012). The formation of excitatory or inhibitory synaptic contacts on dendrites of hippocampal CA3 pyramidal neurons is specifically impaired in mice lacking FGF22 or FGF7, respectively. Specifically, the clustering of vesicles containing the excitatory neurotransmitter glutamate is impaired in FGF22 deficient mice, whereas the clustering of inhibitory, GABA-containing vesicles is reduced in FGF7 deficient mice (Jones and Basson, 2010). These presynaptic defects are rescued by postsynaptic expression of the appropriate FGF, demonstrating that there is an absolute requirement of FGF7 during the formation of inhibitory GABAergic synapses and of FGF22 during excitatory glutamatergic synaptogenesis. The differential effects of FGF22 and FGF7 are likely due to distinct synaptic localizations and employment of different signaling pathways.

The implications for epilepsy are of course opposite. FGF22 knock-out mice (with reduced excitatory synapses) are resistant to epileptic seizures, whereas FGF7 knock-out mice (with reduced inhibitory synapses) are prone to seizures (Terauchi et al., 2010; Lee et al., 2012). In addition, increased neurogenesis and mossy fiber sprouting have been reported in FGF7 knock-out mice during post-SE epileptogenesis, both events that may also favor susceptibility to epilepsy development in these mice (Lee et al., 2012). Therefore, FGF7 activation may be capable of decreasing vulnerability to epilepsy by multiple mechanisms. In contrast, epileptogenesis-associated aberrant neurogenesis and cell death in the hippocampal dentate gyrus hilus are suppressed in FGF22 knock-out mice (Lee and Umemori, 2013), suggesting that inhibition of FGF22 may alleviate epileptogenesis.

FGF8 AND FGF17

FGF8 and FGF17 are implicated in two epileptogenic human neurological disorders. Recently, *FGF8* gene mutations have been identified in recessive holoprosencephaly and in septo-optic dysplasia (Moebius syndrome), and *FGF17* gene chromosomic deletion in Dandy–Walker malformation (McCabe et al., 2011; Zanni et al., 2011). In all these diseases, patients can experience spontaneous seizures.

FHF4

The intracrine FHF4 (FGF14 according to the old nomenclature) also seems to be involved in the morpho-functional alterations associated with epilepsy. De-repression of *FGF14* gene expression is obtained in conditional neuron-restrictive silencer factor (NRSF) knock-out mice (Hu et al., 2011). In fact, the degree of up-regulation of FHF4 following kainate-induced SE is significantly increased in the cortex of NRSF knock-out mice compared with controls. In the kindling model, these mice exhibit dramatically accelerated seizure progression, prolonged after-discharge duration and increased mossy fiber sprouting compared with controls. Thus, FHF4 appears to favor epileptogenesis. The mechanism of this effect remains unknown, but it can be hypothesized that it depends on increased neurogenesis and/or synaptogenesis (Wang et al., 2002; Hu et al., 2011).

α-KLOTHO

Pathological activation of α-Klotho may be implicated in phenylketonuria (PKU), an autosomal recessive disorder caused by a deficiency of phenylalanine hydroxylase, an enzyme that

catalyzes the conversion of phenylalanine to tyrosine. The resultant hyper-phenylalaninemia causes mental retardation, seizures, and abnormalities in behavior and movement. The mechanism of this disease remains incompletely understood, but an implication of the FGF family has been proposed based on the observation of an α-Klotho-dependent increase in Na^+/K^+-ATPase activity (Park et al., 2009; Itoh, 2010).

FGF2 AND EPILEPSY

EXPRESSION IN THE BRAIN

FGF2 (basic FGF, bFGF) expression is developmentally regulated. It is highly expressed in neurons in the fetal brain and, later in development, in glia cells (Caday et al., 1990; Torelli et al., 1990); its levels increase progressively in early postnatal life, and then remain high in the adult and aged rat brain (Riva and Mocchetti, 1991).

BIOLOGICAL ACTIVITIES

FGF2 is thought to play a critical role in cell-cell signaling between neurons, astrocytes and microglia during development (Gremo and Presta, 2000). In adults, FGF2 can regulate proliferation of neural stem cells and neuronal survival (Bikfalvi et al., 1997; Hefti, 1997). Furthermore, it enhances axonal branching (Waliche, 1988; Aoyagi et al., 1994; Abe et al., 2001; Szebenyi et al., 2001) and synaptogenesis (Li et al., 2002) in neurons.

FGF2 exerts neuroprotective effects against a wide variety of insults, reducing brain cellular damage and improving functional recovery in experimental models of stroke, epilepsy, traumatic brain and spinal cord injury (Liu and Holmes, 1997a; Teng et al., 1999; Li and Stephenson, 2002; Zucchini et al., 2008). This neuroprotective effect seems to depend on interference with a number of signaling pathways, including expression and gating of N-methyl-D-aspartate (NMDA) receptors, maintenance of Ca^{2+} homeostasis, regulation of reactive oxygen species (ROS) detoxifying enzymes and strengthening of anti-apoptotic pathways (Acharya et al., 2008).

In vitro, FGF2 increases survival, proliferation and differentiation of hippocampal neurons (Vicario-Abejon et al., 1995; Lowenstein and Arsenault, 1996). Interestingly, low FGF2 levels predominantly lead to the generation of neurons, whereas high levels generate glia and neurons (Vescovi et al., 1993; Qian et al., 1997). Similar effects are also observed *in vivo*. Neurogenesis is inhibited by injection of an anti-FGF2 antibody and increased by FGF2 injection in P1 rats (Tao et al., 1997; Cheng et al., 2002). The effects of exogenous FGF2 is also observed in the adult brain, in areas of constitutive neurogenesis, i.e., subgranular zone of the dentate gyrus and subventricolar zone (Wagner et al., 1999). FGF2 is the most potent known mitotic agent for adult neural stem and progenitor cells. Moreover, it regulates astroglial cell differentiation, functions, and transition to the "reactive" phenotype observed after lesions (Reuss et al., 2003). As in FGF5 deficient mice, a reduction of GFAP protein levels has been observed in the perivascular astroglial endfeet of FGF2 knock-out mice (Reuss et al., 2003), which results in increased BBB permeability.

FGF2 accelerates bifurcation and growth of axonal branches in cultured rat hippocampal neurons (Aoyagi et al., 1994). A greater number of axon branches is expected to produce a greater number of synaptic contacts and, indeed, local application of FGF2 has been found to increase the number of morphologically mature and functionally active excitatory synapses between hippocampal neurons (Li et al., 2002).

FGF2 AND EPILEPSY

Based on its biological properties, FGF2 seems particularly likely to be involved in epileptogenesis. Indeed, (1) seizures increase FGF2 mRNA and protein levels in specific brain areas and up-regulate the expression of FGFR1 receptors; (2) acute intra-hippocampal injection of FGF2 causes seizures, while chronic i.c.v. infusion of low dose FGF2 does not affect kainate seizures but promotes behavioral recovery and reduces hippocampal damage; (3) kainate seizure severity is not altered in FGF2 knock-out mice, but is increased in FGF2 over-expressing mice.

FGF2 gene expression is induced with similar patterns in different acute seizure models (Riva et al., 1992; Bugra et al., 1994; Follesa et al., 1994; Gall et al., 1994; Riva et al., 1994; Gómez-Pinilla et al., 1995; Kondratyev et al., 2002). This phenomenon is fast, marked and transient, peaking at 6–24 h in different hippocampal subfields and in the cortex. In the hippocampus of naive rats, FGF2 is expressed diffusely in astrocytes and in CA2 pyramidal neurons (Ernfors et al., 1990; Gomez-Pinilla et al., 1992; Woodward et al., 1992). Following acute seizures, increased *FGF2* mRNA levels in these cell populations, as well as new expression in CA1 pyramidal neurons and in dentate gyrus granule cells have been observed (Gall et al., 1994; Riva et al., 1994). In the kindling model, induction of *FGF2* mRNA expression is observed in a more pronounced manner after a single after-discharge, not accompanied by behavioral seizures, than after a fully kindled, generalized tonic-clonic seizure lasting more than a minute (Simonato et al., 1998; Bregola et al., 2000). In addition, FGF2 expression in limbic regions has been reported to be more pronounced after partial than after generalized electroshock seizures (Follesa et al., 1994). These observations suggest that the duration and intensity of seizures within a specific area does not necessarily correlate with the magnitude of *FGF2* mRNA level increase, and that FGF2 may be more directly implicated with epileptogenesis than with generalized seizure expression. Induction of mRNA for FGF2 is typically followed by an increase in FGF2 protein: FGF2-like immunoreactivity is detectable 6 h following seizures, peaks after about 24 h and may remain elevated up to 30 days, being mainly localized in the nuclei of astrocytes (Humpel et al., 1993; Gómez-Pinilla et al., 1995; Ballabriga et al., 1997; Gwinn et al., 2002). Increased levels are mainly observed for the high molecular weight isoforms of FGF2, which contain nuclear targeting sequences, and therefore may enter the nucleus and influence gene regulation, activating programs for cellular plasticity or proliferation (Gwinn et al., 2002). Therefore, the transient pattern of *FGF2* mRNA elevation may have prolonged translational effect influencing long-term plasticity changes. Finally, seizure-induced increases in the neuronal and astrocytic expression of a high-affinity FGF receptor (FGFR1) have been found in an epilepsy model (Bugra et al., 1994; Van Der Wal et al., 1994; Gómez-Pinilla et al., 1995). A strong FGFR3 staining has also been found in reactive microglia in several brain

areas, including the hippocampus, 30 days after kainate injection (Ballabriga et al., 1997). These observations lead to the notion that epileptogenic seizures co-ordinately increase the expression of FGF2 and of its receptor(s). The hypothesis that these events may take part in the plastic changes associated with epilepsy has been pharmacologically and genetically investigated.

Injection of FGF2 into the dentate region of the ventral hippocampus causes an immediate excitatory effect culminating in EEG and behavioral seizures (Liu and Holmes, 1997b). In contrast, the chronic infusion of low FGF2 doses into the cerebral ventricles does not modify latency and duration of kainate seizures, but prevents seizure-induced hippocampal cell loss and improves long-term behavioral recovery (Liu et al., 1993; Liu and Holmes, 1997a). This neuroprotective action may depend on the induction of activin A (ActA), a cytokine belonging to the transforming growth factor-beta superfamily. When co-injected with kainate in the hippocampus, FGF2 prevents the loss of CA3 neurons in mice. In mice treated with kainate and FGF2, but not in those treated with kainate alone, ActA-immunoreactivity is high in pyramidal neurons (Tretter et al., 2000) and FGF2 fails to protect CA3 neurons against kainate-induced death in the presence of the ActA-neutralizing protein follistatin (Tretter et al., 2000).

Studies in FGF2 knock-out and transgenic mice have extended these pharmacological findings. The FGF2 knock-out mice (Ortega et al., 1998) are viable, fertile and without any obvious phenotypical difference from their wild-type littermates, but exhibit a significant reduction in the number of neurons in the neocortex and a delayed would healing. The susceptibility to seizures of FGF2 knock-out mice has been studied in the kainate model (Yoshimura et al., 2001). The severity of kainate seizures did not differ between knock-out and wild-type mice. However, while an increase in FGF2 protein levels and neuroproliferation was observed in the hippocampus of wild-type mice, very low levels of neurogenesis were observed in the knock-outs (Yoshimura et al., 2001). High FGF2 levels were restored in the mutant mice using a viral gene delivery system, leading to levels of neurogenesis comparable to those of wild-type littermates. These data suggest that seizure-induced FGF2 overexpression is necessary and sufficient to prime proliferation of neural progenitor cells in the adult hippocampus (Yoshimura et al., 2001).

In an attempt to further elucidate the effect of FGF2 in epilepsy, we studied transgenic mice (TgFGF2) expressing the human FGF2 (Coffin et al., 1995; Fulgham et al., 1999). By gross examination, these mice are affected by skeletal malformations, such as shortening and flattening of long bones and moderate macrocephaly (Coffin et al., 1995). In addition, without having any spontaneous vascular defect, they exhibit a predisposition to angiogenic reactions with subsequent amplified angiogenesis (Fulgham et al., 1999). TgFGF2 mice display increased FGF2 expression in hippocampal pyramidal neurons and dentate granule cells. Increased density of glutamatergic synaptic vesicles is observed in the hippocampus, and electrophysiological data confirm an increase in excitatory inputs to CA1, suggesting the presence of a latent hyperexcitability (Zucchini et al., 2008). Indeed, TgFGF2 mice display increased susceptibility to kainate-induced seizures compared with wild-type littermates, in that latency to generalized seizure onset is reduced, while behavioral seizure scores and lethality are increased. Wild-type and TgFGF2 mice with similar seizure scores were employed for examining seizure-induced cellular consequences. Neurogenesis and mossy fiber sprouting are not significantly different between the two groups. By contrast, cell damage is significantly lower in TgFGF2 mice, especially in the areas of FGF2 overexpression (CA1 and CA3), indicating reduction of seizure-induced necrosis and apoptosis. These data are in good agreement with the neuroprotective action of FGF2 in injury models, and it can be hypothesized (as above) that seizures prompt the over-production of FGF2 in astrocytes and neurons and, in turn, this newly produced FGF2 enhances the production of ActA in selected neurons. In the hippocampus, ActA may reach high levels in CA1 and CA3 neurons, protecting these cell types from injury (Mattson, 2000). Accordingly, in FGF2 transgenic mice we observed the preservation from degeneration of CA1 and CA3 neurons, but not of those in the hilus of the dentate gyrus. Finally, we explored possible long-term synaptic rearrangements, like the sprouting of mossy fibers. Under control condition, neither wild-type of TgFGF2 mice display sprouting of the mossy fibers. Thirty days after kainate treatment, sprouting is observed in similar grade both in transgenic and in control mice (Zucchini et al., 2008).

Altogether, these data suggest that FGF2 may be implicated in seizure susceptibility and in seizure-induced plasticity, exerting different, and apparently contrasting effects: favoring acute seizures but reducing seizure-induced cell death. Coherent with this idea, FGF2 has been suggested to be implicated in the preconditioning effect of brief, non-injurious seizures that can protect against cell death induced by otherwise harmful insults, like adrenalectomy or kainate-induced SE (Kelly and McIntyre, 1994; Masco et al., 1999; Kondratyev et al., 2001). These apparently contrasting effects could depend on activation of different receptor subtypes (Simonato et al., 2006), a hypothesis not yet explored.

In addition to rodent studies, the implication of FGF2 has been investigated in human epilepsy-associated malformations of cortical development by autoptic analysis and corticectomy specimens. The data support the notion that FGF2 favors epilepsy development by altering gliogenesis and maturation of cortical neurons from migrating neuroblasts (Ueda et al., 2011).

CONCLUSIONS

The data described in this review clearly demonstrate that the FGFs are highly involved in the generation of the morpho-functional alterations in brain circuitries associated with epileptogenesis, that will eventually lead to unbalanced control of excitability and spontaneous seizures, i.e., epilepsy. As noted, information is still very incomplete, with many FGF members still unexplored and with very limited information on the FGF receptors. Moreover, these findings should be integrated with others on the involvement of other NTF families (Simonato et al., 2006).

Altogether, it seems likely that the morphology and function of adult brain circuits is regulated in a highly redundant manner. With reference to epilepsy, the emerging picture is impressively complex: multiple NTFs implicated at different levels and in different areas, producing synergic or antagonistic effects on

biological parameters that, in turn, may facilitate or oppose the transformation of a normal tissue in epileptic. Therefore, even if, in principle, identifying key elements in these phenomena may lead to effective therapies, reaching this goal will require confronting this huge complexity.

Dissecting out this situation is not trivial at all. One first step could be to generate a sort of "neurotrophicome" listing all NTFs (and NTF receptors) that are active during epileptogenesis. This would require identification of the extent to which each NTF is active (concentrations at the site of action); how it is active (local representation of receptor subtypes, including low-affinity receptors); when in the natural history of disease this occurs; how the NTF at hand will possibly interact with other NTFs. Once a sufficiently realistic picture of this "neurotrophicome" will become available, it will still be needed to determine a strategy for intervention and to develop appropriate tools to manipulate (and possibly correct) the pathological situation.

All this is extraordinarily challenging but, at the same time, is extremely fascinating and holds the promise of outstanding rewards: a better understanding of the brain function, and maybe a cure for epilepsy and other neurological diseases.

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Canonical Wnt signaling protects hippocampal neurons from Aβ oligomers: role of non-canonical Wnt-5a/Ca^{2+} in mitochondrial dynamics

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Alzheimer's disease (AD) is the most common type of age-related dementia. The disease is characterized by a progressive loss of cognitive abilities, severe neurodegeneration, synaptic loss and mitochondrial dysfunction. The Wnt signaling pathway participates in the development of the central nervous system and growing evidence indicates that Wnts also regulate the function of the adult nervous system. We report here, that indirect activation of canonical Wnt/β-catenin signaling using Bromoindirubin-30-Oxime (6-BIO), an inhibitor of glycogen synthase kinase-3β, protects hippocampal neurons from amyloid-β (Aβ) oligomers with the concomitant blockade of neuronal apoptosis. More importantly, activation with *Wnt-5a*, a non-canonical Wnt ligand, results in the modulation of mitochondrial dynamics, preventing the changes induced by Aβ oligomers (Aβo) in mitochondrial fission-fusion dynamics and modulates Bcl-2 increases induced by oligomers. The canonical Wnt-3a ligand neither the secreted Frizzled-Related Protein (sFRP), a Wnt scavenger, did not prevent these effects. In contrast, some of the Aβ oligomer effects were blocked by Ryanodine. We conclude that canonical Wnt/β-catenin signaling controls neuronal survival, and that non-canonical Wnt/Ca^{2+} signaling modulates mitochondrial dysfunction. Since mitochondrial dysfunction is present in neurodegenerative diseases, the therapeutic possibilities of the activation of Wnt signaling are evident.

