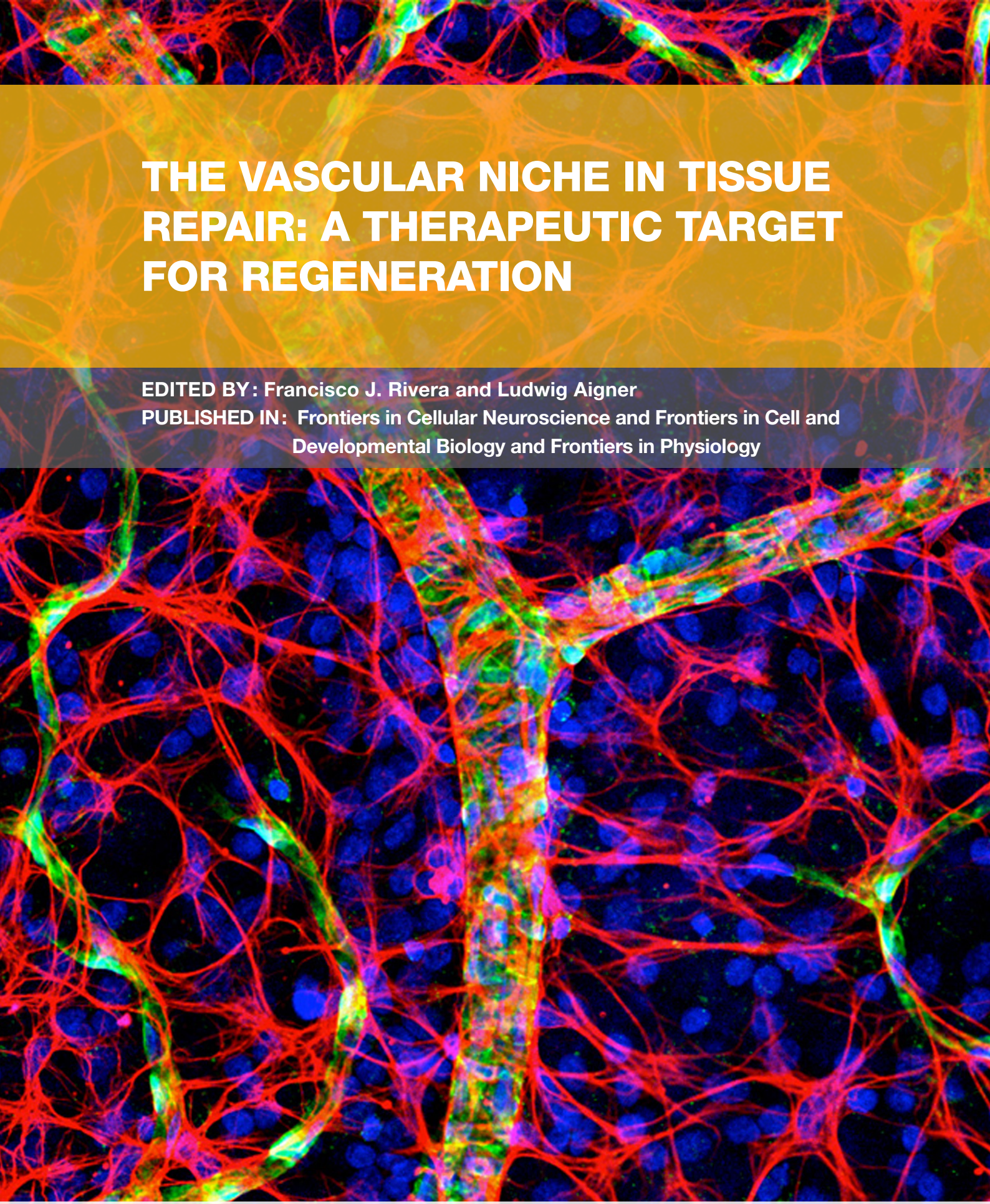

THE VASCULAR NICHE IN TISSUE REPAIR: A THERAPEUTIC TARGET FOR REGENERATION

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THE VASCULAR NICHE IN TISSUE REPAIR: A THERAPEUTIC TARGET FOR REGENERATION

Topic Editors:
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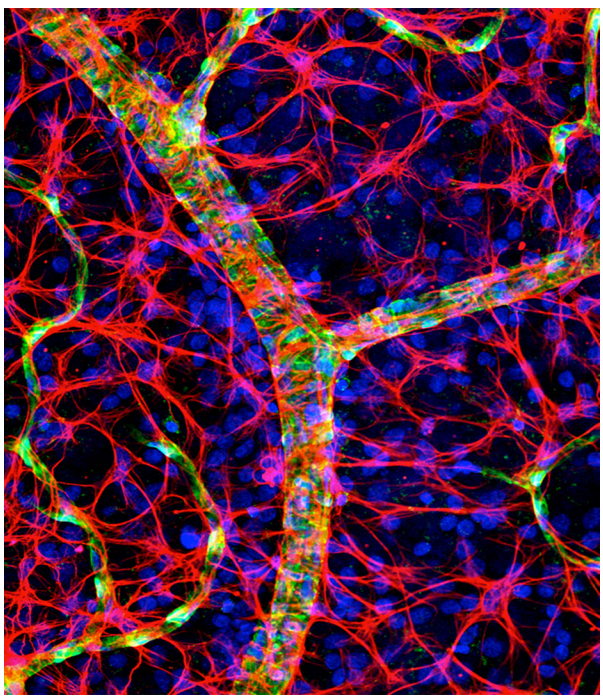

The Neurovascular Niche in the Central Nervous System: Vascular cells (pericytes in green) directly interact with glial cells (astrocytes in red), contributing and stabilizing the blood-brain/retina-barrier.

Image by Dr. Andrea Trost.

Tissues and organs have, although sometimes limited, the capacity for endogenous repair, which is aimed to re-establish integrity and homeostasis. Tissue repair involves pro- and anti-inflammatory processes, new tissue formation and remodelling. Depending on the local microenvironment, tissue repair results either in scar tissue formation or in regeneration. The latter aims to recapitulate the original tissue structure and architecture with the proper functionality. Although some organisms (such as planarians) have a high regenerative capacity throughout the body, in humans this property is more restricted to a few organs and tissues.

Regeneration in the adult is possible in particular through the existence of tissue-resident pools of stem/progenitor cells. In response to tissue damage, these cells are activated, they proliferate and migrate, and differentiate into mature cells. Angiogenesis and neovascularization play a crucial role in tissue repair. Besides providing with oxygen and nutrients, angiogenesis generates a vascular niche (VN) consisting of different blood-derived elements and endothelial cells surrounded by basement membrane as well as perivascular cells. The newly generated VN communicates with the local stem/progenitor cells and contributes to tissue repair. For example, platelets, macrophages, neutrophils, perivascular cells and other VN components actively participate in the repair of skin, bone, muscle, tendon, brain, spinal cord, etc. Despite these observations, the exact role of the VN in tissue repair and the underlying mechanisms are still unclear and are awaiting further evidence that, indeed, will be required for the development of regenerative therapies for the treatment of traumatic injuries as well as degenerative diseases.