Keywords: Wnt-5a signaling, Wnt/ Ca^{2+}, mitochondria, Aβ oligomers, hippocampal neurons

INTRODUCTION

Wnt proteins are involved in regulating axon guidance, dendrite morphogenesis, and synapse formation (Inestrosa and Arenas, 2010; Budnik and Salinas, 2011; Park and Shen, 2012). Wnts have also been implicated in synaptic plasticity and modulation of long-term potentiation (LTP) in mouse hippocampal slices (Chen et al., 2006; Cerpa et al., 2011). Moreover, the expression of Wnts in the mature nervous system suggests that Wnt signaling plays a key role in neuroprotection and synaptic plasticity (De Ferrari and Moon, 2006; Toledo et al., 2008).

The canonical Wnt pathway is activated by the binding of the ligand to its receptor, Frizzled (Fz), leading to glycogen synthase kinase-3β (GSK-3β) inactivation and the dissociation of β-catenin from the destruction complex (Gordon and Nusse, 2006; Angers and Moon, 2009). Under these conditions, β-catenin is accumulated in the cytoplasm and translocate to the nucleus where it associates with the TCF/LEF transcription factor and regulates Wnt target gene expression (Arrázola et al., 2009; Inestrosa et al., 2012; Nusse and Varmus, 2012). This pathway plays a key role in pre-synaptic assembly of central synapses (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). There are at least two β-catenin-independent pathways: the planar cell polarity (PCP) pathway and the Ca^{2+} pathway. The PCP pathway regulates tissue polarity and cell migration, and it is known as the Wnt/JNK pathway. The activation of the Wnt/Ca^{2+} pathway triggers the increase in intracellular Ca^{2+} levels and activates the protein kinases Ca^{2+}/Calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC) (Toledo et al., 2008; Angers and Moon, 2009).

At the post-synaptic compartment, Wnt signaling modulates the assembly of the post-synaptic apparatus (Farías et al., 2009; Varela-Nallar et al., 2010). It was observed that activation with *Wnt-5a* induces rapid changes in the clustering of the post-synaptic density protein (PSD-95), through a JNK-dependent signaling pathway, indicating that the Wnt-5a/JNK pathway modulates the post-synaptic region of the mammalian synapse (Farías et al., 2009).

Neurons are highly dependent on mitochondrial function for energy supply. Mitochondria in neurons are dynamic; they can migrate, divide, and fuse. These processes are thought to facilitate energy distribution throughout neuronal projections and to sites of high-energy demand such as synapses, to maintain

bioenergetic functionality (Westermann, 2010). In addition to energy supply, mitochondria also play a critical role in synaptic plasticity through the maintenance of calcium homeostasis in the synaptic microenvironment by calcium buffering. Mitochondria are remarkably dynamic and mitochondrial morphology is controlled by a dynamic balance between fission and fusion (Chan, 2012). The first observation of mitochondrial fission and fusion events was made in yeast (Nunnari et al., 1997; Hoppins et al., 2007). Regulation of mitochondrial division is critical for normal cellular function (Chan, 2012) and excess division is linked to numerous diseases, including neurodegenerative diseases like Alzheimer's disease (AD), Parkinson and Huntington (Cho et al., 2010; Johri and Beal, 2012; Manji et al., 2012; Itoh et al., 2013).

It has been previously demonstrated that Ca^{2+} influx leads to mitochondrial fission through activation of PKC, CaMKIα, and calcineurin, which activate Dynamin-related protein 1 (Drp1), a critical protein in mitochondrial dynamics (Smirnova et al., 2001; Qi et al., 2011). In fact, over the last few years, compelling evidence has demonstrated the relevance of the Wnt/Ca^{2+} pathway in several cellular processes (Kohn and Moon, 2005; Varela-Nallar et al., 2010). Considering the intracellular calcium increase, in response to the stimulation of the non-canonical Wnt/Ca^{2+} pathway, we previously analyzed and found that Wnt-5a might modulate mitochondrial dynamics through the possible activation of the Wnt/Ca^{2+} signaling pathway (Silva-Alvarez et al., submitted).

Here we investigated whether Wnt signaling protects neurons exposed to Aβ oligomers (Aβo) as was previously demonstrated in the case of Aβ fibrils. Also, we studied the role of the non-canonical Wnt-5a ligand on mitochondrial fission-fusion, and in particular its effect on neurons exposed to Aβ oligomers. Wnt signaling protects neurons from Aβ oligomers, in particular, we found that Wnt-5a prevents changes in mitochondrial fission-fusion dynamics and also Bcl-2 exposure on the mitochondrial surface in rat hippocampal neurons.

MATERIALS AND METHODS

PRIMARY CULTURED RAT HIPPOCAMPAL NEURONS

Rat hippocampal cultures were prepared from Sprague-Dawley rats at embryonic day 18. At day 2, cultured neurons were treated with 2 μM cytosine arabinoside (AraC) for 24 h, to remove the number of glial cells. This method resulted in highly enriched neuron cultures (95% neurons) (Cerpa et al., 2008; Farías et al., 2009).

GENERATION OF CONTROL MEDIA AND Wnt LIGAND CONDITIONED MEDIA

Control and *Wnt* ligand conditioned media were prepared from L Cells (ATCC CRL-2648), L *Wnt-3* (ATTC CRL-2814) cells and L *Wnt-5a* (ATTC CRL-2814) cells. Cells were grown until 90% confluence, approximately, and the culture medium was replaced with neurobasal medium without supplement and antibiotics. After 60 h of incubation, the media was recovered, centrifuged, sterile filtered and stored at 4°C until use (Alvarez et al., 2004; Arrázola et al., 2009; Cuitiño et al., 2010).

FORMATION OF AMYLOID-β OLIGOMERS

Synthetic $Aβ_{1–42}$ peptide corresponding to wild type human Aβ was obtained from Genemed Synthesis, Inc. (San Francisco, CA). Aβ peptide stock solution was prepared by dissolving freeze-dried aliquots of Aβ in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma H-8508) at 1 mM, incubated at room temperature for 1 h, and lyophilized. For Aβo preparation, peptide film was dissolved in dimethyl sulfoxide (DMSO, Sigma D2650) at 5 mM and then diluted into distilled water to a final concentration of 100 μM. The preparation was incubated overnight for Aβo formation (Klein, 2002). Aβo were visualized by electron microscopy and analyzed by Tris-Tricine SDS gel electrophoresis, as previously described (Dinamarca et al., 2010, 2011).

NEURONAL VIABILITY ASSAYS

Hippocampal neurons plated on polylysine-coated coverslips (30,000 neurons/cover) were treated with different concentrations of Aβo (1–20 μM) for 24 h. Live and dead neurons were analyzed in non-fixed cells with the LIVE/DEAD Viability/Cytotoxicity Kit (stock N° L3224) for mammalian cells (Molecular Probes, Carlsbad, CA).

MITOCHONDRIAL LENGTH MEASUREMENTS

Hippocampal neurons were labeled with 50 nM Mitotracker Orange CMTMRos (Molecular Probes, M-7510) for 20 min at 37°C and photographed under confocal microscopy. Mitochondrial length was measured with Image J software (NIH). For comparison purposes mitochondria were classified into three different categories of length ranging from less than 0.5, 1–2, and greater than 3 μm. A minimum of 10 micrographs were made for each treatment and scored (Silva-Alvarez et al., submitted; Zolezzi et al., 2013).

IMMUNOFLUORESCENCE

Hippocampal neurons were plated on polylysine-coated coverslips (30,000 neurons/cover). Cells were rinsed twice in ice-cold PBS and fixed with a freshly prepared solution of 4% paraformaldehyde in PBS for 20 min, and permeabilized for 5 min with 0.2% Triton X-100 in PBS. After several rinses in ice-cold PBS, cells were incubated in 1% BSA in PBS (blocking solution) for 30 min at room temperature, followed by an overnight incubation at 4°C with primary antibodies. Cells were extensively washed with PBS and then incubated with Alexa-conjugated secondary antibodies (Molecular Probes) for 30 min at 37°C (12). *Primary antibodies:* rabbit anti-β-catenin (Santa Cruz Biotechnology, Inc.); mouse anti-βIII-Tubulin (Promega Corporation); anti- Aβ 17–24 (4G8 clone)(EMD Millipore Corporation); and rabbit anti-Bcl2 (Cell Signaling Technology).

RYANODINE (Ry) AND THAPSIGARGIN INCUBATION

Hippocampal neurons were incubated with blocking concentrations of Ry (20 μM) to inhibit Ry receptors (RyRs), since Ry could inhibit or activate RyRs in a concentration dependent manner (Adasme et al., 2011), or 5 μM of thapsigargin in order to inhibit SERCA, for different times, up to 5 h (Hom et al., 2007).

QUANTIFICATION AND STATISTICAL ANALYSIS

The data represents the mean and SD or SEM from 4–6 independent experiments, where *n* stands for each independent experiment. *P*-values were obtained using Student's *t*-test. A *p*-value $^{*} < 0.05$ was considered and was indicated on the graph by an asterisk. Error bars indicate SEM. $^{*}p < 0.01$; $^{**}p < 0.001$.

RESULTS

CHARACTERIZATION OF Aβo AND THEIR EFFECT ON NEURONAL VIABILITY

Since some variability has been observed in various studies carried out so far with Aβ aggregates, we first characterized our Aβo preparation. **Figure 1A**, shows a blot made using an anti-4G8 antibody where the pattern of dimers, trimers and tetramers were observed (**a**). Roughly 50% of the initial monomer became oligomer (**b**). Under examination using an electron microscope they appear as spheres, exactly as described previously (Dinamarca et al., 2010, 2011) (**c**). In cultured hippocampal neurons, control and challenged with Aβo, mitochondria were stained with Mitotracker and Aβo were detected with the same antibody (4G8) as used in **Figure 1B**. The Aβ oligomers became attached to the somatodendritic region of pyramidal neurons, as previously described by Lacor et al. (2004). Our Aβo preparation was proved to be neurotoxic with the Calcein/Ethidium method (**Figure 1C**, green, calcein stain; red, EthD1). Calcein and ethidium stains were decreased and increased, respectively, in neurons treated with 5 μM Aβ oligomers (**a**). The quantification of viability decreased by 50% (35 ± 2%) when we used 5 μM Aβo compared with the control (72 ± 3%) and when we used 20 μM Aβo the viability decreased 90% (7 ± 1%) compared to the control (**Figure 1Cb**).

A GSK-3β INHIBITOR, BROMOINDIRUBIN-30-OXIME (6-BIO), STABILIZES β-CATENIN, AND PREVENTS NEUROTOXIC EFFECTS OF Aβ OLIGOMERS

Earlier work in our laboratory (Alvarez et al., 2004; Farías et al., 2007) showed that activation of the canonical Wnt signaling

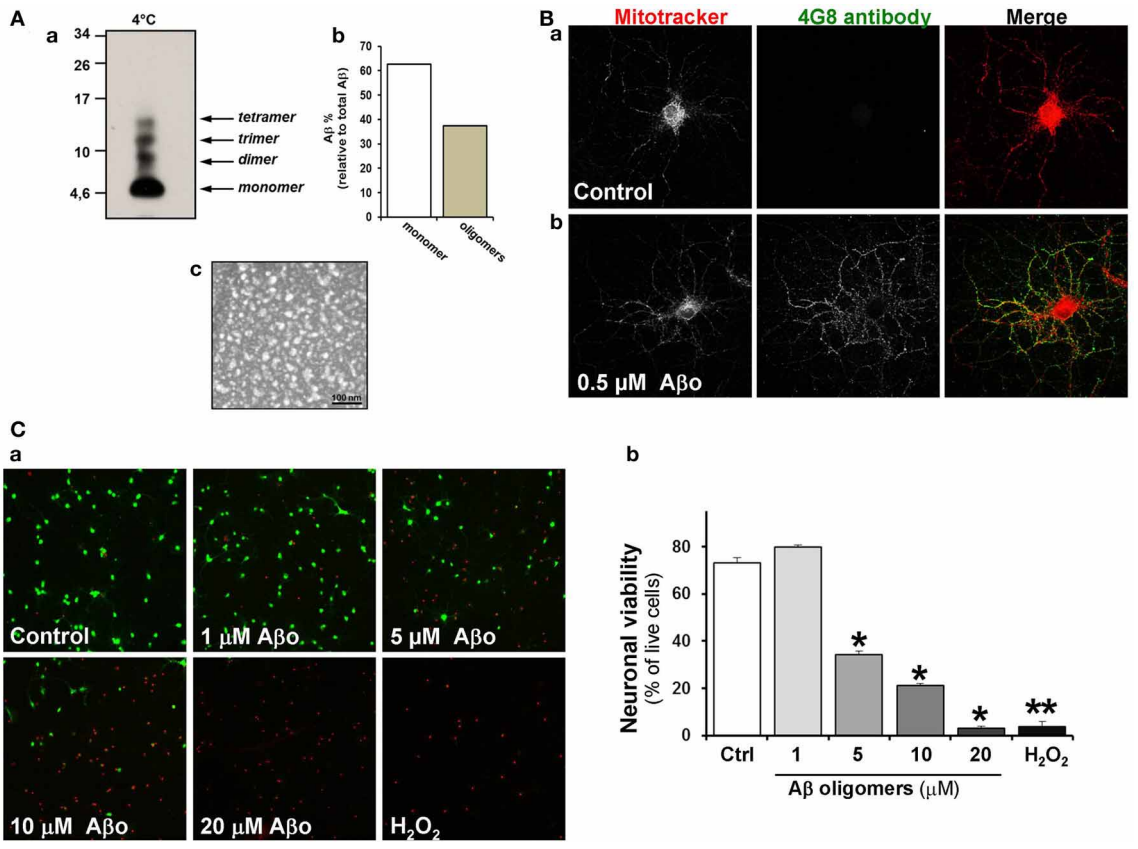

FIGURE 1 | Aβ oligomers are attached to the somatodendritic region of hippocampal neurons. (A) Characterization of Aβ oligomer species. **a**, Different forms of Aβo were analyzed by Tris-Tricine SDS gels using anti-4G8 antibody. **b**, Densitometric measurement represent relative percentage of oligomers to total Aβ. **c**, Electron microscopy shows the Aβ oligomer preparation obtained under negative staining, scale bar: 100 nm. **(B)** Hippocampal neurons were treated with Aβ oligomers and then stained with Mitotracker (red) and 4G8 antibody against $Aβ_{17–24}$ (green). **a**, Control neurons; **b**, Neurons exposed to 500nM of Aβ oligomers for 24 h. **(C)** The viability of hippocampal neurons was measured in non-fixed cells using LIVE/DEAD kit assay. Neurons were treated for 24 h with various concentrations of Aβ oligomers (1–20 μM) **a**, Treated neurons were stained with Calcein-AM/EthD1. Calcein detects live cells (green) and ethidium stain dead cells (red). 0.5 mM H_2O_2 was used as a positive control for cell death **b**, Statistical analysis represents the neuronal viability using LIVE/DEAD assay. Results are the mean ± SEM. $n = 3$ experiments, Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.005$.

pathway with either Wnt-3a or Wnt-7a in hippocampal neurons prevents the neurotoxicity triggered by Aβ aggregates formed by amyloid fibrils. In the present study, we assessed the capacity of canonical Wnt signaling activation to prevent the neurodegenerative effects of Aβ oligomers. In these experiments, to activate the canonical Wnt signaling pathway, we used 6-BIO an inhibitor of GSK-3β at low concentrations (10 nM) (Meijer et al., 2004; Polychronopoulos et al., 2004). In cultured neurons we evaluated the integrity of neuronal branching with βIII-tubulin and β-catenin levels under treatment of 5 μM Aβ oligomers plus 10 nM 6-BIO. This compound prevented almost all the Aβ oligomer-induced neurotoxicity and also recovered β-catenin levels in the neuritic branches and particularly at the soma, as shown in the graph (**Figure 2A**, graph: **b**). In addition, we also studied the effect of Aβ oligomers and 6-BIO on apoptosis, using Hoechst staining. In hippocampal neurons treated with 5 μM of Aβ oligomers, several picnotic nuclei were observed (**Figure 2Ba**), however, the apoptotic nuclei were not observed when 6-BIO was present. The quantification shows a 2.5 fold increase in apoptotic nuclei over the control, and this phenomenon was decreased to control levels when 6-BIO was present (**Figure 2Bb**). These studies with 6-BIO indicate that GSK-3β inhibition induces β-catenin stabilization and the consequent activation of the canonical Wnt/β-catenin signaling, which in turn protects neurons from the apoptotic effects of Aβ oligomers.

Wnt-5a MODULATES MITOCHONDRIAL DYNAMICS ON HIPPOCAMPAL NEURONS

Changes in mitochondrial morphology was quantified by measuring the length of mitochondria stained with Mitotracker Orange, a reduced probe that fluoresces only when it enters living cells, where it is oxidized and then sequestered into the mitochondria. This is why it is widely used as a mitochondrial membrane potential (MMP) indicator. Pictures were obtained using a confocal fluorescence microscope. As discussed in the Materials and Methods section, we selected three types of Mitotracker-stained

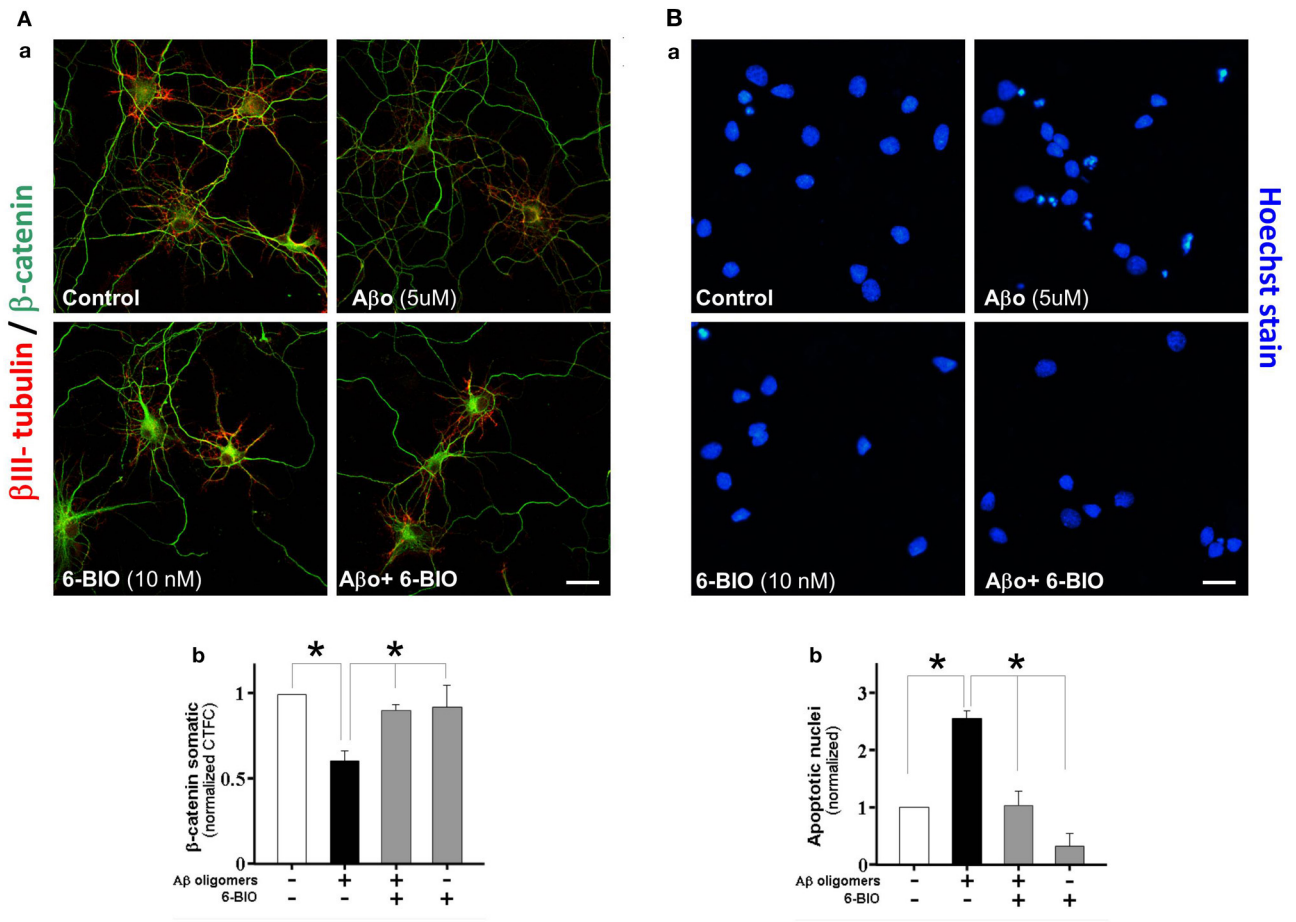

FIGURE 2 | Activation of canonical Wnt/β-catenin by a GSK-3b inhibitor (6-BIO) induces β-catenin stabilization and protects hippocampal neurons from Aβ oligomers damage. (A) The micrographs show representative neurons stained with βIII-tubulin (green) and β-catenin (red), **a**, control hippocampal neurons, and treatment with Aβ oligomers, 6-BIO or 6-BIO plus Aβ oligomers; **b**, the graph shows the somatic fluorescence of β-catenin in the neuronal soma. **(B)** The micrograph shows neurons stained with Hoechst to visualize apoptotic nuclei; **a**, Hippocampal neurons were treated under the same early conditions; **b**, the graph shows the number of apoptotic nuclei under conditions specified. Results are the mean ± SEM. $n = 4$–6 experiments, Student's *t*-test, $^*p < 0.05$. Bar represent 10 μm.

particles according to their different lengths: less than 0.5 μm representing mitochondria undergoing fission; particles of 1–2 μm that represent mitochondrial intermediate length in equilibrium between fission-fusion processes; and particles of more than 3 μm representing long mitochondria after the fusion process (Zolezzi et al., 2013). In control hippocampal neurons 15 days *in vitro* (DIV), after 2 h in control medium, mitochondria stained with Mitotracker showed an intermediate size morphology (**Figure 3A**, magnification in **a′**), however when neurons were treated with Wnt-5a for 30 min (**Figure 3Ab**), mitochondria looked smaller which is consistent with morphological changes related to fission events (**Figures 3Aa**, vs. **3Ab**; **Figure 3B**, white bar 90 ± 8%). Later, after 1 h of incubation with Wnt-5a, a fusion process became apparent (Silva-Alvarez et al., submitted). When neurons were co-incubated with Wnt-5a plus a scavenger sFRP (Rattner et al., 1997), the mitochondrial changes observed in the presence of Wnt-5a alone were abolished (**Figure 3C**: light gray bar). At the same time, neurons exposed to a canonical control Wnt ligand, Wnt-3a, showed no change in mitochondrial morphology (**Figure 3C**: gray bar). These results suggest that mitochondrial dynamics are specifically stimulated by the Wnt-5a ligand (**Figure 3C**: dark bar; 15 min, 0.75 ± 0.15; 30 min, 0.4 ± 0.15; 60 min, 2.5 ± 0.2 μm).

Wnt-5a PROTECTS MITOCHONDRIA FROM DAMAGE BY Aβ OLIGOMERS

Considering Aβ-mediated neurotoxicity in AD (Haass and Selkoe, 2007; Ballard et al., 2011), we evaluated the effect of Aβo and the participation of Sarco/Endoplasmic reticulum Ca^{+2}-ATPases (SERCA) in this process. We exposed hippocampal neurons to 0.5 μM Aβ oligomers for 2 h. Similarly, cultures were exposed to *Wnt-5a* and 5 μM Thapsigargin, a SERCA inhibitor, for the same period of time. Mitochondria were stained with Mitotracker and examined using a Confocal Microscope. As expected, Aβ oligomers induced a significant alteration of the size of the mitochondrial population compared to the control (**Figure 4Aa** vs. **4Ab**), with an increase in the number of small rounded mitochondria (see magnification **a′**, **b′**). After treatment with Wnt-5a,

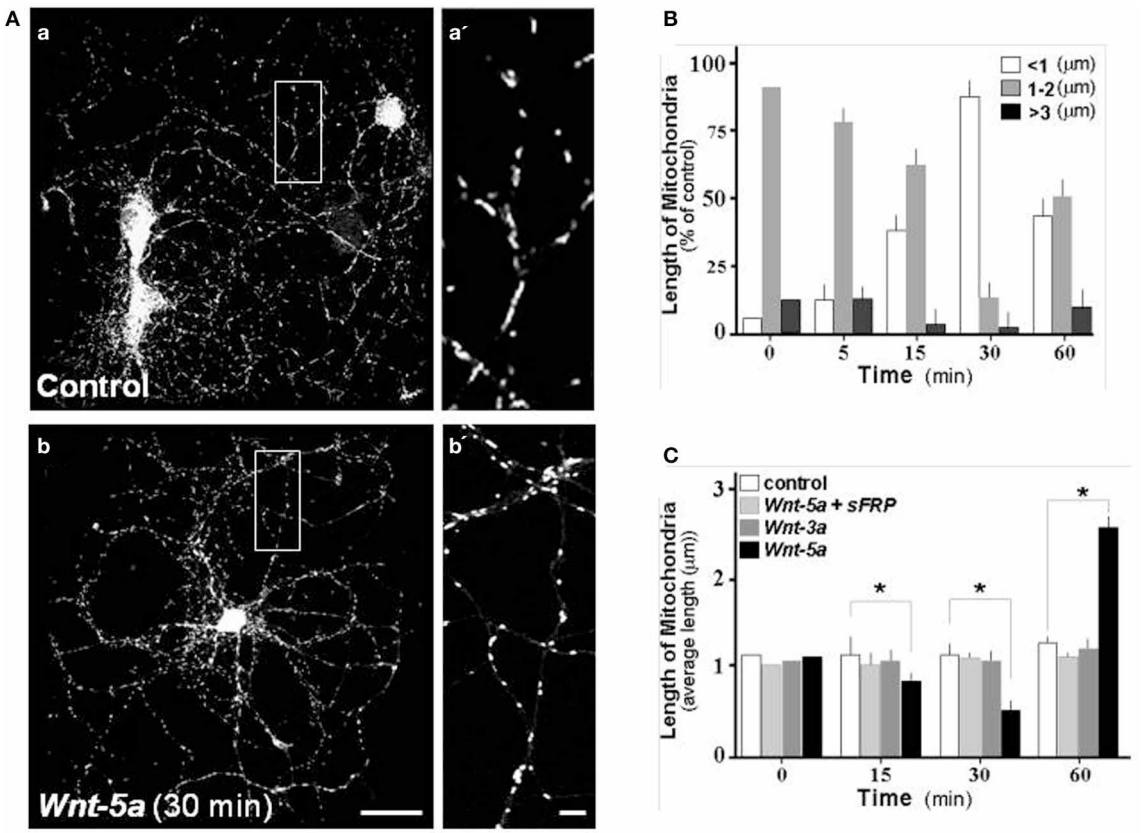

FIGURE 3 | Wnt-5a modulates mitochondrial dynamics in hippocampal neurons. Hippocampal neuron cultures of 15 days *in vitro* were labeled with Mitotracker and treated with different Wnt ligands. **(A)** Photographs show mitochondria staining with Mitotracker in control and treated neurons, **a**, Control neurons; **a′**, magnification of mitochondria, **b**, treated with Wnt-5a, **b′**, magnification of mitochondria treated with Wnt-5a for 30 min. **(B)** Various populations of mitochondria were selected and measured in control and treated neurons. The graph shows the average mitochondrial length distribution observed in a time course experiment with Wnt-5a. The quantitative analysis considered a mitochondrial length ranging from <1 μm (white bars), 1–2 μm (gray bars) and greater than 3 μm (black bars). **(C)** Quantitative analysis of mitochondrial dynamics in neurons treated with different ligands. The graph shows the average mitochondrial length distribution observed in a time course treatment with control (white bars), *Wnt-5a* plus sFRP (light gray bars), Wnt-3a (dark gray bars), Wnt-5a (black bars), bar **(a,b)**, 10 μm; bar **(a′,b′)**, 1 μm. Results are the mean ± SEM. $n = 4$–6 experiments, Student's *t*-test, $^*p < 0.05$.

mitochondria looked rather normal (**Figure 4Ac**), however in the presence of Thapsigargin, only small sized mitochondria were observed, indicating some degenerative changes (**Figure 4Ad**) (see also magnification **c′** and **d′**), as this has been described previously in ER-calcium release studies (Jiang et al., 1994; Hom et al., 2007; Friedman et al., 2010; San Martín et al., 2012).

To examine whether or not the effects of Aβo on the number of mitochondria were concentration dependent, hippocampal neurons were incubated with increasing amounts of Aβo (**Figure 4Ba**). After 5 h of treatment with 50 nM Aβo, almost no effect was observed, however, a transient increase was apparent at 1 h. In addition, a rather small change was observed with 250 nM Aβ oligomers. However, at 0.5 and 1.0 μM of Aβo, a clear decrease of the mitochondria/neuron ratio was apparent (**Figure 4Ba**, 3 h: 40 ± 5, 6.5 ± 5%), probably due to the reduction of MMP in response to Aβo, and therefore a decrease in the number of functional mitochondria. In order to compare the putative protective effect of Wnt ligands, neurons were pre-incubate with Wnt-3 and Wnt-5a ligands by separate, before 0.5 μM Aβo challenge (**Figure 4Bb**). Wnt-5a, a non-canonical Wnt ligand, clearly

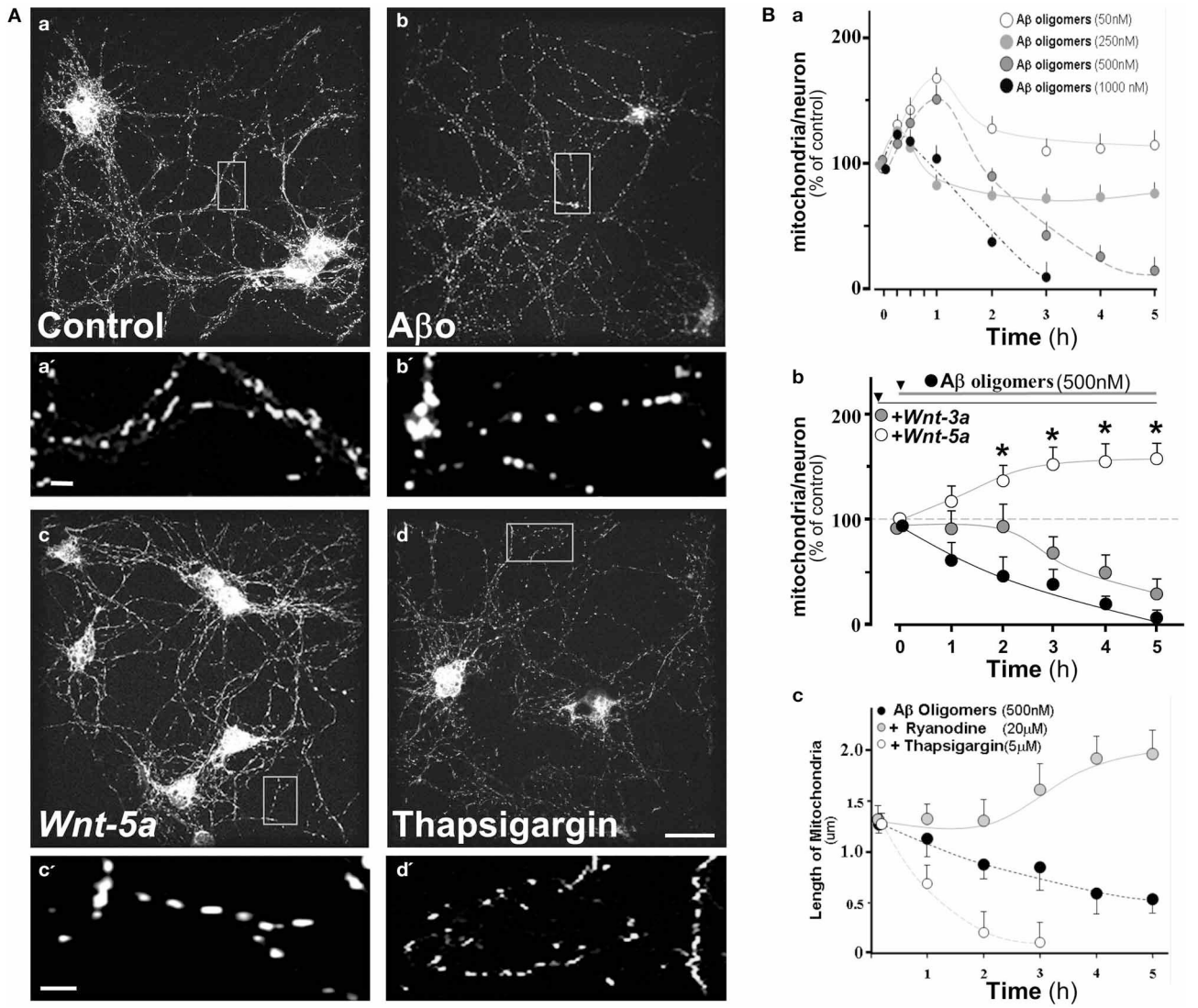

FIGURE 4 | Aβ oligomers modify mitochondrial dynamics in hippocampal neurons, Wnt-5a ligand protects and stabilizes mitochondrial membrane potential from such effects. 15 day *in vi*tro hippocampal neuron cultures were labeled with Mitotracker orange and treated with various concentrations of Aβ oligomers. **(A)** Photographs show mitochondria stained with Mitotracker in control and treated neurons, **a**, Control neurons; **a′**, magnification of mitochondria; **b**, treated with Aβ oligomers; **b′**, magnification of mitochondria treated with Aβ oligomers, **c**, treated with Wnt-5a, **c′**, magnification of mitochondria treated with Wnt-5; **d**, treated with 5 μM Thapsigargin. **(B)** Quantitation of mitochondrial dynamics under different conditions; **a**, Graph shows mitochondria/neuron ratio in a time course experiment under various concentrations of Aβ oligomers; **b**, Neurons pretreated with Wnt-5a or Wnt-3a and then challenged with Aβ oligomers; **c**, Length of mitochondria under inhibitors of RyR and SERCA. Primary cultures of rat embryo hippocampal neurons (15 DIV) were treated at 37°C. bar **(a,b,c,d)**, 10 μm; bar **(a′,b′,c′)**, 2 μm. Results are the mean ± SEM. $n = 4$–6 experiments, Student's *t*-test, $^*p < 0.05$.

protected from the toxic effect of the Aβ oligomers from very early on and the mitochondria/neuron ratio increased almost 50% after 3 h of co-treatment (**Figure 4Bb**, white circles, 3 h: 152 ± 5%). This suggests that Wnt-5a protects or increases the number of functional mitochondria in neurons. This effect was sustained over time and was also capable of preserving MMP in these neurons during the analyzed period. A different situation was observed with Wnt-3a, a canonical *Wnt* ligand, which during the first 2 h of treatment was able to prevent the Aβo effect, however, after 3 h a similar decay to the one observed with oligomers treatment was apparent (**Figure 4Bb**, gray circles, 3 h: 75 ± 5%), indicating that canonical Wnt signaling was not able to sustain a long-term protection/preserving MMP of these mitochondria. Previous evidence indicates that the Wnt-3a ligand enhances mitochondrial biogenesis through Insulin receptor substrate-1 (IRS-1) producing, as a consequence, an increase of reactive oxygen species (ROS) levels and oxidative damage in non-neuronal cells (Yoon et al., 2010).