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EDITORIAL
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Editorial: The Vascular Niche in Tissue Repair: A Therapeutic Target for Regeneration

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Keywords: vascular niche, tissue repair, regeneration, angiogenesis, stem cells, endothelial cells, pericytes, platelets

Editorial on the Research Topic

The Vascular Niche in Tissue Repair: A Therapeutic Target for Regeneration

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In mammals, although regeneration is quite restricted to a number of tissues and organs, this particular healing process is possible through the existence of tissue-resident stem/progenitor cells. Upon injury, these cells are activated, they proliferate, migrate, and differentiate into tissue-specific cells and functionally replace the damaged or lost cells. Besides this, angiogenesis and neovascularization play crucial roles in tissue repair. Blood vessels (BV) together with the resident surrounding cells create a vascular niche which is central to local and distant signaling thereby shaping the regenerative response.

The Frontiers Research Topic "The Vascular Niche in Tissue Repair: A Therapeutic Target for Regeneration" encompasses 14 articles highlighting various aspects of the vascular niche (VN) in health and disease. This research topic first describes *ex vivo* methodological aspects to study the role of the VN in regeneration, second addresses the VN cellular composition and roles during regeneration, third described local as well as distant signaling mechanisms regulating the VN in regeneration and, finally addresses the VN responses in pathology.

VASCULAR NICHE: METHODOLOGICAL INSIGHTS IN THE STUDY OF ANGIOGENESIS AND REGENERATION

Two papers highlight special methods to study the VN. Hutter-Schmid et al. present organotypic brain slices cultures as a tool and a system to study angiogenesis in the brain. This is certainly a very valuable method as it presents cells in their physiological context and provides a screening tool for the search for factors to modulate angiogenesis and neovascularization in the brain.

Chavez et al. present the zebrafish as an organism to study angiogenesis in development and regeneration *in vivo*. In addition, this review summarizes also other currently available *in vitro* and *in vivo* experimental models to study angiogenesis.

VASCULAR NICHE: CELLULAR COMPOSITION AND ROLES IN REGENERATION

Over the past years, the view of the vascular system as a sole provider for oxygen and nutrients has dramatically changed. First, endothelial cells, which form the inner layer of the blood vessel wall, are in direct contact at the luminal face with blood-derived elements and on the outer face with other various parenchymal cell types facilitating the communication between all these components, creating a functional VN.

Koutsakis and Kazanis highlight in their article the role of the vascular system in neural stem cell (NSCs) biology and neurogenesis mainly from an evolutionary and developmental point of view. During early development, the generation and specification of NSCs apparently does not require the presence of BV, as specification of neuroectoderm and of neuroepithelial cells precedes angiogenesis. In contrast, in the adult central nervous system (CNS), NSCs are in direct contact or at least in close proximity to BV, allowing for a direct interaction between these two cell types. Even though signals derived from endothelial cells can modulate NSC proliferation and differentiation *in vitro*, BV mainly influence and support migration of neuronal precursors and young immature neurons toward their final destination. The concept addressed by these authors is well supported by the fact that BV serve endogenous oligodendrocyte progenitor cells (OPCs) as scaffolds for migration during myelination (Tsai et al., 2016). This, of course, may provide opportunities for the future design of approaches to target progenitors to specific areas of need.

Besides endothelial cells, pericytes have recently moved into the focus of attention as an essential player for different roles of the VN. Trost et al. review the current knowledge on brain and retinal pericytes. Classically, pericytes contribute to vascular homeostasis such as vessel stabilization, blood flow regulation, and the formation of the blood brain barrier. However, a number of studies have shown that pericytes display unexpected functions, beyond vascular homeostasis, as this cell type supports tissue repair and regeneration. Indeed, pericytes are multipotent (Crisan et al., 2008) being able to give rise to cells that form the fibrotic scar after acute injuries (Goritz et al., 2011). In addition, pericytes may act as stromal cells and modulate the function of neighboring local stem and progenitor cells in their regenerative activities. Supporting this concept exposed by Trost et al., we have recently shown that in response to demyelination pericytes modulate CNS-resident OPC function during myelin regeneration (De La Fuente et al., 2017). The different modes of action of pericytes might be explained by the existence of heterogeneous subpopulations with different functions, as their embryonic origin can either be neuroectodermal/neural crest or mesodermal. However, this issue needs further investigation. Finally, considering the similarities between pericytes and mesenchymal stem cells (MSCs) Schimke et al. review the clinical potential of MSCs-based therapies to favor tissue regeneration and in which extend aging might affect this therapeutic feature. The review stretches from cell phenotyping to cell therapy concepts.

Traditionally, the vasculature and the ventricular/spinal canal have been considered to be the main fluid systems in the CNS for supply and for drainage. Here, Guerra et al. highlight the importance of the subcommisural organ and other periventricular secretory structures in regulating neurogenesis. These structures contribute to the cerebrospinal fluid, which is essential in the regulation of NSC activities. More recently, it has been revealed that the brain and the spinal cord do have also a lymphatic system. In line with the previous, Kaser-Eichberger et al. provide a descriptive immunohistological study on the lymphatic system with a focus on the spinal cord. The lymphatic system in the CNS needs to be explored more in detail, as it provides a mechanism for clearance of substances and any malfunction might lead to detrimental consequences for the brain.

Another entity of the VN in the CNS seems to be the platelets. While the main function of platelets is to control blood clotting and thereby repair of vascular damage, more recent data demonstrate that platelets might be involved in the regulation of tissue repair and regeneration. Rivera et al. point out in an opinion paper that platelets are involved in neuroinflammation and hence might promote degenerative processes in the CNS. On the other hand, they might as well be beneficial as they promote regeneration through acting on endogenous progenitors. Besides their effects on neural progenitors, platelets and platelet derived factors affect angiogenesis, this particular topic is illustrated in the review article by Martinez et al. Nevertheless, the contribution of platelets in tissue repair and regeneration is quite unexplored.

In huge contrast to most regenerative niches of the body, the tendon has very little vasculature. Tempfer and Traweger describe the tendons as an atypical niche since they are almost avascular and angiogenesis and neovascularization seem to hamper their regeneration. Therefore, this review article opens the idea that depending on the tissue, one might either use pro- or anti-vascularization approaches to promote regeneration.

VASCULAR NICHE: MOLECULAR SIGNALING IN REGENERATION

Blood circulating factors modulate tissue regeneration through the VN. This enables local as well as distant signaling. Regenerative soluble factors can either be produced locally or at distal sites reaching the target tissue through the blood stream. Similarly, cells produced at distant locations might reach the target tissue through the vasculature, and might then modulate local regeneration. Wallner et al. review on the role of granulocyte colony stimulating factor (G-CSF) in angiogenesis and neuroregeneration upon spinal cord injury and during Amyotrophic Lateral Sclerosis. G-CSF is a multimodal and pleiotropic factor modulating a wide spectrum of events including neurogenesis and angiogenesis, eventually, boosting tissue regeneration leading to an improvement of neurological function in patients.