Early studies carried out in our laboratory indicate that Wnt-5a induces a rapid increase in intracellular calcium, as predicted by the Wnt/Ca^{2+} pathway (Varela-Nallar et al., 2010). The main targets of intracellular calcium are the SERCA and the Ryanodine receptor (RyR), both located in the ER, that control the mitochondrial calcium and the local feedback of intracellular calcium homeostasis (Paula-Lima et al., 2011). To evaluate the role of these receptors, we used specific inhibitors for each one, including Thapsigargin (SERCA) and Ryanodine (RyR).

The graph shows the mitochondria length in the presence of RyR and SERCA inhibitors in a time-course experiment (**Figure 4Bc**). Aβ oligomers decreased mitochondrial length, held constantly during the 5 h period of study (**Figure 4Bc**: black circle, 0.55 ± 0.2 μm), however when we blocked RyR (20 μM Ryanodine), the mitochondrial length appeared normal and eventually increased (**Figure 4Bc**: gray circle, 5 h: 1.9 ± 0.2 μm), suggesting that Ryanodine blocks the mitochondrial fragmentation induced by Aβo (Paula-Lima et al., 2011). When the hippocampal neurons were co-incubated with Aβ oligomers plus Thapsigargin, mitochondria entered a fragmentation process, from where they did not recover (**Figure 4Bc**: white circle, 2 h: 0.25 ± 0.2 μm). These results indicate that RyR and SERCA are involved in calcium homeostasis and desregulation of this homeostasis is protected by Wnt-5a through RyR clustering associated with the ER (Silva-Alvarez et al., submitted). Wnt-5a, a non-canonical Wnt/Ca^{+2} pathway, activates a physiological signal which triggers mitochondrial dynamics, protecting neurons from the neurotoxic effects of the Aβ oligomers.

Wnt-5a PREVENTS Bcl-2 INCREASE INDUCED BY AβO IN MITOCHONDRIAL COMPARTMENTS

To determine the role of Wnt-5a as a neuroprotective agent against Aβo damage, we stained the mitochondria with Mitotracker and examined Bcl-2 levels, a Wnt-target gene and anti-apoptotic protein (Fuentealba et al., 2004; Fuenzalida et al., 2007), by immunofluorescence in neurons exposed to 500 nM Aβo in the presence of Wnt-5a (**Figure 5A**). As expected, there was a weak positive reaction to Bcl-2 in control neurons (first row) and neurons treated with Wnt-5a (second row). Neurons treated with the Aβ oligomers showed an increase in Bcl-2 staining in the mitochondrial compartment (third row). This effect was almost completely prevented when neurons were co-incubated with Wnt-*5a* in the presence of Aβo (fourth row). Similar experiments were carried out in a time-dependent manner in the presence of Aβo, and an increase in the deposition of Bcl-2 on mitochondria was significant (**Figure 5B**: black circle, 3 h: 1.6 ± 0.3). It was apparent that soon after the application of Wnt-5a, Bcl-2 levels were normalized up to 2 h of treatment (**Figure 5B**: gray circle, 2 h: 1 ± 0.1). These results indicate that Wnt-5a modulates the apoptotic changes induced by Aβo to prevent neuronal death.

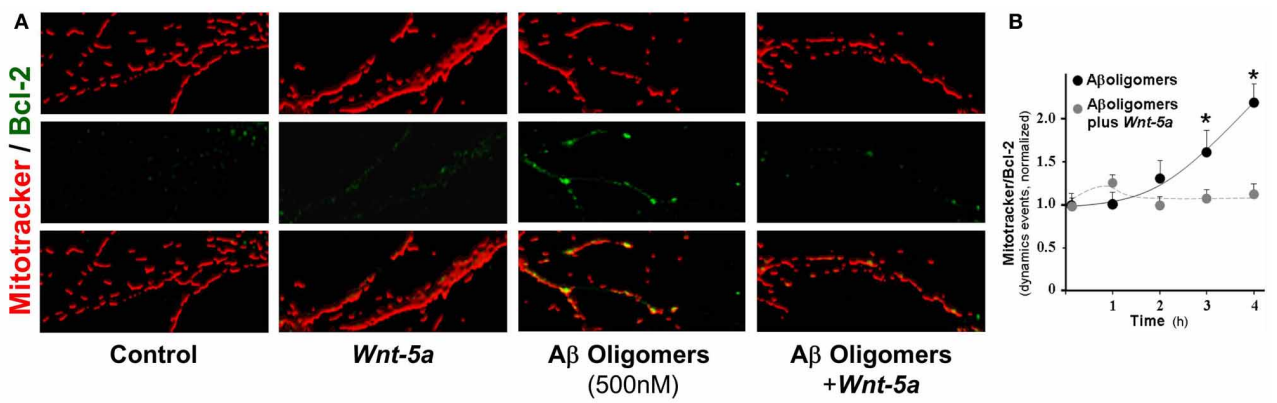

FIGURE 5 | Wnt-5a prevents Bcl-2 increase triggered by Aβo in mitochondrial compartments. The micrographs show representative mitochondria stained with Mitotracker orange and Bcl-2 (green). **(A)** control neurons, neurons treated with Wnt-5a for 2 h, neurons treated with Aβ oligomers (500 nM) and neurons treated with Aβ oligomers plus *Wnt-5a*. **(B)** graph shows the co-localization of Bcl-2 staining over mitochondria (dynamic events) in the presence of Aβ oligomers with and without Wnt-5a. Primary cultures of rat embryo hippocampal neurons (15 DIV) were treated at 37°C. Results are the mean ± SEM. $n = 4$–5 experiments, the number of dynamics events were measured in 100 μm of dendrites. Student's *t*-test, $^*p < 0.05$.

DISCUSSION

Mitochondria are dynamic organelles that constantly fuse and divide. The correct modulation of the mitochondrial fission–fusion equilibrium is critical to regulating cell death, mitophagy, and organelle distribution (Chan, 2012). Excessive mitochondrial fission is associated with pathologies such as Charcot-Mary-Tooth-IIa, neurodegenerative diseases and diabetes (Detmer and Chan, 2007; Yoon et al., 2011; Itoh et al., 2013). In AD, it is possible to recognize several defects in mitochondrial functions (Wang et al., 2009), for instance, impaired mitochondrial fission or fusion, can produce oxidative damage (Kageyama et al., 2012) and may also produce local bioenergetics failure in neuronal processes lacking mitochondria. Currently, there is considerable evidence suggesting a key role for the morphological abnormalities and/or dysfunctional mitochondria in the pathogenesis of AD (Lin and Beal, 2006; Cho et al., 2010; Manji et al., 2012; Itoh et al., 2013), indicating mitochondria as a central organelle and a new potential therapeutic target against AD.

Mitochondrial dysfunction is a prominent feature on AD neurons. Quantitative morphometric analysis of mitochondria shows increased abnormal and damaged mitochondria in the AD brain (Hirai et al., 2001; Baloyannis, 2006). Aβ causes rapid and severe impairment of mitochondrial transport (Rui et al., 2006), and Aβ overproduction causes abnormal mitochondrial dynamics in neurons (Wang et al., 2008). Interestingly, Aβ oligomers caused mitochondrial fragmentation and reduced mitochondrial density in neuronal processes. Also Aβ oligomer-induced synaptic change correlates with abnormal mitochondrial distribution (Wang et al., 2009).

In a previous study, we demonstrated that activation of the canonical Wnt signaling pathway in hippocampal neurons prevents the neurotoxicity triggered by Aβ aggregates formed by amyloid fibrils (Alvarez et al., 2004). In the present study we showed for the first time that the activation of canonical and non-canonical Wnt signaling exerts a neuroprotective effect against Aβ oligomers at different levels: (1) firstly, the canonical Wnt/β-catenin pathway primarily affects the whole neuronal repertoire; (2) secondly, the non-canonical Wnt pathway through the Wnt-5a ligand specifically affects the mitochondrial compartment. In order to activate the canonical Wnt pathway we incubated hippocampal neurons with a GSK-3β inhibitor (6-BIO) (Meijer et al., 2004). In these studies, the pre-incubation with 6-BIO, in a concentration selected to inhibit GSK-3β (Polychronopoulos et al., 2004) prevented the decrease of β-catenin accumulation observed in neurons treated with Aβ oligomers, indicating that 6-BIO acts through the activation of the canonical Wnt/β-catenin pathway protecting neurons from Aβ oligomer toxicity. On the other hand, our previous work (Silva-Alvarez et al., submitted) together with these results, suggest that the non-canonical Wnt pathway activation, using Wnt-5a ligand, protects neurons against the toxicity induced by Aβ oligomers, producing a regulation of calcium homeostasis, which is altered by the presence of Aβ oligomers.

Both Wnt/$Ca^{2+}$and Wnt/JNK, activated by the Wnt-5a ligand, modulate NMDA receptors (Cerpa et al., 2010, 2011, Varela-Nallar et al., 2010). Furthermore, Wnt-5a signaling via the Wnt/Ca^{2+} pathway stimulates dendritic spine morphogenesis in hippocampal neurons (Varela-Nallar et al., 2010). Wnt-7a, a canonical Wnt ligand, also has an effect in dendritic spines (Ciani et al., 2011). Finally, *Wnt-5a* regulates inhibitory synapses, inducing the surface expression and maintenance of the GABAA receptor in the membrane of hippocampal neurons as well as the recycling of functional GABAA receptors through activation of CaMKII (Cuitiño et al., 2010).

We recently established that Wnt-5a -induces mitochondrial dynamics in an ER-calcium related mechanism (Silva-Alvarez et al., submitted), suggesting that one of the underlying molecular mechanisms related to Wnt-5a effects might be due to the modulation of the balance between mitochondrial fission and fusion. The role of the Wnt/Ca^{2+} pathway in several neuronal processes has been well established (Kohn and Moon, 2005; Angers and Moon, 2009). In the Wnt/Ca^{2+} pathway, Wnt-5a can activate both PKC and CaMKII, by increasing the intracellular Ca^{2+} concentration coming from internal stores (Kuhl et al., 2000; Kohn and Moon, 2005). Considering that the ER is the main cellular calcium reserve (Bravo et al., 2012), the specific inhibition of RyR and SERCA studied here, suggests that Ca^{2+} certainly comes from the ER and supports the idea that ER-related calcium lies downstream of Wnt-5a effects, as illustrated in **Figure 6**.

The recovery of mitochondrial length observed in the presence of ryanodine, suggests that calcium from the ER, via RyR, contributes to the imbalance produced by Aβ oligomers leading to mitochondrial fragmentation as seen with the inhibition of RyR in the present study. The relationship between mitochondrial fragmentation and ER-mediated calcium release, has been previously reported in hippocampal neurons challenged with Aβo (Paula-Lima et al., 2011), and with the direct agonist of RyR 4-CMC, that reproduced the mitochondrial fragmentation effects observed with Aβo (San Martín et al., 2012). There is strong evidence for the critical role of calcium balance on mitochondrial functions and fate. In this context, we have described that tetrahydrohyperforin (THH), a drug that prevents several toxic effects observed in a transgenic AD mouse model (Inestrosa et al., 2011), is able to avoid mitochondrial calcium overloading and at the same time can control alterations of mitochondrial fission-fusion dynamics in injured hippocampal neurons (Zolezzi et al., 2013). On the other hand, the anti-apoptotic Bcl-2 protein is a target gene of the canonical Wnt/β-catenin signaling pathway (Fuentealba et al., 2004; Fuenzalida et al., 2007; Youle and Strasser, 2008), which is activated in response to cell injury, such as exposure to Aβ oligomers or oxidative stress to prevent apoptosis. Several studies have revealed that Bcl-2 proteins could act on mitochondrial Ca^{2+} homeostasis on neural cells, allowing for more mitochondrial Ca^{2+} uptake without any mitochondrial respiratory impairment, suggesting that Bcl-2 can protect mitochondria from Ca^{2+} overload, although no consensus exists concerning the mechanisms underlying this function (Murphy et al., 1996; Zhu et al., 1999). In this direction, our results show that Wnt-5a is able to modulate the increase in the anti-apoptotic protein on the outer

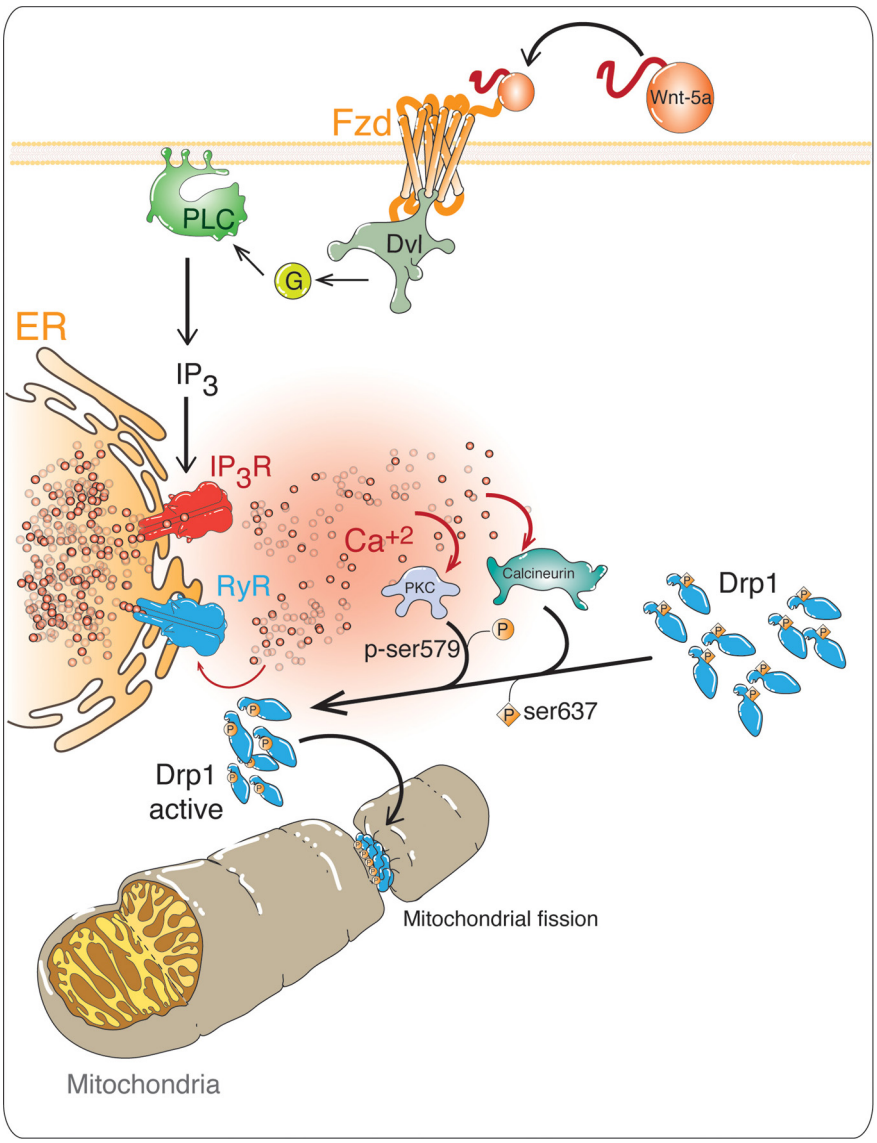

FIGURE 6 | Possible role of non-canonical *Wnt/Ca*$^{2+}$ pathway in mitochondrial dynamics. In the non-canonical Wnt/Ca^{2+} pathway, the binding of the ligand to its receptor Fz, activates Dishevelled (Dsh), which allows the activation of a trimeric G protein. The G protein activates phospholipase C (PLC), increasing the levels of inositol triphosphate (IP_3), which increases the intracellular Ca^{2+} concentrations, coming from the ER. This Ca^{2+} induces further Ca^{2+} releases through RyRs. The high levels of Ca^{2+} activate Ca^{2+}-dependent proteins such as protein kinase C (PKC) and the phosphatase Calcineurin. These enzymes regulate the activation of Dynamin-related protein 1 (Drp1), phosphorylation by PKC and CaMK and dephosphorylation by calcineurin, as a result Drp1 is translocated from the cytoplasm to the outer mitochondrial membrane (OMM), which is a signal for mitochondrial fission.

mitochondrial membrane of neurons, suggesting that Wnt-5a protects neurons from the early apoptotic effect induced by Aβ oligomers.

We conclude that the neuroprotective properties of Wnt-5a not only affect mitochondrial dynamics, but also might control molecular mechanisms related to apoptotic processes that take place in the living mitochondria.

Considering the importance of the regulation of intracellular calcium levels mediated by the activation of the Wnt/Ca^{2+} pathway, we propose a model for the possible mechanism of action of *Wnt-5a* on mitochondrial dynamics (**Figure 6**). In this model, Wnt-5a binds to the Fz receptor activating phospholipase C, which generates inositol-3-phosphate (IP_3) and binds to its receptor (IP3 R) releasing intracellular Ca^{2+} from the ER. This Ca^{2+} could activate the RyRs and induce the Ca^{2+} release (Fill and Copello, 2002). In a previous work, we established that mitochondrial dynamics induced by Wnt-5a, was abolished by the use of the ER-calcium specific inhibitors of IP3R and RyRs, Xestospongin C and high concentrations of Ry respectively, supporting the idea that ER-related calcium is part of the signal transduction of Wnt-5a required to induce mitochondrial dynamics (Silva-Alvarez et al., submitted). The increased

calcium activates several calcium dependent proteins such as PKC and Calcineurin. Our model suggests that these enzymes could activate Drp1, which controls mitochondrial fission by PKC-phosphorylation at Ser579 (Qi et al., 2011) and Calcineurin-dephosphorylation at Ser637 residues (Cereghetti et al., 2008), triggering Drp1 translocation from the cytoplasm to the mitochondrial outer membrane inducing mitochondrial fission.

Our research indicates that non-canonical Wnt signaling, activated by Wnt-5a ligand, regulates mitochondrial dynamics and/or preserves the MMP and prevents the mitochondrial fragmentation induced by Aβ oligomers and the exposure of Bcl-2, an anti-apoptotic mitochondrial protein, suggesting that Wnt components might be used to control mitochondrial dysfunction, such as the one observed in AD.

Considering that neurons are among the most energy-consuming cell types, it is essential to keep a stable metabolic-energetic stage during synaptic function, through normal mitochondrial functions. Therefore it becomes important to further study the role of the Wnt-5a ligand in mitochondrial functions in order to consider the mitochondria as a possible therapeutic target for AD treatment.

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Characterization of Wnt/β-catenin and BMP/Smad signaling pathways in an *in vitro* model of amyotrophic lateral sclerosis

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Different pathways activated by morphogens of the early embryonic development, such as the Wnt and the Bone Morphogenetic Protein (BMP) ligands, are involved in diverse physiological and pathological conditions of the nervous system, including neurodegeneration. In this work, we have analyzed the endogenous activity of the canonical Wnt/β-catenin and BMP/Smad-dependent pathways in an *in vitro* model of amyotrophic lateral sclerosis (ALS), given by motor neuron-like NSC34 cells stably expressing wild-type or G93A mutated forms of human Cu/Zn superoxide dismutase-1 (SOD1). As ALS-derived motor neurons, NSC34 cells expressing mutated hSOD1 show a decreased proliferation rate, are more susceptible to oxidation-induced cell death and display Golgi fragmentation. In addition, they display an impaired ability to induce the expression of the motor neuronal marker Hb9 and, consistently, to morphologically differentiate into a motor neuronal phenotype. Regarding signaling, our data show that the transcriptional activity associated to the Wnt/β-catenin pathway is decreased, a finding possibly associated to the cytosolic aggregation of β-catenin. In turn, the BMP-dependent phosphorylation of Smad1 and the transcriptional activation of the BMP/Smad pathway is increased in the pathologic model. Together, these findings suggest that Wnt/β-catenin and the BMP-dependent pathways could play relevant roles in the neurodegeneration of motor neurons in the context of ALS.

Keywords: Wnt, BMP, ALS, motor neurons, beta catenin, smad proteins

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective degeneration and subsequent death of motor neurons, those in the motor cortex and brainstem (superior motor neurons), as well as the ones located in the spinal cord (inferior motor neurons; Goodall and Morrison, 2006; Pasinelli and Brown, 2006). Even though most ALS cases are sporadic, a 5–10% of them have a genetic linkage, as they are associated to point mutations of several proteins, such as Cu/Zn superoxide dismutase-1 (SOD1), TAR DNA-binding protein 43 (TDP43), Fused in Sarcoma (FUS), senataxin (SETX), amongst others (Bruijn et al., 2004; Schymick et al., 2007; Robberecht and Philips, 2013). Remarkably, a 20% of familial ALS cases have been linked to point mutations of SOD1 (Rosen, 1993). Indeed, the G93A mutation of SOD1 has been widely used to generate model systems of ALS, either animals or *in vitro*, as they mimic the main clinical, pathological, and cellular features of the disease (Gurney et al., 1994; Pasinelli et al., 1998; Arciello et al., 2011). The mechanisms by which these mutations cause neuronal death have not been fully described; however, it is believed that rather than changes in protein activity, conformational changes due to these mutations lead to the formation of toxic intracellular protein aggregates (Bruijn et al., 1998; Durand et al., 2006; Bendotti et al., 2012). In spite of the fact that clinical manifestations of ALS occur in the adult life, several research lines indicate that alterations of the motor neuronal network begin at early development of the nervous system, marking the onset of the disease (Bendotti et al., 2012; van Zundert et al., 2012; Robberecht and Philips, 2013). However, the molecular players involved in these early phenotypes have not been fully described.

Cumulative evidence in the last few years reveals that signaling pathways triggered by morphogens of the early embryonic development, such as the Wnt and Bone Morphogenetic Protein (BMP) families, play key roles on different features of the nervous system (Henriquez et al., 2011; Benavente et al., 2012; Henriquez and Salinas, 2012; Salinas, 2012). Wnt ligands signal through their cognate Frizzled (Fz) receptors to activate several different pathways. Upon activation of the "canonical" Wnt pathway, the key intracellular effector β-catenin escapes proteosomal degradation, becomes accumulated in the cytosol and traslocates to the nucleus activating the expression of Wnt target genes (Gordon and Nusse, 2006; Kim et al., 2009). In addition to its role in Wnt signaling, β-catenin plays crucial roles in cell–cell contacts (Nelson and Nusse, 2004). In turn, BMP ligands bind to pre-formed heteromeric complexes of BMP receptors type I and type II (BMPRI and BMPRII) to induce the phosphorylation of the cytosolic Smad proteins that migrate to the nucleus to induce target genes (Nohe et al., 2002; Shi and Massague, 2003; Miyazono et al., 2005). In addition, BMPs also have the ability to trigger Smad-independent pathways (Sieber et al., 2009; Bragdon et al., 2011). Signaling through

Wnt and BMP ligands exert a wide range of effects that are highly dependent on the cell type; therefore, indentifying critical factors such as co-receptors, antagonists and intracellular regulatory proteins, is required to understand the potential contribution of these signaling pathways in different physiological contexts.

Wnt pathways are not only involved in the formation of neuronal connectivity, but also in synaptic function and plasticity (Cerpa et al., 2011; Salinas, 2012). Remarkably, several lines of research support the idea that a decrease in Wnt signaling activity is associated to the pathogenesis of some neurodegenerative diseases, such as Alzheimer's and Parkinson's (Inestrosa and Arenas, 2010; L'Episcopo et al., 2011). Consistent with this notion, activation of Wnt signaling plays neuroprotective roles in models of Alzheimer's disease, either *in vivo* or *in vitro* (De Ferrari et al., 2003; Alvarez et al., 2004; Cerpa et al., 2010; Purro et al., 2012). In this regard, recent evidence shows that some Wnt ligands are up-regulated in motor neurons of ALS model mice (Chen et al., 2012; Li et al., 2013; Wang et al., 2013). Regarding BMP-dependent signaling, it has been demonstrated that the BMP2 ligand is up-regulated in damaged motor axons of the facial nerve, suggesting that changes in the activity of BMP pathways could be involved in protection or regeneration of motor neurons (Wang et al., 2007; Henriquez et al., 2011).

In this work, we first characterized motor neuron-like NSC34 cells stably expressing wild-type or G93A mutated forms of human SOD1 (Gomes et al., 2008). ALS-like cells displayed Golgi fragmentation, as well as impaired morphological differentiation and lower expression levels of the motoneuron marker Hb9 than control cells. Also, cell death was significantly higher in differentiated cells expressing mutant hSOD1. Regarding signaling, Wnt-dependent transcription was inhibited in these cells, a finding likely associated to an altered distribution of β-catenin. In turn, the BMP/Smad-dependent pathway was increased in ALS-like cells. Our findings suggest that Wnt and BMP-dependent pathways could play relevant functions in the context of motor neuronal cell death occurring in ALS.

MATERIALS AND METHODS

CELL CULTURE

Neuroblastoma × spinal cord cells NSC34 (Cashman et al., 1992) stably expressing human wild-type SOD1 (NSC34hSOD1WT cells) or mutant SOD1 (NSC34hSOD1G93A cells) were a gently gift of Dr. Julia Costa at ITQB, Oerias, Portugal (Gomes et al., 2008). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Hy-Clone, South Logan, UT, USA) supplemented with 15% fetal bovine serum (FBS) 1% penicillin/streptomycin solution and 0.4 mg/ml G418 at 37°C in a 5% CO2 atmosphere. Cells were grown on plastic or glass surfaces previously coated with 0.01% poli-L-lysine (Sigma Aldrich, Saint Louis, MO, USA) for 24 h at 37°C, and 0.5% gelatin (Sigma) for 30 min at 37°C. Cells were induced to differentiate in Neurobasal medium (Invitrogen, Grand Island, NY, USA) without FBS for 24–36 h.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Total RNA from NSC34 cells was obtained using Trizol reagent (Invitrogen). For reverse transcription-polymerase chain reaction (RT-PCR), 1 μg of RNA was pretreated with DNase I (Fermentas, ON, Canada) and further incubated in a buffer containing 10 μM oligo dT, reverse transcription buffer (0.5 M Tris–HCl, pH 8.3, 0.75 M KCl, 0.03 M MgCl2), 20 U RNase inhibitor (NEB, Ipswich, MA, USA) and 1 mM dNTPs (Invitrogen) at 37°C for 5 min. Stratascript reverse transcriptase (Stratagene, Baltimore, MD, USA) was added (160 U) and the mix was further incubated at 42°C for 1 h. Parallel reactions were performed in the absence of reverse transcriptase to control for the presence of contaminant DNA. For amplification, a cDNA aliquot in a volume of 12.5 μl containing 20 mM Tris buffer pH 8.4, 50 mM KCl, 1.6 mM MgCl2, 0.4 mM dNTPs, and 0.04 U Taq polymerase (Kapabiosystems, Boston, MA, US) was incubated 95°C for 5 min, 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s for 35 cycles. Primers were Hb9_S: GTACCTGTCTCGACCCAAGC, Hb9_AS: CCATTGCTGTACGGGAAGTT (expected product 327 bp), GAPDH_S: GGAGCCAAACGGGTCATCATCTC, GAPDH_AS: GAGGGGCCATCCACAGTCTTCT (expected product 233 bp) BMPRII_S: TTTGCAGCCTGTGTGAAGTC, BMPRII_AS: CACAAGCTCGAATCCCTAGC (expected product 403 bp). PCR products were separated by 1.2% agarose gel electrophoresis and visualized following ethidium bromide staining.

WESTERN BLOT

Cells were lysed in Tris-HCl 50 mM, pH 7.5; NaCl 100 mM, Triton X-100 0.5 % v/v buffer. Equal amounts of protein were resolved on SDS-polyacrylamide gels, transferred onto PVDF membranes (Millipore, Billerica, MA, USA) and subjected to Western blot analyses. Antibodies against α-tubulin (Sigma-Aldrich, St. Louis, MO, USA), Id1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-Catenin (Santa Cruz), phospho-Smad 1/5/8 (pSmad) (Cell Signaling Technologies, Frankfurt, Germany), and Hb9 (Abcam, Cambridge, UK) were used for immunoblotting. Bound antibodies were visualized using horseradish peroxidase-coupled secondary antibodies (Jackson InmunoResearch, West Grove, PA, USA) followed by development under a chemiluminescence kit (Perkin Elmer, Waltham, MA, USA).

IMMUNOFLUORESCENCE MICROSCOPY

For immunostaining, NSC34 cells were grown on 18 mm glass coverslips. When indicated, cells were treated with 40 mM lithium chloride for 6 h. The medium was removed and cells were rinsed with cold PBS, fixed with 4% paraformaldehyde for 30 min, or acetone/methanol (1:1) for 5 min, at 4°C and subsequently permeabilized with 0.1% Triton X-100 in Tris-phosphate buffer. Cells were rinsed with Tris-phosphate and then incubated with primary antibodies diluted in blocking solution (1% BSA in Tris-phosphate buffer), 15 h at 4°C. Primary antibodies were anti-Islet1 (1:10; Developmental Studies Hybridoma Bank); anti-MAP1B (1:450; Santa Cruz); anti-β-Catenin (1:200; Santa Cruz), and anti Hb9 (1:100; Abcam). Corresponding Alexa-488 and -546 conjugated secondary immunoglobulins (Invitrogen) were incubated for 2 h at room temperature. Images were acquired with a laser confocal LSM700 Zeiss microscope at the CMA Bio–Bio facility (Universidad de Concepción, Concepción, Chile).

TRANSIENT TRANSFECTION

Cells were incubated in OptiMEM medium (Invitrogen) and transfected using a Lipofectamine and Plus Reagent mix (Invitrogen), according to the indications of the manufacturer. For different luciferase reporter assays, the amounts of plasmid DNAs were: pId1-luc (Lopez-Rovira et al., 2002) 1.8 μg, pRL-SV40 180 ng, and TOPFlash 0.7 μg, pRL-SV40 70 ng. To determine the morphology of the Golgi apparatus, cells were transfected with 700 ng the FU-GolgimRFP plasmid, coding for the Golgi β-galactosiltransferase protein fused to red fluorescent protein (a gently gift of Dr. P. Zamorano, Universidad de Antofagasta, Chile).

LUCIFERASE ASSAYS

Transfected cells in 35 mm plates were split 24 h after transfection and induced to differentiate in serum free Neurobasal medium for the indicated times. Luciferase activity was quantified using the Luciferase Assay System (Promega, Madison, WI, USA). Results were normalized against a Renilla luciferase control reporter vector (Promega).

CELL VIABILITY ASSAY

Cells grown in 35 mm plates were treated with 0.2 mM hydrogen peroxide for 30 min at 37°C. After washing, cells were incubated with growing or differentiation medium and returned to the incubator for additional 6 h. Cell viability was measured by the LDH release assay based on the instructions of the manufacturer (VALTEK, Santiago, Chile). LDH activity was quantified in conditioned media and in cell extracts obtained in buffer Tris-HCl 50 mM, pH 7.5, NaCl 100 mM, Triton X-100 0.5% v/v, at the indicated times.

ADHESION ASSAY

Cell culture substrates were prepared by coating Petri plates with nitrocellulose by dissolving 5 cm^2 of nitrocellulose in 6 ml of methanol (Lagenaur and Lemmon, 1987). Droplets of 2 μl containing substrates were applied. Substrates were 3.6 mg/ml collagen, 1% gelatin, 2 mg/ml poli-L-lysine, 1 mg/ml laminin (Sigma). After 1 h at 37°C, plates were washed and subsequently blocked with 1 % BSA. Cells were added and incubated for 2 h at 37°C. After washing, bound cells were fixed with 4% paraformaldehyde for 30 min at 4°C. Images were acquired with an Olympus CK40 microscope.

PROLIFERATION ASSAY

Plastic 35 mm plates were seeded with 250,000 cells and trypsinized every day for three consecutive days. Cells were manually counted with a hemacytometer using an Olympus CK40 microscope. Results correspond to the average ± SEM of three independent experiments.

IMAGE ANALYSIS

Acquired images were analyzed using the ImageJ software. The number of differentiated cells and the length of their neurites were determined in cells having at least one neurite with a minimum size equal to the cell soma diameter. For each condition, 20–25 fields from three different experiments were evaluated. The mean ± SEM intensity was plotted.

STATISTICAL ANALYSIS

One-way ANOVA was used for comparison among three or more groups followed by Bonferroni's *post hoc* analysis for multiple comparison between different groups. Neurite length and TOPFlash activity were compared using unpaired *t*-test. $p < 0.05$ was considered to indicate statistical significance.

RESULTS

CULTURE CONDITIONS FOR NSC34hSOD1 CELLS

As NSC34 cells stably expressing human SOD1, either the wild-type or the G93A mutated form, adhere poorly to plastic or glass culture surfaces, we first searched for a homogeneous and reproducible surface coating that allows good cell adhesion and differentiation. We begun evaluating commonly used coating substrates for cell adhesion such as gelatin, collagen, and poly-L-lysine, using bovine serum albumin and laminin as negative and positive controls, respectively. Quantification of the data shows that poly-L-lysine and gelatin were the best substrate of the series, as they allowed the adhesion of significantly higher amounts of cells than the control BSA (see table in **Figure 1A**). As we observed that cells seeded on poly-L-lysine showed a round and refringent aspect, whereas cells on gelatin assumed a flattened polygonal morphology (see table in **Figure 1A**), we tested mixtures of substrates. **Figure 1** (two lower panels) shows that cells seeded on a surface containing poly-L-lysine plus gelatin adhered in suitable number and morphology, as compared to cells plated onto poly-L-lysine alone (**Figure 1**, two upper panels). In addition, we found that cells seeded on poly-L-lysine plus gelatin also displayed better morphological differentiation than cells plated on poly-L-lysine alone, characterized by cells extending processes which origin and end are clearly distinguishable (**Figure 1**, 24 h). This morphological feature was better observed after decreasing the amount of plated cells (**Figure 1**, lowest panel). In summary, we setup optimal conditions for the amplification, adhesion, and differentiation of NSC34hSOD1 cells.