Besides the classical growth factor/cytokine mediated signaling, communication via extracellular vesicles/exosomes has recently moved into the center of interest. Batiz et al. provide a comprehensive overview on: (i) the adult neurogenic niches and their cellular components; (ii) classical signaling mechanisms regulating the neurogenic niches such as neurotransmitters, hormones, cerebrospinal fluid-derived as well as blood-derived factors, and; (iii) exosome mediated signaling at the neurogenic niche.

VASCULAR NICHE: REGENERATION IN DISEASE

The VN is massively altered in diseases and often, vascular changes are crucial in the process of pathogenesis. Fierro et al. present novel data on how hypoxic pre-conditioning leads to an increased infiltration of endothelial cells into scaffolds for dermal regeneration. Here, MSCs were seeded in scaffolds and angiogenic features *in vitro* and after implantation *in vivo* were studied. This is of relevance in particular for the field of biomaterials and tissue engineering for repair and regeneration.

Original data were also presented by Klein et al., who describe how microglia-mediated neuroinflammation induced by allergic conditions impact in adult hippocampal neurogenesis. Very surprisingly, acute allergic reactions in the periphery caused an elevation of neurogenesis.

AUTHOR CONTRIBUTIONS

FR and LA conceived the idea and prepared the manuscript. MS designed, corrected and edited the manuscript.

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
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Organotypic brain slice cultures as a model to study angiogenesis of brain vessels

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Brain vessels are the most important structures in the brain to deliver energy and substrates to neurons. Brain vessels are composed of a complex interaction between endothelial cells, pericytes, and astrocytes, controlling the entry of substrates into the brain. Damage of brain vessels and vascular impairment are general pathologies observed in different neurodegenerative disorders including e.g., Alzheimer's disease. In order to study remodeling of brain vessels, simple 3-dimensional *in vitro* systems need to be developed. Organotypic brain slices of mice provide a potent tool to explore angiogenic effects of brain vessels in a complex 3-dimensional structure. Here we show that organotypic brain slices can be cultured from 110 μm thick sections of postnatal and adult mice brains. The vessels are immunohistochemically stained for laminin and collagen IV. Co-stainings are an appropriate method to visualize interaction of brain endothelial cells with pericytes and astrocytes in these vessels. Different exogenous stimuli such as fibroblast growth factor-2 or vascular endothelial growth factor induce angiogenesis or re-growth, respectively. Hyperthermia or acidosis reduces the vessel density in organotypic slices. In conclusion, organotypic brain slices exhibit a strong vascular network which can be used to study remodeling and angiogenesis of brain vessels in a 3-dimensional *in vitro* system.

Keywords: organotypic slice, vibrosection, vessel, capillary, vascular, pericyte

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Introduction

The organotypic brain slice model resembles partly the *in vivo* condition of a high density cell system. In slices, individual cells are in close contact and do not lose density dependent regulatory mechanisms, 3-dimensional architecture as well as tissue specific transport and diffusion probabilities. The model has been introduced by Gähwiler and colleagues (Gähwiler and Hefti, 1984; Gähwiler et al., 1997), modified by Stoppini et al. (1991) and meanwhile used and characterized by several research groups including ours (Gähwiler et al., 1990; Ostergaard, 1993; Robertson et al., 1997; Ullrich et al., 2011; Daschil et al., 2015).

Brain capillaries constitute the blood-brain barrier (BBB) and innervate all areas of the brain. A first description of vasculature in organotypic brains slices has been given in 1975 (Wolff et al., 1975). Subsequently, Renkawek et al. (1976) characterized brain capillaries in organotypic cultures by relative unselective butyryl-cholinesterase stainings. We were one of the first to demonstrate that organotypic brain slices contain a strong network of laminin$^+$ brain capillaries (Moser et al., 2003, 2004). Laminin is a well-established basement membrane marker which excellently stains the

vascular structures of the brain. We demonstrated that capillaries survive in organotypic sections without any circulation (Moser et al., 2003). Although, the capillaries are not functional any longer and do not display any blood flow, it is likely, that they express and secrete a cocktail of different molecules which may influence other cells within the slices including nerve fiber innervations (Moser et al., 2003). Furthermore, we have recently shown that brain vessels in organotypic brain slices can re-grow between specific areas, when exogenously stimulated (Ullrich and Humpel, 2009). Meanwhile, brain vessels are well studied in organotypic cultures and particularly the neurovascular unit (NVU) is intensively explored in this complex 3-dimensional network (Morin-Brureau et al., 2013; Chip et al., 2014). The use of growth factors, especially vascular endothelial growth factor (VEGF) is important to study angiogenesis and revascularization in organotypic slices (Morin-Brureau et al., 2011). Recently, the interaction of vascular cells with astrocytes and particularly with pericytes within 3-dimensional organotypic slices came into intense investigation (Mishra et al., 2014). Zehendner et al. (2013) provided a detailed characterization of such a novel organotypic *in vitro* model of the NVU in the developing cortex.

Thus, there is clear evidence that organotypic brain slices are suitable to explore angiogenesis, vascularization or re-growth of vessels. It is hypothesized that *in vitro* vessels react upon stimulation with growth factors or pharmaceutical drugs and may represent a situation, which may also be found *in vivo*. The aim of the present work is to first summarize published experiments (including own data) regarding modulation of the vascular network in organotypic brain slices. Second, we add novel data and show for the first time how pericytes can be studied in such brain slices.

Material and Methods

Organotypic Slices and Vibrosections

Adult or postnatal day 8–10 mice (C57BL/6N, Charles River, wild-type, WT) were used. Postnatal animals were rapidly sacrificed with a large scissor and adult animals were first injected with a lethal dose of thiopental. Then the head was quickly transferred in 70% ethanol, the brains dissected and sagittally cut. The brains were glued (Glue Loctite) onto the chuck of a water cooled vibratome (Leica VT1000A) and triggered close to a commercial shave racer. Under aseptic conditions, 110 μm thick vibrosections were cut and collected in sterile medium. The organotypic vibrosections were carefully placed onto a 0.4 μm membrane insert (Millipore PICM03050) within a 6-well plate (Greiner). Optional, slices were placed first onto a sterile 0.4 μm pore extramembrane (Millipore HTTP02500). Vibrosections (1–3 per well) were cultured in 6-well plates at 37°C and 5% CO_2 with 1.2 ml/well of the following culture medium (Stoppini et al., 1991): 50% MEM/HEPES (Gibco), 25% heat inactivated horse serum (Gibco/Lifetech, Austria), 25% Hanks' balanced salt solution (Gibco), 2 mM $NaHCO_3$ (Merck, Austria), 6.5 mg/ml glucose (Merck, Germany), 2 mM glutamine (Merck, Germany), pH 7.2. Vibrosections were incubated for minimal 2 weeks and medium was changed 1–2x per week. At the end of the experiment, vibrosections were fixed for 3 h at 4°C in 4% paraformaldehyde (PAF)/10 mM phosphate buffered saline (PBS) and then stored at 4°C in PBS/sodium acide until use. Alternatively, cortical brain pieces were cut into 400 μm thick sections using a MacIllwain tissue chopper and 6–8 slices were cultured on the membrane. Vibrosections or brain slices were either cultured with or without 100 ng/ml VEGF or FGF-2 for 2 or 4 weeks. Some slices were subjected immediately after dissection to 42°C (hyperthermia) or pH 6.0 (acidosis) overnight and then cultured for 2 weeks in normal medium. To study vessel re-growth, a cut was made with a scalpel through a whole vibrosection, starting from parietal cortex ending at the mesencephalon.