NSC34hSODG93A CELLS AS A MODEL OF ALS MOTOR NEURONS

We next conducted a series of experiments aimed to assess the reliability of NSC34hSOD1 cells to be used as a model of ALS motor neurons. First, we evaluated the effect of the stable expression of hSOD1 on cell proliferation by counting the number of cells seeded onto uncoated plastic dishes every 24 h. While both NSC34hSOD1 cell lines grow 0.5-fold during the first 48 h, only NSC34hSOD1WT cells continued proliferating at 72 h (2.01 ± 0.1 times, compared to 24 h; $^{**}p < 0.05$). Indeed, the number of NSC34hSODG93A cells remained constant and was significantly lower than NSC34hSOD1WT cells at the same time point (**Figure 2A**), revealing that the stable expression of hSODG93A results in an impaired proliferation rate of NSC34 cells.

Second, considering that ALS cells are more sensitive to oxidative stress than controls (Wijesekera and Leigh, 2009), we evaluated the survival of NSC34hSOD1 cells in basal and oxidative conditions (0.2 mM hydrogen peroxide) by quantifying LDH activity in conditioned media and extracts of both cell lines. In basal conditions, cell survival was not different in growing and differentiated conditions of both cell lines (**Figure 2B**). However, exposure to an oxidative condition induces a strong increase in

A

	NSC34hSODWT		NSC34hSODG93A	
	Mean ± SEM	Morphology	Mean ± SEM	Morphology
BSA	43,9 ± 3,6	-/-/-	49,2 ± 4,7	-/-/ -
Collagen 3,6mg/ml	210,7 ± 23,6*	-/-/-	229 ± 16,1	-/-/ -
Gelatin 1%	180 ± 17,5**	-/+/+	190,8 ± 13,4	-/+/+
Poly-L-Lysine 0,01%	241,5 ± 49,1**	-/-/+	437,1 ± 14,3	-/-/+
Laminin 1mg/ml	327,9 ± 26,5***	+/+/+	400,4 ± 25,3	+/+/+

B

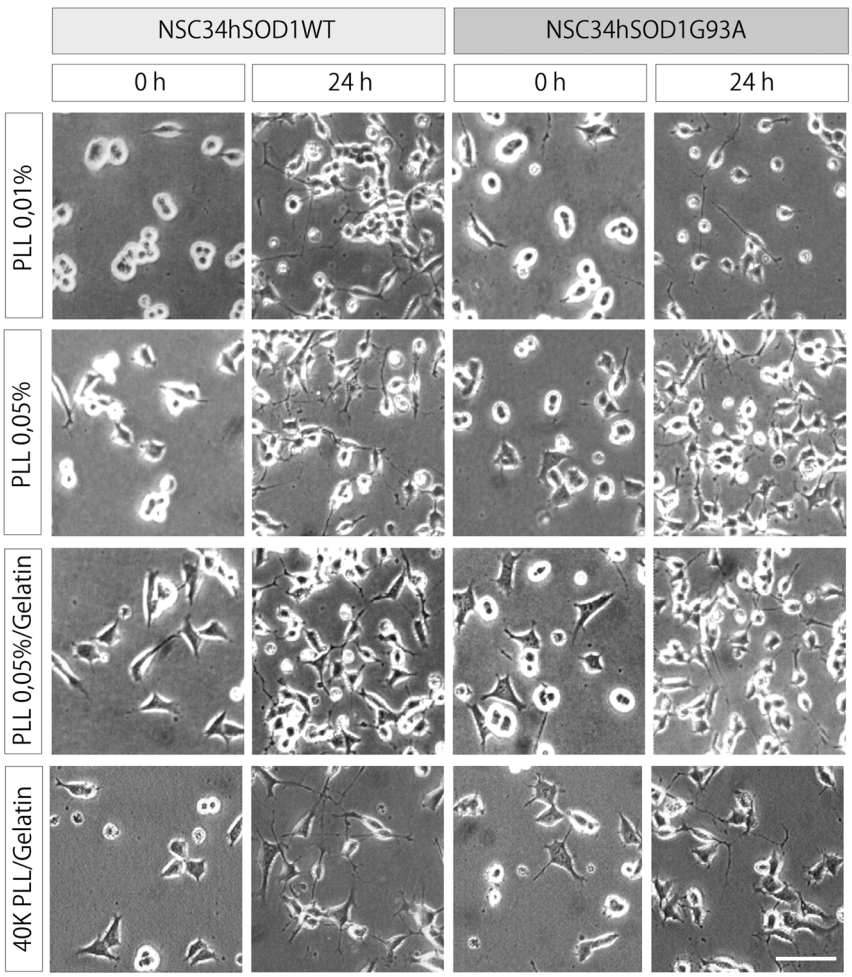

FIGURE 1 | PLL/gelatin coating is a good adhesion and differentiation substrate for NSC-hSOD1 cells. NSC34hSOD1 cells bind to different adhesion substrates. **(A)** Culture dishes were coated with methanol-solubilized nitrocellulose. Droplets containing collagen, gelatin, PLL, BSA, and laminin were applied to the surface of the dishes and dried. NSC34hSOD1 cells were then incubated for 2 h and examined by phase contrast microscopy. Table represents the average ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.001$, ***$p < 0.001$, ANOVA) as well as a morphological evaluation of the adhesion. **(B)** Culture dishes were coated with the indicated concentrations of PLL for 24 h. When indicated, gelatin was added for 30 min. Cells were allowed to adhere for 36 h, and differentiated for additional 24 h and analyzed by phase contrast microscopy. A total of 60,000 cells were seeded in every condition, except for the lowest panel pictures, where the number was diminished to 40,000 cells. Bar, 50 μm.

LDH release of differentiated NSC34hSODG93A cells compared to those cultured in growing conditions; indeed, extracellular LDH is significantly higher in differentiated NSC34hSODG93A than control NSC34hSODWT cells (**Figure 2C**). Thus, NSC34 cells expressing hSODG93A are more susceptible to oxidation-induced cell death.

Third, we considered that ALS motor neurons display fragmentation of their Golgi apparatus (Mourelatos et al., 1994; Turner et al., 2005; Gomes et al., 2008; Sundaramoorthy et al., 2013). In order to determine the temporal appearance of Golgi disruptions, cells were transfected with a plasmid coding for a β-galactosyltransferase fused to mRFP and subsequently differentiated. In NSC34hSOD1WT cells, the Golgi staining distributes, as expected, in the periphery of the nucleus, either in undifferentiated cells as well as in those differentiated for 24 h (**Figure 3**). Remarkably, even though the labeling also distributes in the perinuclear region of undifferentiated NSC34hSOD1G93A cells, Golgi portions are also disseminated within cytoplasmic regions (**Figure 3**). In differentiated NSC34hSOD1G93A cells, Golgi fragments were not concentrated in any particular subcellular region and appear regularly distributed within the cytoplasm (**Figure 3**). Taken together, our results show NSC34hSOD1 cells phenocopy what has been observed in patients as well as in validated models of ALS. Also, they suggest that Golgi disruption could be an early event of ALS pathogenesis.

Fourth, we evaluated the ability of NSC34hSOD1 cells to differentiate into a neuronal-like phenotype (**Figure 4**). Cells were seeded onto coated glass coverslips, differentiated for 24 h and subsequently stained against MAP1B (Benavente et al., 2012). Even though both cell lines extend neurites after 24 h, this feature is impaired in NSC34hSODG93A cells (see **Figures 1** and **4A**, lowest panel). Indeed, quantification of the data reveals an expected significant increase of NSC34hSOD1WT cells bearing neurites upon differentiation (from 12.1 ± 7.1 to $54.0 \pm 6.7\%$; $^{**}p < 0.01$), while the proportion of NSC34hSOD1G93A cells having neurites was not significantly higher than undifferentiated cells (**Figure 4B**). Similarly, the average length of the neurites was higher in control NSC34hSOD1WT than in NSC34hSOD1G93A cells, either before (77.4 ± 4.9 vs. 66.8 ± 6.4 μm; $^{*}p < 0.05$) and after differentiation (95.3 ± 10.3 vs. 76.6 ± 7.5 μm; $^{*}p < 0.05$; **Figure 4C**). These results suggest that the constitutive expression of hSOD1G93A impairs the ability of NSC34 cells to differentiate.

Our findings thus far show that several features of the physiopathology of ALS are evident at particular time points of the differentiation of NSC34hSOD1 cells. Even though NSC34 cells have been extensively used as a valid model of motor neurons (Cashman et al., 1992), their molecular identity has not been fully described. In this context, we first analyzed the expression of Islet-1, a transcription factor early expressed and necessary for the differentiation of motor neurons (Ericson et al., 1992; Pfaff et al., 1996). Immunocytochemical analyses showed that Islet-1 is constitutively expressed in both cell lines, in growing and differentiated conditions, and becomes mainly concentrated in the cell nuclei (**Figure 5A**), suggesting that the early commitment of NSC34 cells to the motor neuron phenotype is not affected by the stable expression of hSOD1. We then followed the expression of Hb9, a transcription factor selectively expressed

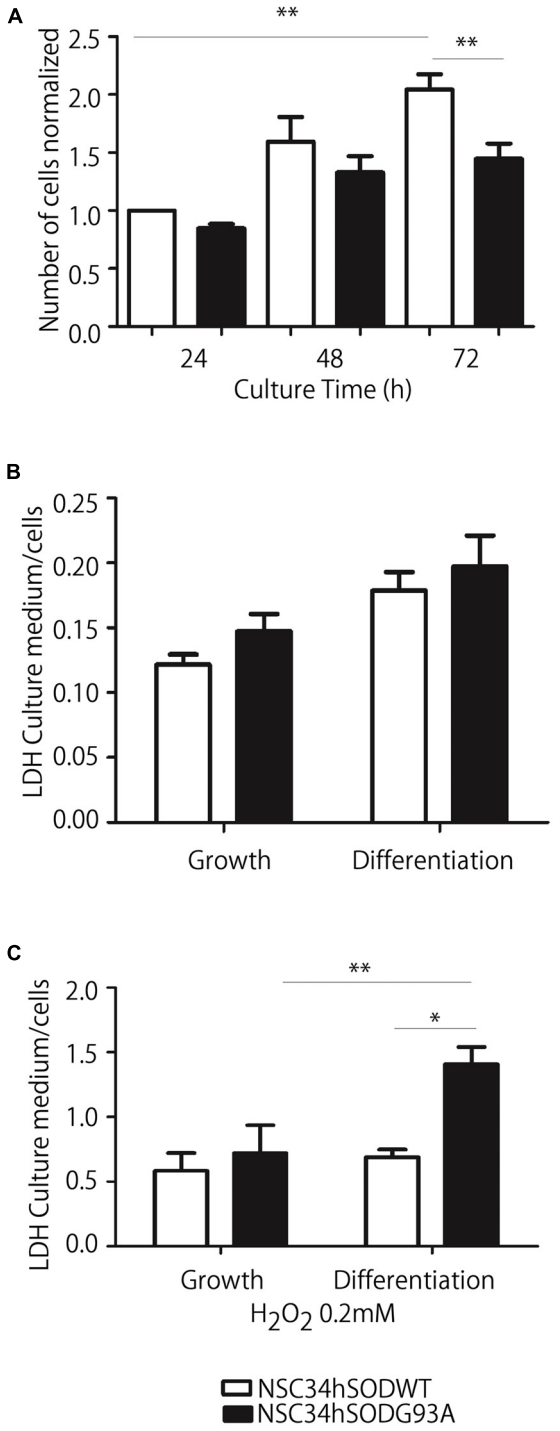

FIGURE 2 | NSC34-hSOD1G93A cells proliferate less efficiently and are more susceptible to oxidation-induced cell death. (A) Cells were seeded in p35 plastic dishes and kept in growing medium for up to 72 h. Cells were tripsinized every 24 h and counted. Plot represents the average ± SEM of three independent experiments performed by triplicate ($^{**}p < 0.001$, ANOVA). **(B,C)** NSC34-hSOD1 cells were seeded in PLL/gelatin-coated plastic dishes and differentiated **(B)** or treated with H2O2 and differentiated **(C)**. Plots represent the ratio of LDH concentration in conditioned media against that in cell extracts, and is the average ± SEM of three experiments ($^{*}p < 0.05$, ANOVA).

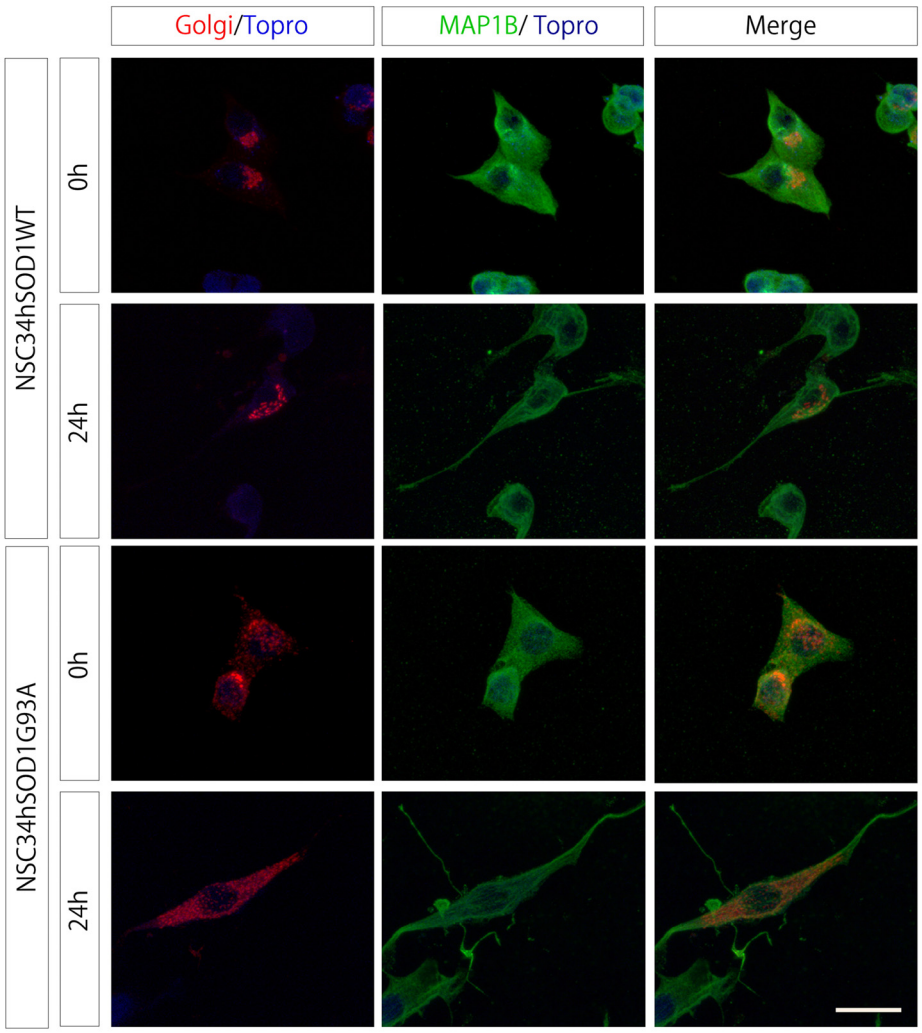

FIGURE 3 | NSC34hSOD1G93A cells display Golgi disruption. NSC34hSOD1 cells were transfected with the FU-Golgi-mRFP vector, and fixed at 0 and 24 h of differentiation to determine the subcellular localization of Golgi fragments. Immunocytochemistry against MAP1B was used to stain the cell cytoplasm and nuclei counter staining Topro-3. Bar, 20 μm.

by developing motor neurons (Pfaff et al., 1996). Hb9 expression suffices to induce the differentiation of post-mitotic cells, whereas its absence results in misslocalized motor axons in distal muscle regions (Arber et al., 1999). PCR analyses showed that the expression of Hb9 mRNA is increased at 24 h of the differentiation of NSC34hSOD1WT cells, but not in NSC34hSOD1G93A cells (**Figure 5B**). These results were corroborated at the protein level following Western blot analyses (**Figure 5C**). Quantification of the data reveals that Hb9 expression is significantly higher in differentiated NSC34hSOD1WT, compared to NSC34hSOD1G93A cells (**Figure 5C**, lower panel). Consistent with these findings, our immunocytochemical analyses showed that, even though all cells distribute Hb9 in the nucleus, the intensity of the staining is reduced in differentiated NSC34hSOD1G93A cells (**Figure 5D**). These findings suggest that an ALS condition could interfere with the ability of neuronal precursors to acquire the motor neuronal fate during early embryonic development.

Wnt- AND BMP-DEPENDENT SIGNALING IN NSC34hSOD1WT AND NSC34hSODG93A CELLS

Disfunction of Wnt signaling has been associated to neurodegenerative conditions such as Alzheimer's and Parkinson's disease (Inestrosa and Arenas, 2010; L'Episcopo et al., 2011; Marchetti et al., 2013). More recently, the expression levels of mRNAs coding for proteins related to Wnt signaling have also been proven to be altered in mouse models of ALS (Yu et al., 2013).

We first analyzed the transcriptional activation of the Wnt/β-catenin pathway following the activity of a Wnt reporter gene. Our findings show a significant increase during the differentiation of control NSC34hSOD1WT cells, whereas such activation was not observed in NSC34 cells expressing the G93A mutation of hSOD1 (**Figure 6A**). Based on these findings, we next studied the expression profile of the key Wnt mediator β-catenin. Western blot analyses showed that the total amount of β-catenin

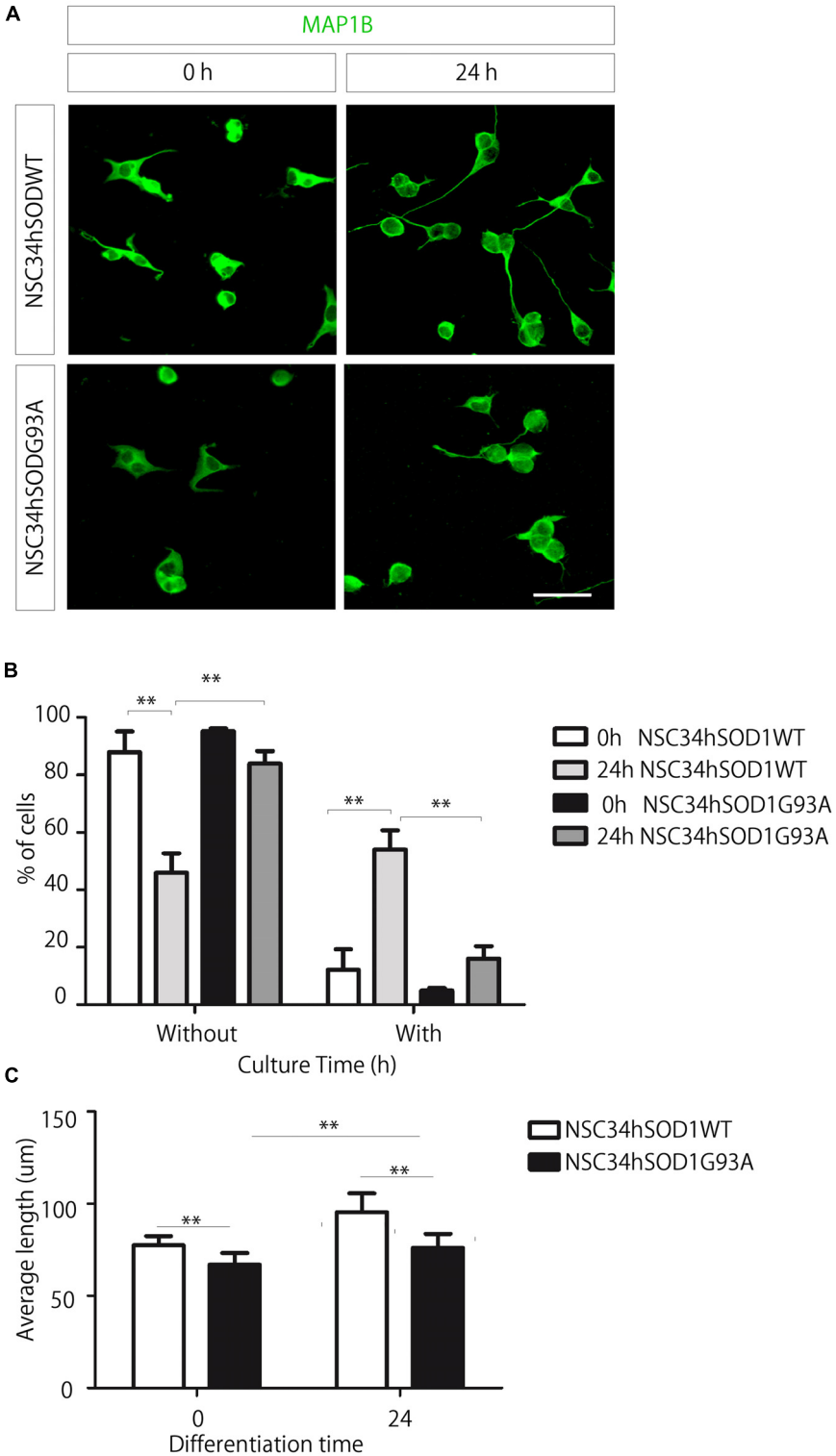

FIGURE 4 | A small proportion of NSC-hSOD1G93A cells display neurite outgrowth. NSC34hSOD1WT and NSC34hSOD1G93A cells were plated in PLL/gelatin-coated coverslips and differentiated for 24 h. Cells were fixed and stained against MAP1B **(A)**. Different parameters of morphological differentiation, including percentage of cells with or without neurites **(B)** and average neurite length **(C)** were measured using ImageJ. Plots are the average ± SEM of three independent experiments (**$p < 0.01$, ANOVA; *$p < 0.05$, t test). Bar, 50 μm.

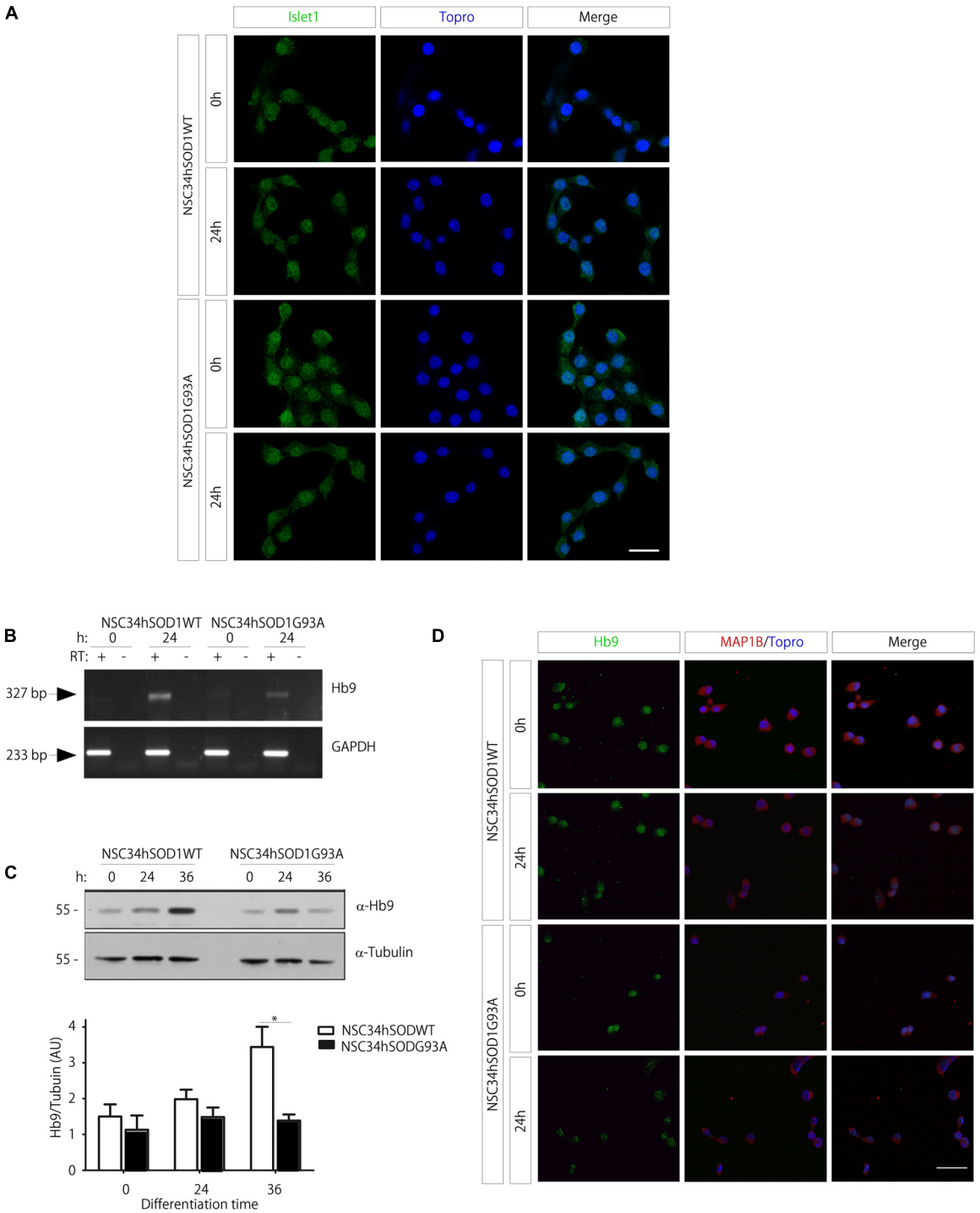

FIGURE 5 | Expression of motor neuron cell markers in NSC34hSOD1 cells. (A) NSC34hSOD1 cells were seeded onto glass coverslips and differentiated for 24 h. Immunocytochemical analyses show that Islet-1 is localized at the cell nuclei, which were counterstained with ToPro-3. **(B)** Total RNA was extracted at 0 and 24 h of NSC34hSOD1 cell differentiation, and subsequently subjected to reverse transcription (RT^+) to obtain cDNA. As a control, the same reaction was performed in the absence of reverse transcriptase (RT^-). PCRs were performed with specific primers to amplify Hb9 and the internal control GAPDH. **(C)** Western blot analyses were performed with specific antibodies against Hb9 and the internal control α-tubulin. The bottom panel shows quantification of the data, which is expressed as the mean ± SEM of four independent experiments ($*p < 0.05$). **(D)** Immunocytochemical analyses show that Hb9 is localized in the nuclei of all cells, but the intensity of the staining is less evident in differentiated NSC34hSOD1G93A cells. Cells were also stained with MAP1B and nuclei with ToPro-3. Bar, 20 **(A)** and 50 μm **(D)**.

was not significantly modified during the differentiation of both NSC34hSOD1 cell lines (**Figure 6B**). Next, we analyzed the distribution of β-catenin following activation of the pathway with lithium chloride (Alvarez et al., 2004). The intensity of nuclear β-catenin staining becomes strongly increased in the control cell line upon lithium treatment, being more evident in differentiated cells (**Figure 6C**). In turn, the localization of β-catenin in the nuclei of ALS-like cells was virtually absent in undifferentiated cells and very faint in differentiated ones (**Figure 6C**). Nevertheless, the distribution of β-catenin in the cytoplasm displayed pronounced modifications. In control cells most β-catenin is found in focal points of cell–cell contacts, either in differentiation and growing conditions. In turn, these structures are more abundant and larger in NSC34hSOD1G93A cells, a feature that is particularly evident in undifferentiated cells (**Figures 6C,D**). In isolated cells, aggregates of cytosolic β-catenin were only detected in NSC34hSOD1G93A cells (arrows in **Figure 6D**). Together, these findings suggest that canonical Wnt signaling is impaired in ALS-like cells, possibly due to the altered distribution of the key effector β-catenin.

Our recent findings show that BMP-dependent pathways could play relevant roles on the differentiation of motor neurons (Benavente et al., 2012). As a first hint to analyze BMP signaling in the context of ALS, we studied the expression of the mRNA coding for the type II BMP receptor (BMPRII), which signals through either Smad and non-Smad pathways (Sieber et al., 2009; Henriquez et al., 2011). Our results show that BMPRII expression was not modified during the differentiation of both NSC34hSOD1 cells, as compared to the internal control GAPDH (**Figure 7A**). Next, we performed western blot analyses to detect the phosphorylation of Smad1/5/8, a crucial step in the activation of the Smad-dependent BMP pathway (Henriquez et al., 2011). Similar to our previous findings in parental NSC34 cells (Benavente et al., 2012), pSmad is down-regulated during the differentiation of both NSC34hSOD1 cells; however, pSmad basal levels are significantly higher in undifferentiated NSC34hSODG93A cells, compared to controls (**Figure 7B**). Then, in order to correlate pSmad levels with BMP-dependent transcription, we analyzed the expression and activity of the Smad-dependent early responsive gene Id1, a negative regulator of the differentiation of neurogenic precursors (Ying et al., 2003; Vinals et al., 2004). Immunodetection experiments showed that Id1 levels in undifferentiated cells were significantly higher in NSC34hSOD1G93A cells compared to controls, whereas its expression is similarly down-regulated during the differentiation of both cell types (**Figure 7C**). Finally, we transiently transfected undifferentiated NSC34hSOD1 cells with a luciferase Smad-responsive Id1 reporter gene (Lopez-Rovira et al., 2002). Consistent to our findings on pSmad and Id1 expression levels, **Figure 7D** shows that the basal Id1-dependent transcription is twofold higher in NSC34hSOD1G93A cells compared to control NSC34hSOD1WT cells, whereas this activity is strongly diminished after 24 and 48 h of differentiation in both cell types. Together, these findings support the notion that BMP/Smad signaling varies inversely with motor neuron differentiation; in addition, they also suggest that this signaling pathway could be up-regulated in ALS motor neuron precursor cells.

DISCUSSION

Amyotrophic lateral sclerosis is a neurodegenerative disease that leads to progressive paralysis and death after around 4 years from the onset of the disease. Since its approval, the only treatment for ALS is Riluzole, a drug that inhibits the release of glutamate by inactivation of voltage-sensitive sodium channels (van Zundert et al., 2012), extending 3 months on average. Therefore, it is critical to generate model systems to describe the physiopathology of the disease and where to assess more effective therapeutic alternatives.

Because of its origin, NSC34 cells exhibit several features of motor neurons (Cashman et al., 1992), and are a more suitable *in vitro* model to study ALS than other neuronal cell lines that have also been used for similar analyses such as the Neuro-2a and SH-SY5Y cells (Pasinelli et al., 1998; Arciello et al., 2011). In their initial characterization, both NSC34hSOD1 cell types adhered well to Matrigel/poly-D-lysine matrices (Gomes et al., 2008). As Matrigel contains most of the components of the extracellular matrix, likely influencing cell behavior (Hughes et al., 2010; Ventre et al., 2012), we looked for simpler coating molecules. We found that that the poly-L-lysine plus gelatin mixture allowed good cell adhesion and differentiation of NSC34hSOD1 cells. In agreement with our results, both poly-L-lysine and gelatin have been extensively used by their ability to significantly increase cell adhesion in various cell types while not affecting cell proliferation or differentiation (Alvarez Perez et al., 2012; Kuo and Chung, 2012).

Several molecular mechanisms are likely to converge to allow motor neuronal degeneration, the main feature of ALS (Pasinelli and Brown, 2006; Wijesekera and Leigh, 2009; Gordon and Meininger, 2011; Bendotti et al., 2012). In this regard, our results revealing that NSC34hSOD1G93A cells are more prone to oxidation-induced cell death are supported by previous findings showing that NSC34 cells expressing hSOD1G93A increase reactive oxygen species production, while decreasing their viability (Li et al., 2003). In addition, other detrimental stimuli, such as staurosporine treatment, induced the death of NSC34hSOD1G93A cells (Gomes et al., 2008), reinforcing the notion that the expression of mutant SOD1 increases the vulnerability of motor neurons to cell damage.

Another hallmark of ALS neuropathology is the fragmentation of their Golgi apparatus, a feature that has been observed in ALS patients (Mourelatos et al., 1994), as well as in various model systems, such as NSC34 cells expressing or treated with hSOD1G93A (Mourelatos et al., 1994; Gomes et al., 2008), and transgenic mice expressing hSOD1G93A (Turner et al., 2005). Interestingly, our present findings reveal that Golgi fragmentation occurs prior to neuronal differentiation. In this regard, it has been shown that mutated hSOD1 is released from NSC34hSOD1G93A cells, as well as from primary astrocyte cultures derived from hSOD1G93A transgenic mice, via exosomes (Gomes et al., 2007; Basso et al., 2013). These vesicles are then endocytosed by neuronal cells inducing Golgi fragmentation (Sundaramoorthy et al., 2013). Considering these previous findings supporting the notion that ALS-inducing stimuli could be spread between cells, our present results strongly suggest that such mechanism could take place from early steps of the pathogenesis of the disease.