Immunohistochemistry

Immunohistochemistry was performed as described in detail (Daschil et al., 2013). Brain slices were processed free-floating and were washed with PBS and incubated in PBS/0.1% Triton (T-PBS) for 30 min at room temperature (RT) while shaking. After incubation, the sections were blocked in T-PBS/20% horse serum (GIBCO Invitrogen)/0.2% BSA (SERVA) for 30 min at RT while shaking. Following blocking, brain sections were incubated with primary antibodies (collagen-IV, 1:500, abcam ab6586; alpha smooth muscle actin, αSMA, 1:1000, Novus Biologicals NB300-978; laminin, 1:500, Sigma L9393; PDGFRβ 1:250, Novus Biologicals NB110-57343) in T-PBS/0.2% BSA over 2–3 days at 4°C. The sections were then washed and incubated with fluorescent Alexa (-488, -546-; Invitrogen-Life Tech, Vienna, Austria) secondary antibodies in T-PBS/0.2% BSA for 1 h at RT while shaking. Finally, the sections were washed with PBS, then mounted onto glass slides and cover-slipped with Mowiol 4-88 (Roth, Austria). Some brain slices were processed using the chromogenic substrate diaminobenzidine DAB as described earlier (Ullrich et al., 2011).

Confocal microscopy was performed using a SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a HCX PL APO 63x/1.3 NA glycerol objective. Imaging was performed with an argon laser line for AlexaFluor 488, a DPSS561 nm laser for AlexaFluor 546 or thiazine red, and a HeNe 633 nm laser for AlexaFluor 647. Emission of each fluorophore was detected from 493 to 556 nm (AlexaFluor 488), 566 to 628 nm (AlexaFluor 546, thiazine red), and 638 to 750 nm (AlexaFluor 647). Images were acquired using the LAS AF acquisition software, version 2.1., and further processed with Huygens Deconvolution and Imaris V6.4 software.

Western Blot

Western blot analysis was performed as previously described by us (Hohsfield et al., 2014). Slices (adult and postnatal) were incubated for 2 weeks, and all slices from three wells were taken and pooled in an Eppendorf tube, then dissolved in 100 μl ice-cold PBS containing a protease inhibitor cocktail (P-8340, Sigma), homogenized using an ultrasonic device (Hielscher Ultrasonic Processor, Germany) and then centrifuged at 14,000 × g for 10 min at 4°C. Then, 20 μl of the extracts were loaded onto 10% Bis-Tris SDS-polyacrylamide gels, separated for 25 min at 200 V and finally electrotransferred to nylon-PVDF Immobilon-P^{SQ} membranes for 90 min at 30 V in 20%

methanol blotting buffer. The Western Breeze Chromogenic System was used for the detection of specific proteins in cortical extracts. Briefly, blots were blocked for 30 min in blocking buffer, incubated with primary antibodies against PDGFRβ (1:2000) or actin (1:1000) at 4°C overnight, washed, and then incubated in alkaline phosphatase conjugated anti-rabbit IgG for 30 min. After washing, bound antibodies were detected using an enhanced chemiluminescence (ECL) system. As a control purified cultured brain capillary endothelial cells (BCEC) were isolated, extracted and loaded.

Data Analysis and Statistics

The vascular density was counted in a 6 × 6 grid (see **Figure 2C**). Briefly, a digital picture was taken under the microscope at a 10x magnificiation. The digital picture was overlaid with a 6 × 6 grid using Photoshop (Adobe Photoshop Elements 2.0) and the number of all vessels crossing all lines was counted. Statistical analysis was performed by One-Way ANOVA and subsequent Fisher LSD *post-hoc* test. Statistical results were considered significant at $p < 0.05$.

Results

The Vascular Network in Brain Slices

Organotypic brain vibrosections can be cultured from postnatal or adult mice and exhibit a strong vascular network all over the brain (**Figure 1A**). The vessels are well structured and express collagen IV (**Figures 1B**, **2B**) as well as laminin (**Figure 2A**). The vessels are intact and represent tube-like formations as seen in the confocal microscope (**Figure 1C**).

Effects on Angiogenesis

In control brain slices, approximately 100 vessel crossings were counted in a 6 × 6 grid (**Figure 2D**). Incubation of postnatal brain slices with 100 ng/ml FGF-2 but not VEGF significantly increased the vessel density in the cortex (**Figure 2D**). When postnatal slices were pre-treated overnight with pH 6.0 (acidosis) but not with heat (42°C) the laminin$^+$ vessel density significantly declined (**Figure 2D**).

Effects on Re-growth of Vessels

In order to study re-growth, adult vibrosections were cut with a scalpel directly after transferring them on a semipermeable membrane. The number of laminin$^+$ vessels crossing this cut was low (2 per field) after incubating for 4 weeks (**Figures 3A,C**). In contrast, when slices were incubated with 100 ng/ml VEGF, the crossing of vessels over the cut markedly increased (**Figures 3B,C**).

Pericytes in Brain Slices

Pericytes specifically stained with an antibody against PDGFRβ, exhibit an intense staining along the vessel wall (**Figure 4B**). A control staining without antibody showed only background (**Figure 4A**). Western Blot analysis confirmed that postnatal as well as adult organotypic brain slices expressed an approximately 80–100 kDa PDGFRβ protein. As a control, purified cultured BCEC were negative. Actin was used as a loading control

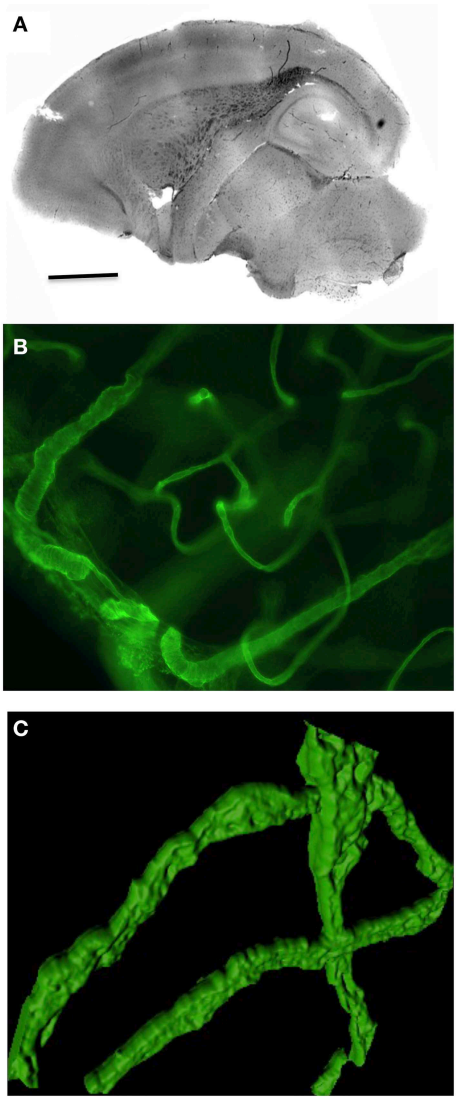

FIGURE 1 | Overview of vasculature in postnatal organotypic vibrosections. Vibrosections (110 μm) were prepared from a 10 day postnatal wild-type mouse and cultured for 2 weeks, postfixed in paraformaldehyde and stained for collagen IV using a chromogenic DAB substrate **(A)** or fluorescence Alexa-488 **(B,C)**. Panel **(C)** shows a high power confocal microscopic picture. Scale bar in A = 1500 μm **(A)**, 17 μm **(B)**, 12 μm **(C)**.