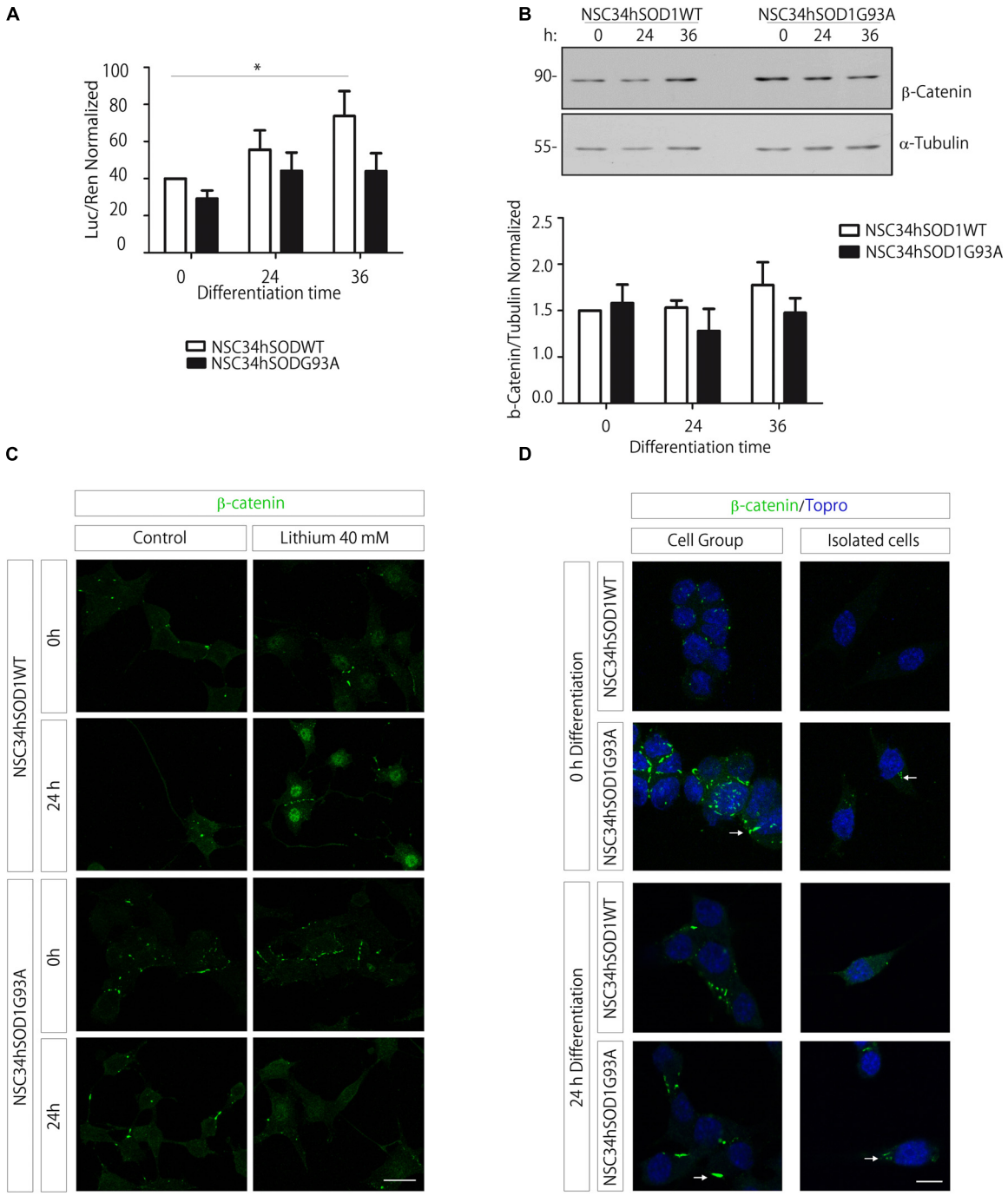

FIGURE 6 | The Wnt/β-catenin signaling increases in control NSC34hSOD1WT cells but not in ALS-like NSC34hSOD1G93A cells, possibly due to the presence of β-catenin aggregates. (A) Cells were seeded on PLL/gelatin-coated dishes and transfected with the Wnt reporter TOPFLASH vector plus the pRLSV40 Renilla control plasmid, and subsequently differentiated for the indicated times. The graph shows the normalized reporter gene activity (luciferase/Renilla). Data are expressed as the average ± SEM of three independent experiments performed by quadruplicate (*$p < 0.05$, paired t test). **(B)** Total protein extracts were obtained from differentiating NSC34hSOD1 cells at 0, 24, and 36 h and were subjected to western blot analyses using an anti β-catenin antibody. An anti α-tubulin antibody was used as loading control. The graph shows the quantification of β-catenin normalized to α-tubulin bands, and represent the average ± SEM of three independent experiments. **(C)** Undifferentiated and differentiated NSC34hSOD1 cells were treated with 40 mM lithium chloride for 6 h and stained with an anti β-catenin antibody. Lithium treatment increases the nuclear staining of β-catenin in control cells, a feature that is not evident in ALS-like cells. **(D)** NSC34hSOD1 cells were fixed before and after 24 h of differentiation and subsequently subjected to immunocytochemical analyses using an anti β-catenin antibody. Grouped and isolated cells are shown for each condition. Nuclei were counterstained with ToPro-3. Bar, 20 **(C)** and 10 μm **(D)**.

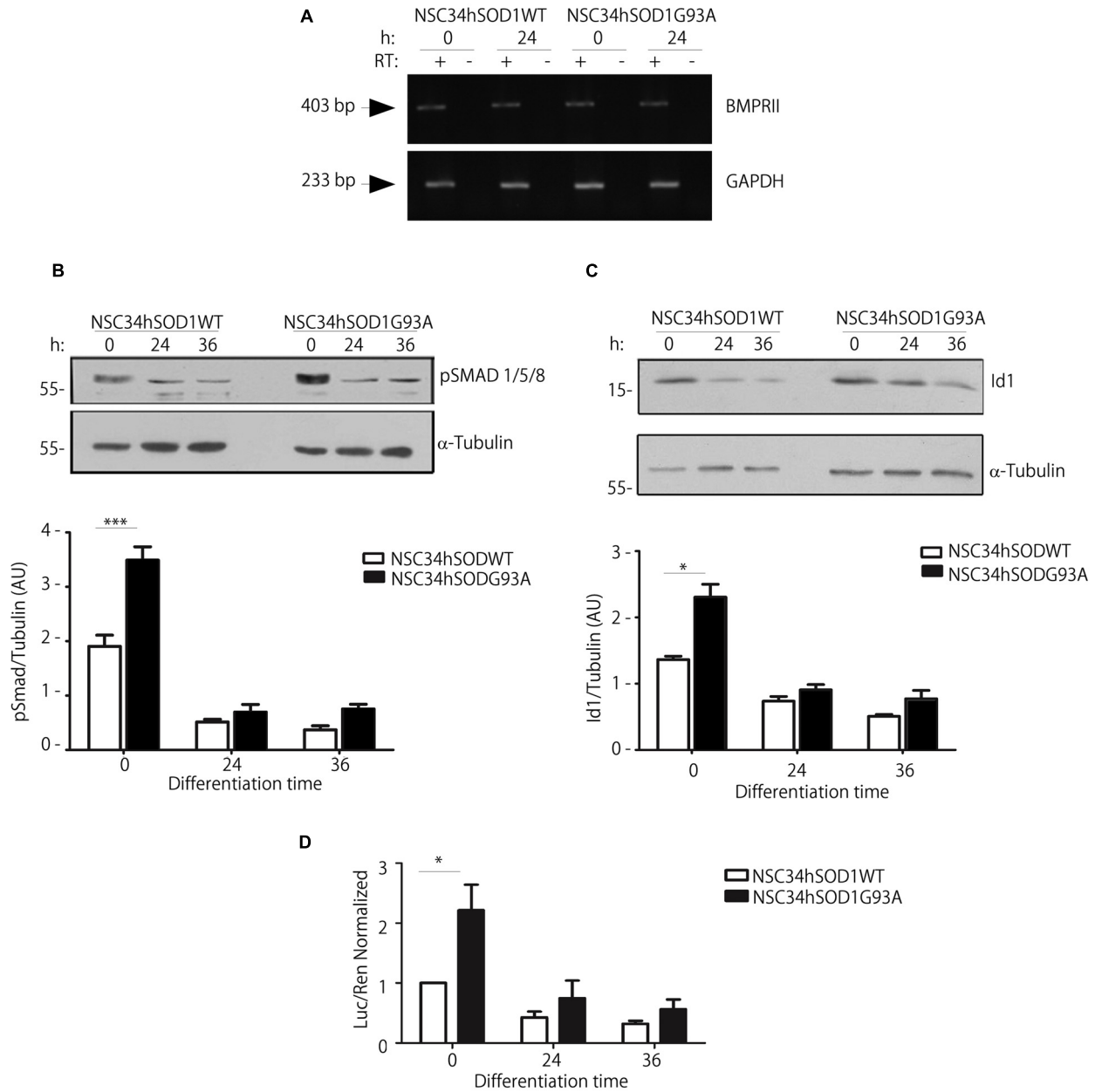

FIGURE 7 | Undifferentiated NSC34hSOD1G93A cells display increased activity of the BMP/Smad signaling pathway. (A) Total RNA was obtained from NSC34hSOD1 cells at 0 and 24 h of differentiation and BMPRII mRNA levels were analyzed by RT-PCR. As loading control GAPDH was amplified. Negative control reactions without reverse transcriptase (RT^-) are also shown. **(B,C)** Western blot analysis using anti pSmad **(B)** or anti Id1 **(C)** antibodies in protein extracts of NSC34hSOD1 cells obtained at the indicated times of differentiation. Anti α-tubulin was used as loading control. Data analyses of band intensities are expressed as the mean ± SEM of three independent experiments ($*p < 0.05$; $***p < 0.01$). **(D)** NSC-34 cells were transfected with an Id1 promoter luciferase (Luc) reporter gene. Enzyme activity was measured before or after 24 and 36 h of differentiation and normalized by Renilla (Ren) luciferase activity. Data are the mean ± SD of three independent experiments performed in quadruplicate ($*p < 0.05$).

Consistent with previous findings showing that NSC34 cells expressing hSOD1G93A display impaired morphological differentiation (Gomes et al., 2008; Magrane et al., 2009), and decreased expression of the neuronal marker MAP2 (Lee et al., 2002), our data show that these cells have a lower percentage of differentiated cells that bear shorter neurites than controls. What is the molecular mechanism underlying this altered phenotype? Even though NSC34 cells were obtained from neuronal precursors from the spinal cord, and have been validated as a cholinergic system (Cashman et al., 1992; Maier et al., 2013), their identity as motor neurons has not been evaluated in detail. Islet-1 is considered an early marker of differentiation of motor neurons and their

absence impairs the ability of neuronal precursors to differentiate into motor neurons (Pfaff et al., 1996). In turn, Hb9 is a transcription factor expressed in post-mitotic motor neurons, which suggest that it functions later in the differentiation process (Pfaff et al., 1996; Tanabe et al., 1998). Our findings show that control cells display a constitutive expression of Islet-1 and induce the expression of Hb9 upon differentiation; however, whereas Islet-1 levels are similar in both cell lines, the expression of Hb9 is significantly impaired in ALS-like cells, indicating that the consolidation of motor neuronal identity might be compromised. In this regard, recent evidence show that motor neuron-like cells driven from iPS cells obtained from hSOD1G93A mice express several motor neuron markers, such as choline acetyl transferase and Hb9, and exhibit shorter neuronal projections than cells derived from control mice (Yao et al., 2013).

Several lines of research support the idea that a decrease in Wnt signaling activity is associated to the pathogenesis of some neurodegenerative diseases, such as Alzheimer's and Parkinson's (Inestrosa and Arenas, 2010; L'Episcopo et al., 2011). When hippocampal neurons are treated with the Aβ peptide (which extracellular accumulation is a hallmark of Alzheimer's disease), there is an increase in cell apoptosis as well as in GSK-3β activity, whereas the intracellular levels of β-catenin and the transcription of Wnt-target genes are reduced (Garrido et al., 2002; De Ferrari et al., 2003; Alvarez et al., 2004). Consistent with this notion, activation of Wnt signaling plays neuroprotective roles in models of Alzheimer's disease either *in vivo* or *in vitro* (De Ferrari et al., 2003; Alvarez et al., 2004; Cerpa et al., 2010; Purro et al., 2012). Indeed, the treatment of hippocampal slices with antibodies against the Wnt inhibitor DKK1 reverts the adverse effects observed in AD brains (De Ferrari et al., 2003; Alvarez et al., 2004; Cerpa et al., 2010; Purro et al., 2012). Together, these findings provide a strong support to a crucial role for Wnt signaling on neurodegeneration.

Recent findings show that the motor neurons of ALS model mice display altered expression of some Wnt effectors, such as Wnt1, Wnt2, Wnt5a, Fz1, and Fz2 (Chen et al., 2012; Li et al., 2013; Wang et al., 2013). Consistently, following a systematic microarray approach, Yu et al. (2013) have shown that many Wnt effectors, including Wnt ligands, Fz receptors, endogenous antagonists, downstream effectors, and target genes modify their expression in the spinal cord of ALS mice. Remarkably, when we analyzed Wnt signaling, we found that the ALS-like motor neurons did not activate the β-catenin-dependent Wnt pathway during their differentiation, as we observed in control cells.

Neurodegenerative diseases are characterized by the formation of toxic protein aggregates, either because they block cell functions or because they sequester other proteins (Wood et al., 2003; Bergemalm et al., 2010). In *in vitro* models of Alzheimer's disease, activation of the Wnt pathway with lithium chloride results in an increase in total β-catenin levels (De Ferrari et al., 2003; Alvarez et al., 2004), as well as in increased neuronal survival (De Ferrari et al., 2003). Our present findings show that lithium treatment induced the localization of β-catenin in the nuclei of control cells, whereas this staining was significantly reduced in cells expressing mutated hSOD1. Remarkably, the intracellular distribution of β-catenin was notoriously affected,

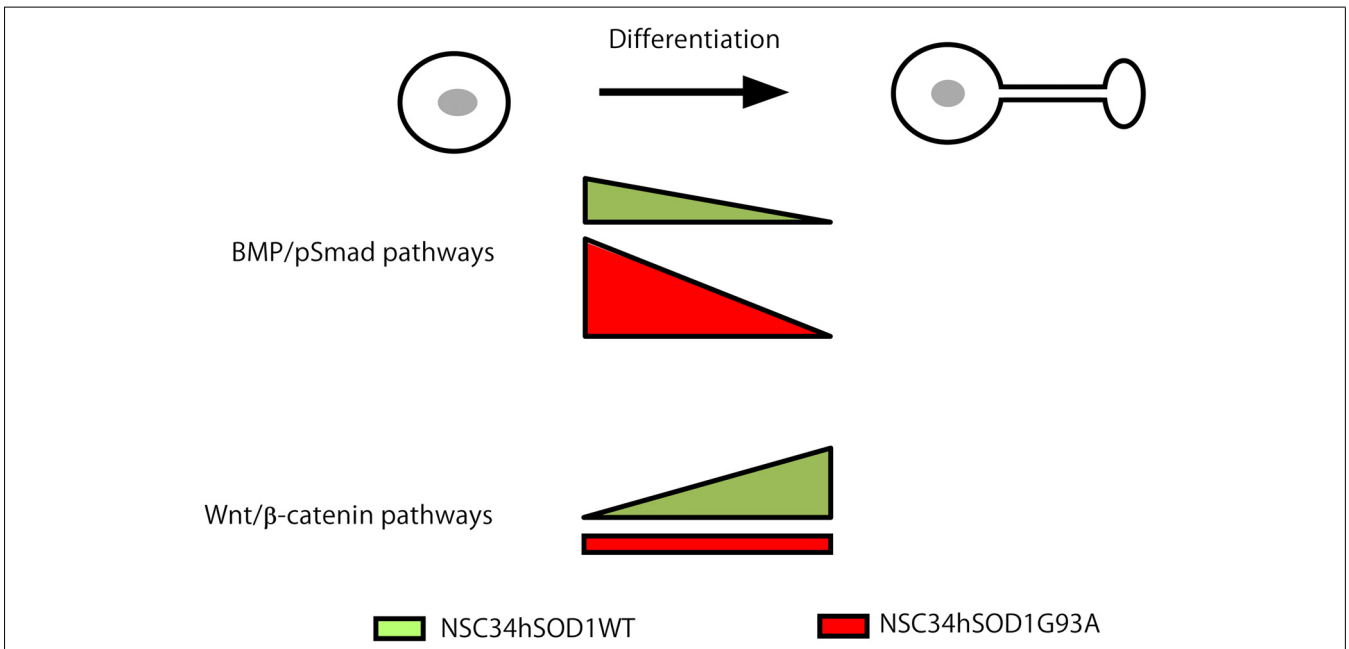

FIGURE 8 | Working model for Wnt and BMP pathways as potential mediators of ALS pathogenesis. Wnt and BMP-dependent signaling pathways display opposite alterations at different stages of the development of ALS motor neurons. Based on our present findings we propose that BMP/Smad signaling is up-regulated at early stages of the differentiation of ALS motor neurons. In turn, the differentiated cells display an impaired ability to activate Wnt/β-catenin signaling.

evidenced by the presence of intracellular aggregate-like structures in cell–cell contacts of NSC34hSOD1G93A cells. Interestingly, aggregates of β-catenin have been detected in *in vitro* models of AD (Ghanevati and Miller, 2005). Also, β-catenin is slightly detected in hSOD1-containing hyaline inclusions in the spinal cord of ALS model mice (Zhang et al., 2006). Therefore, it is plausible to interpret our findings as that the abnormal distribution of β-catenin alters its nuclear translocation, thus reducing transcriptional activation. In support of this view, β-catenin aggregates have been observed in the brain of patients and mice models of the neurodegenerative Huntington's disease; moreover, the silencing of β-catenin rescued neuronal degeneration in this model system (Godin et al., 2010), suggesting that β-catenin aggregation could be crucial in the pathogenesis of ALS.

Similar to Wnt pathway, BMP signaling is also altered in the damaged adult nervous system (Wang et al., 2007; Bayat et al., 2011). We found a strong induction of Id1, Smad phosphorylation and Smad-dependent transcription in undifferentiated NSC34hSOD1G93A cells, suggesting that BMP signaling could be up-regulated during early stages of the ALS condition. Interestingly, the levels of the BMP2 ligand are up-regulated upon induction of neuronal damage (Wang et al., 2007; Matsuura et al., 2008). In this regard, we have recently demonstrated that activation of Smad-dependent signaling by treatment of NSC34 cells with the BMP2 ligand correlates with decreased morphological differentiation; however, it also induces the expression of the type II BMP receptor, which could be required for subsequent steps of regeneration (Benavente et al., 2012). In our ALS model system, the decreased morphological differentiation that correlates with up-regulation of Smad-dependent signaling was not accompanied by changes in the expression of the type II BMP receptor. Similarly, in Drosophila models of several motor diseases, including ALS, signaling mediated by the BMPRII is impaired (Ratnaparkhi et al., 2008; Hirth, 2010), thus suggesting that signaling through this receptor could also play important roles on the pathogenesis of ALS.

Altogether, our findings fit with a model (**Figure 8**) where signaling through Wnt and BMP morphogens act as potential mediators of the pathogenesis of ALS, with opposite alterations at different stages of development; whereas BMP/Smad signaling could be up-regulated during early stages of the differentiation of ALS motor neurons, the differentiated cells could display an impaired ability to activate Wnt/β-catenin signaling. Remarkably, cumulative evidence reveals that these two signaling pathways interact in various developmental and tissue specific contexts to exert many different cellular responses (Itasaki and Hoppler, 2010; Marcellini et al., 2012). Modulating these signaling pathways individually or in a coordinated fashion could then represent interesting therapeutic alternatives to mitigate the progression of ALS.

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