(**Figure 4C**). The collagen IV positive vessels co-expressed alpha-smooth muscle actin, another pericyte marker, as seen in the confocal microscope (**Figures 4D–F**).

Discussion

In the present work we summarize published experiments and present examples of own data on exogenous modulation of the vascular network in organotypic brain slices. We show that brain slices exhibit a dense vascular network and are positive for laminin and collagen IV. We demonstrate that FGF-2 induces angiogenesis, however, that laminin$^+$ staining is down-regulated

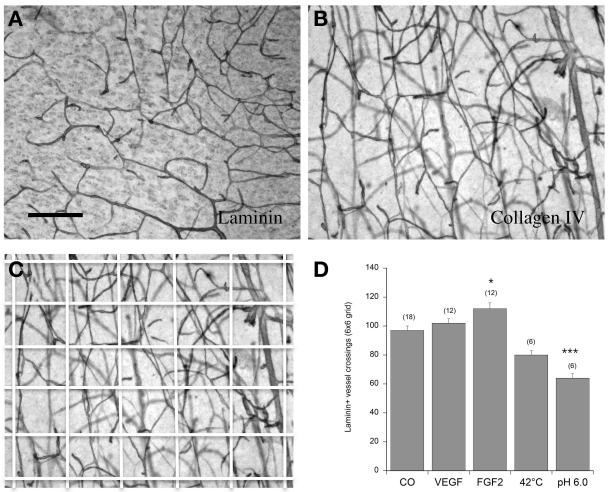

FIGURE 2 | Angiogenesis of vessels in the postnatal organotypic slice. Brain slices from the cortex of 10 day old postnatal mice were cultured for 2 weeks either without (Co) or with 100 ng/ml VEGF or FGF-2 and then postfixed and stained for laminin **(A,D)** or collagen IV **(B,C)**. Some slices were incubated immediately after dissection at 42°C or at pH 6.0 overnight and then cultured for 2 weeks without any growth factors. The vessel density was quantified in a 6 × 6 grid **(C)**. Statistical analysis was performed by One-Way ANOVA with a subsequent Fisher LSD *post-hoc* test. Values are given as mean ± SEM; values in parenthesis give the number of analyzed slices. $^{*}p < 0.05$; $^{***}p < 0.001$. Scale bar in A = 90 μm **(A–C)**.

by acidosis and that vessels can re-grow over a lesion site when stimulated by VEGF. Furthermore, we add novel data on the characterization of pericytes in organotypic brain slices and selective staining by PDGFRβ immunohistochemistry and Western Blot.

The Vascular Network in Organotypic Brain Slices

The brain is almost the only organ within the human body which depends on successive blood flow to ensure the continuous uptake of energy metabolites into neurons (Iadecola, 2004; Winkler et al., 2014a). In case of any interruption of the cerebral blood flow, brain function decreases immediately which leads to irreversible brain damage (Iadecola, 2004). Neurons are known to have high metabolic rates with only limited cellular reserves. Therefore, neuronal and cerebrovascular functions are tightly connected in the brain to guarantee appropriate vascular excess (Winkler et al., 2014a). Thus, almost every neuron within the human brain possesses its own capillary (Zlokovic, 2005). A proper function of the vascular system is essential for normal cerebral function and any alteration within this system leads to neuronal loss, stroke, vascular ischemia or vascular dementia, or Alzheimer's Disease (Ginsberg, 1990; Siesjoe, 1992; Hossmann, 1994).

In the last decades, several researchers investigated the function of the neurovascular system. Beside *in vivo* studies, *in vitro* techniques including organotypic brain slices became an own research focus for neuroscientists. Crain et al. (1982) established organotypic brain slices by using spinal cord-dorsal root ganglia. Moreover, in the following years, some alternative techniques came up comprising amongst others the roller-tube cultures, membrane cultures, and slices grown on culture dishes (Gähwiler and Hefti, 1984; Stoppini et al., 1991; Gähwiler et al., 1997). Recently, we developed a novel organotypic vibrosection model by culturing whole sagittal brain slices from postnatal rats for several weeks (Ullrich et al., 2011). Organotypic slices from different regions of the brain comprising striatum, hippocampus, cortex, cerebellum, and spinal cord are found to be a useful tool for physiological as well as pharmacological investigations (Gähwiler et al., 1997; Cho et al., 2007). Slices are easy to handle and maintain the cytoarchitecture of their originated brain region (Gähwiler et al., 1997; Ullrich et al., 2011). In the last years, *in vitro* studies using acute (dissected and analyzed the same day) brain slices were performed (Peppiatt et al., 2006; Fernández-Klett et al., 2010) However, acute brain slices do not represent the *in vivo* situation, they display an injured tissue with an improper BBB. Moreover, the constant cell death within acute brain slices accompanied with a severe BBB damage may disturb the mechanisms of the neurovascular coupling (Girouard and Iadecola, 2006; Filosa, 2010). Thus, there is clear need to culture slices for prolonged time to reduce any degenerative endogenous stimuli. The present model is such a chronic long-cultured system showing low astrogliosis or inflammation.

Biomarkers to Stain Brain Vessels

To investigate the vascular network, biomarkers like laminin or collagen IV are well established. Since decades, laminin is a well-known basement membrane marker which stains the vascular structures of the brain in an excellent manner (Eriksdotter-Nilsson et al., 1986; Jucker et al., 1996; Sixt et al., 2001). By using immunohistochemistry, several previous studies of our group displayed a strong network of laminin^{+} brain capillaries in organotypic brain slice cultures (Pirchl et al., 2006; Ullrich et al., 2011). Besides laminin, collagen IV is another basement membrane protein and is often used as biomarker for visualizing brain capillaries (Kefalides, 1966; Armulik et al., 2010; Daschil et al., 2015). In this study, we demonstrate that brain slices, which exhibit a dense vascular network are also positive for collagen IV. Using these biomarkers we determined the vessel density in our slices after treatment with different exogenous stimuli, such as growth factors, hyperthermia, or acidosis. It needs to be mentioned, however, that the immunostainings with a single marker only give some limited insight into the cellular processes. Thus, an increased laminin^{+} network could indicate that either the vessel density is increased and reflects angiogenesis or that only the vessel marker is up-regulated. The same is true if the vessel marker is decreased, which could reveal that the vessels are damaged and degenerate or that only the marker is down-regulated.

Effects of Growth Factors VEGF and FGF-2 on Vessel Growth

Vessels are sensitive for degenerative stimuli, such as e.g., ischemia, trauma, stroke, or Alzheimer's Disease. Thus, there is increased importance to explore how vessels can be protected or

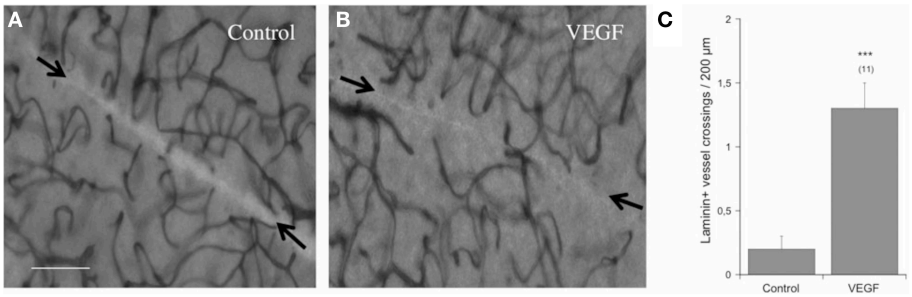

FIGURE 3 | Re-growth of vessels in the adult organotypic vibrosection. Vibrosections were prepared from adult (6 month old) wild-type mice. A knife cut was made through the whole brain. Slices were then incubated for 4 weeks either without or with 100 ng/ml VEGF. Slices were then postfixed and stained for laminin using the chromogenic substrate DAB. The number of vessels crossing the cut (arrows in **A,B**) were counted and are given as crossings per 200 µm distance **(C)**. Statistical analysis was performed by an unpaired *T*-test with equal variance. Values are given as mean ± SEM; values in parenthesis give the number of analyzed slices. $^{***}p < 0.001$. Scale bar in A = 40 µm **(A,B)**.

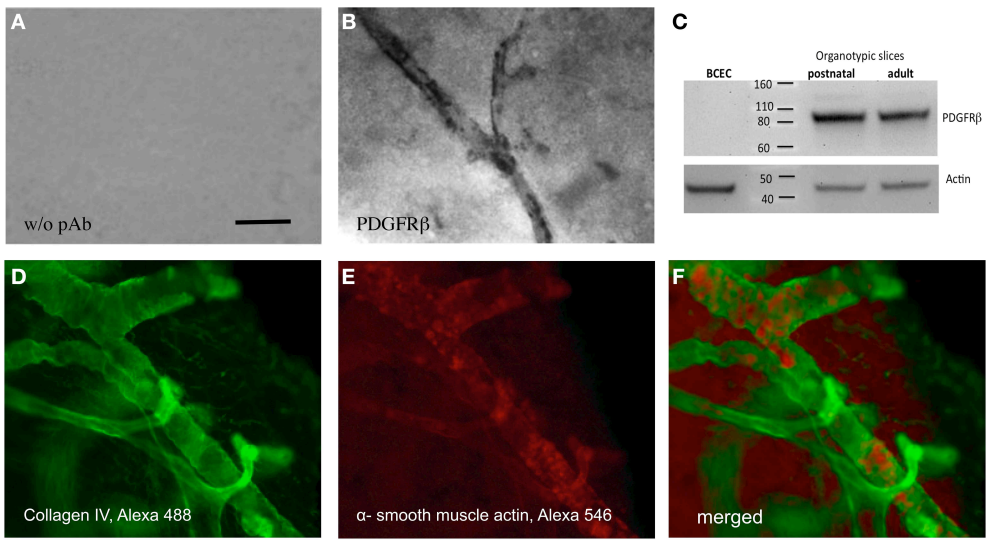

FIGURE 4 | Pericytes in adult organotypic vibrosections. Vibrosections (110 µm) were prepared from the adult mouse, incubated for 2 weeks, then postfixed and stained for PDGFRβ **(A,B)**, collagen IV **(D)** or alpha-smooth muscle actin **(E)**. Slice extracts were subjected for a Western Blot and stained for PDGFRβ **(C)** showing a single band at approximately 80–100 kDa. As a control, extracts from cultured brain capillary endothelial cells (BCEC) or postnatal slices were analyzed. Loading control was performed by staining for actin **(C)**. Slices from panels **(A,B)** were stained using the chromogenic substrate DAB and slices from panels **(D–F)** by fluorescence (Alexa-488 for collagen IV, green and Alexa-548 for alpha-smooth muscle actin, red). Panel **(F)** shows a merged picture. Panels **(D,E)** were high power confocal microscopic pictures. Scale bar in A = 15 µm **(A,B)**, 7 µm **(D–F)**.

how new vessels are formed. Several studies show that especially growth factors exhibit strong protective and growth-promoting effects (Kremer et al., 1997; Rosenstein et al., 1998). VEGF is the most potent factor controlling vascular function and formation to enhance the vasculature (Rosenstein et al., 1998; Carmeliet and Collen, 1999; Jośko et al., 2000). However, several other stimuli influence vessel growth. In our research group, we demonstrated a pro-angiogenic effect of thapsigargin in brain slices which is probably caused by an indirect stimulation of the VEGF expression (Ullrich and Humpel, 2009). In the present study, we show that VEGF has a potent effect on growth of vessels across a lesion site but not in an unlesioned slice.

A second potent vessel-promoting growth factor represents FGF-2 which is expressed in astrocytes. However, FGF-2 binds to fibroblast growth factor receptor-1 (FGFR-1) located on endothelial cells and regulates the survival of other angiotrophic factors (Garberg et al., 1998; Sobue et al., 1999). Several *in vitro* studies showed that decreased levels of FGF-2 lead to a malfunction of the BBB (el Hafny et al., 1996; Reuss et al., 2003). Bendfeldt et al. (2007) demonstrated for the first time that FGF-2 enhances the number of vascular structures in mouse brain in a concentration-dependent manner. Furthermore, FGF-2 promotes the function of the BBB by maintaining inter-endothelial tight junctions (Bendfeldt et al., 2007). In agreement

with others, we show that FGF-2 significantly increased the vessel density in our organotypic brain slices.

Effects of Acidosis and Hyperthermia

Acidosis is an important factor in ischemic brain pathologies including the damage of brain capillaries, NVU, and BBB (Siesjö, 1988; Pirchl et al., 2006). In general, acidosis is mainly provoked by increased CO_2 levels in the tissue as well as dysfunctions within the brain metabolism leading to an abnormal accumulation of acids. As a result of hypercapnia, the pH level within the brain decreases to about 6.6 without affecting cell viability. However, in case of hypoxia and severe ischemia, anaerobic glycolysis leads to a pathological accumulation of acids, resulting in pH levels of about 6.0, which causes massive cellular damage (Rehncrona, 1985). Moreover, it is known that acidosis leads to an increase of iron-catalyzed production of reactive oxygen species by releasing iron from its binding partner ferritin or transferrin (Li and Siesjo, 1997). A few years ago, we (Pirchl et al., 2006) observed a decrease in laminin$^+$ capillaries after incubating organotypic brain slices at low pH; this finding was confirmed in the present study.

Hyperthermia is another negative stimulus for the cerebral vascular system. Temperature rise, e.g., during fever, is a dangerous factor to cause brain damage (Fajardo et al., 1985; Ginsberg and Busto, 1998; Nybo et al., 2002). Our data show that, indeed, a rise of the temperature to 42°C overnight decreases the laminin$^+$ vessel density, although not significant.

Brain Slices Coupled to a Blood-brain Barrier

Isolated and cultured brain slices lack a BBB. In order to couple a synthetic BBB to slices Duport et al. (1998) developed a BBB *in vitro* model by co-culturing an endothelial cell monolayer upon a stationary organotypic slice culture. They (Duport et al., 1998) studied the attendance of tight junctions by using morphological analysis and neuronal activity using electrophysiological approaches. Additionally, they showed that dopamine and glutamate did not pass the synthetic BBB but L-DOPA entered the slices which demonstrated the selective permeability of the BBB. Despite the advantages of the system, a major drawback of this study was the number of unsuccessful cultures (error rate of about 25–30%). This model also failed in our hands due to its complexity and low stability of the confluent endothelial cell layer.

Pericytes in Organotypic Brain Slices

Pericytes are perivascular cells, uniquely located within the NVU and play an important role in the regulation of capillary blood flow, maintenance and formation of the BBB (Sagare et al., 2013; Winkler et al., 2014b). Along the arterial-venous axis, pericytes are mainly found on capillaries, pre-capillary arterioles, and post-capillary venules of many different organs (Dalkara et al., 2011). Especially in the capillary tube of the CNS, pericyte coverage of about 70–80% was found (Winkler et al., 2014b). During angio- and vasculogenesis, a cross-talk between endothelial cells and pericytes was observed leading to proliferation, migration, and attachment of pericytes to adjacent capillaries (Winkler et al., 2014b). Studies with pericyte-deficient mice (i.e., PDGFRβ transgenic mice) revealed that loss of pericytes is accompanied with vascular brain damage due to an increased BBB permeability (Dalkara et al., 2011; Winkler et al., 2014b). In organotypic brain slices, pericytes are not well studied. Zehendner et al. (2013) showed for the first time a detailed characterization of pericytes. Thereby, they observed that moderate hypoxia and inflammation caused caspase-3-mediated pericyte loss. Interestingly, Bell et al. (2010) determined that the loss of pericytes results in an impairment of the BBB and accumulation of several neurotoxic substances. Several biomarkers have been shown to be expressed by pericytes, such as e.g., PDGFRβ, NG2 (chondroitin sulfate), CD13 (alanyl (membrane) aminopeptidase), αSMA (alpha-smooth muscle actin), or desmin (Díaz-Flores et al., 2009; Armulik et al., 2010; Krueger and Bechmann, 2010). In the present study we show that within organotypic brain slices, pericytes can be selectively identified using PDGFRβ immunostaining and co-localize in αSMA$^+$ vessels. Using Western Blot analysis, we confirm these data by showing PDGFRβ as a single band of approximately 80–100 kDa in adult as well as postnatal brain slices but not in endothelial cells.

Organotypic Brain Slices as a Screening Model for Angiogenic Drugs

There is a clear need to develop fast and simple *in vitro* models for a high-throughput screening of pro-angiogenic factors or angiogenic inhibitors (Staton et al., 2004). So far the most useful angiogenic assays include *in vivo* Matrigel plug and sponge and corneal neovascularization, the chick chorioallantoic membrane and aortic arch assays, the *in vitro* cellular (proliferation, migration, tube formation) and organotypic (aortic ring) assays (Auerbach et al., 2003; Staton et al., 2009). Most pro- or anti-angiogenic drugs have been tested in co-cultures of endothelial cells and pericytes or smooth muscle cells forming a tubular network (Evensen et al., 2010, 2013; Fu et al., 2013; Wolfe et al., 2013). Organotypic brain slice cultures have to our knowledge not yet extensively used for screening pharmacological drugs, although this model is simple and provides a potent well preserved 3-dimensional capillary architecture. Using such slices we have recently tested different calcium channel blockers (e.g., nimodipine or nifedipine) and found strong pro-angiogenic activity (Daschil et al., 2015).

Taken together, our data summarize current literature and show examples of own data that organotypic brain slices of mice are an appropriate tool to study brain angiogenesis, vascularization, or vessel-re-growth. We provide evidence that screening of pharmaceutical drugs in such 3-dimensional brain slices may give insights into cellular and molecular processes of vessels, which may also play a role *in vivo*.

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REVIEW
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Zebrafish as an Emerging Model Organism to Study Angiogenesis in Development and Regeneration

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Angiogenesis is the process through which new blood vessels are formed from preexisting ones and plays a critical role in several conditions including embryonic development, tissue repair and disease. Moreover, enhanced therapeutic angiogenesis is a major goal in the field of regenerative medicine and efficient vascularization of artificial tissues and organs is one of the main hindrances in the implementation of tissue engineering approaches, while, on the other hand, inhibition of angiogenesis is a key therapeutic target to inhibit for instance tumor growth. During the last decades, the understanding of cellular and molecular mechanisms involved in this process has been matter of intense research. In this regard, several *in vitro* and *in vivo* models have been established to visualize and study migration of endothelial progenitor cells, formation of endothelial tubules and the generation of new vascular networks, while assessing the conditions and treatments that either promote or inhibit such processes. In this review, we address and compare the most commonly used experimental models to study angiogenesis *in vitro* and *in vivo*. In particular, we focus on the implementation of the zebrafish (*Danio rerio*) as a model to study angiogenesis and discuss the advantages and not yet explored possibilities of its use as model organism.

Keywords: *Danio rerio*, vascular development, vessel regeneration, angiogenesis assay, high-throughput screening assays, endothelial markers

INTRODUCTION

Angiogenesis is the process through which new blood vessels emanate from preexisting vascular structures. It plays a pivotal role in various physiological and pathological conditions and is orchestrated by the tight interaction between endothelial cells and their niche. While inadequate vessel maintenance or growth leads to tissue ischemia; excessive vascular growth or abnormal remodeling promotes cancer, inflammatory disorders, and retinopathies (Pandya et al., 2006).

Angiogenesis is mainly accomplished through vessel sprouting, which may be divided into four main steps: tip cell formation, tubule morphogenesis and lumen creation, adaptation to tissue needs and, finally, stabilization and maturation of the newly formed vessels (Ribatti and Crivellato, 2012; Neufeld et al., 2014). A non-sprouting mechanism of microvascular growth has been also described,

and it involves the increment of vascular surface by insertion of a multitude of transcapillary pillars in a process called "intussusception" (Styp-Rekowska et al., 2011).

Parallel to the study of the angiogenic process, a large number of *in vitro* and *in vivo* assays have been developed to study the cellular and molecular mechanisms involved (Cimpean et al., 2011). Each model has its own advantages and disadvantages, and their adequate combination is key to reveal the impact of the element under analysis within the global process.

In vitro assays have been broadly used to answer questions related to specific behaviors of endothelial cells such as proliferation, differentiation, structural organization, cytokine secretion profiling and chemotaxis, as well as the molecular mechanisms associated with angiogenesis (Irvin et al., 2014). Moreover, *in vitro* systems have helped to identify and validate promising compounds to therapeutically promote or inhibit angiogenesis (Goodwin, 2007), as they are quantitative, easily monitored, reproducible, and provide the confidence necessary for the rapid screening of potential pro- or anti-angiogenic compounds (Weiss et al., 2015). However, important aspects should be considered when assessing the potential of an angiogenic effector using *in vitro* assays such as the decision over the type or tissue-origin of the endothelial cells being used, and the experimental bias of the protocols being followed (for a more comprehensive discussion see Unger et al., 2002; Staton et al., 2009). Finally, common *in vitro* experiments do not consider the influence of the vascular niche, which has been shown to be critical in the process of angiogenesis during tissue regeneration (Ribatti and Crivellato, 2012; Kunisaki and Frenette, 2014; Ramasamy et al., 2015).

The complexities of the formation, function and pathology of blood vessels in the context of the living animal mandate the availability of adequate *in vivo* models in order to confirm the results obtained *in vitro*. Since the 1970s multiple animal models have been developed in order to understand the physiological mechanisms of blood vessel formation, as well as to validate approaches that either enhance or inhibit the angiogenic process. The mouse model is by far the most common used to study angiogenesis *in vivo*, with the advantage of being a mammal that in many ways faithfully recapitulates human physiology. However, this animal model can be laborious and expensive to use, especially for screening purposes. Also, the use of mice limits the evaluation of the outcome to a final time point, since *de novo* or re-vascularization can only be visualized and quantified after euthanizing the animal, hence limiting the understanding of angiogenic dynamics.

As mammalian and most vertebrate tissues are opaque, the introduction of the transparent zebrafish larva as a tool for the examination of the vasculature in the intact animal has gained recent attention. Importantly, several studies have made clear that there is a high degree of molecular conservation in the most important pathways involved in the development and physiology of blood vessels in all vertebrates (reviewed by Baldessari and Mione, 2008; Gore et al., 2012). Furthermore, genetic and pharmacological evidence has shown that there is mutual translatability of findings between zebrafish and human vascular biology (Coultas et al., 2006; Lieschke and Currie, 2007). Thus, the emergence of a simple yet validated discovery and/or screening tool has been welcomed by the community.

In the following sections, we provide a brief overview on the currently available *in vitro* and *in vivo* angiogenic assays, describing their most common uses and their potential advantages and limitations. Additionally, we also provide information on the current and potential uses of zebrafish as model to study angiogenesis.

IN VITRO MODELS

In vitro angiogenesis models study the behavior of endothelial cells within a controlled environment (Ayata et al., 2015). They are designed to recapitulate the different steps of the angiogenic processes, where endothelial cells are involved, such as cell proliferation, migration, extracellular matrix digestion and invasion, morphogenesis and capillary tube formation (Cimpean et al., 2011). **Table 1** summarizes the settings and evaluation parameters of the most commonly used assays focusing on migration, proliferation and tubule formation.

Proliferation Assays

These assays are conceived to evaluate the effects of a test substance, based on the quantitation of endothelial cell proliferation. They are broadly classified into those that determine net cell number and those that evaluate cell-cycle kinetics (Staton et al., 2004). Cell numbers can be estimated either manually or through automated cell counting. Alternatively, metabolic assays, which have shown a linear correlation with cell density (Niles and Riss, 2015), quantification of DNA synthesis or expression of proliferation markers may be used (reviewed by Whitfield et al., 2006). However, since none of these methods have been explicitly developed for vascular-related cells, it is indispensable to address the target specificity of the test substance, as well as its therapeutic impact based on other angiogenesis-related parameters. Furthermore, proliferation assays should be combined with quantitative methods for estimation of cell death, in order to discard the possibility of cytotoxicity of the test-substance (Kepp et al., 2011).

Migration Assays

Migration assays allow the study of endothelial cell motility and chemotaxis. They evaluate the active migration of cells into a specific area or toward a specific direction as a result of a treatment. The main advantage of the exclusion zone assay (Poujade et al., 2007; Gough et al., 2011), where silicone-based structures, so-called "masks" or "stencils," are placed on the well bottom to create an cell-empty area, in comparison to a scratch assay (Coomber and Gotlieb, 1990; Yarrow et al., 2004), where a "wound" is created by physically disrupting an endothelial cell monolayer, is the uniformity and hence reproducibility of the denuded area into which confluent endothelial cells will later migrate (reviewed by Hulkower and Herber, 2011).

Another commonly used migration assay follows the principle of the Boyden chamber, first described in 1962, where a semipermeable membrane that only allows active passage of cells is placed in their migration path (Boyden, 1962), sometimes

TABLE 1 | *In vitro* angiogenesis assays.

Type of assay	Basis	Assay	Setting	References
Proliferation (reviewed by Stoddart, 2011; Niles and Riss, 2015)	Cell number		The effect of test substance is measured by estimation of the increase in viable endothelial cell number over time	Staton et al., 2009
	Cell cycle kinetics	BrdU assay	Bromodeoxyuridine (BrdU), a pyrimidine analog, is incorporated during DNA synthesis and quantified by immunohistochemistry or ELISA	Qin et al., 2006
		Proliferation marker detection assay	Ki-67, expressed during the S, G2 and M phases, or the proliferating cell nuclear antigen (PCNA), overexpressed in the G1 and S-phase are estimated quantitatively	Whitfield et al., 2006
	Metabolism	Tetrazolium salt-assays	Metabolically active cells convert tetrazolium-salt compounds (MTT, XTT, MTS and WST1) into formazan dyes. The colorimetric change is quantified using spectrophotometry and correlated to cell number	Boncler et al., 2014
		Protease activity assay	Protease activity measured using a fluorogenic cell permeable substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC) is correlated to viable cell-number	Niles et al., 2007
		Resazurin assay:	Metabolically active cells reduce resazurin to resorufin, changing the spectrometric properties of the compound. Signal is quantified and correlated with cell number	Larson et al., 1997
		ATP-measurement	Bioluminescence-based ATP-detection assay that uses the linear relationship between viable cell number and ATP-concentration	Wang et al., 2010
	Cell death	TUNEL-assay	Fluorescent labeling of terminal deoxynucleotidyl transferase-dUTP nick end of the 3′-OH region of fragmented DNA is estimated by microscopy or flow cytometry	Goodwin, 2007
		Apoptosis marker detection assay	Expression of apoptosis cell-markers, such as caspase-3 or annexin V, is assessed via microscopy or flow cytometry	Köhler et al., 2002
		LDH assay:	The release of lactate dehyrogenase (LDH) as a consequence of loss of cell membrane integrity can be quantified to through a colorimetric reaction	Smith et al., 2011
Migration (reviewed by Hulkower and Herber, 2011)	Wound assay	Scratch assay	A tip or needle is used to remove cells to form a denuded area in a confluent endothelial cell monolayer, in which cell migration can be quantitatively estimated after a specific time interval	Steinritz et al., 2015
		Exclusion zone assay	Stencils are placed in culture plates prior to cell-seeding in order to create uniformly sized wounds in an intact confluent monolayer, in which invasion by the patterned cells can be quantitatively assessed	Gough et al., 2011
	Chemotaxis/ chemoinvasion	Boyden chamber assay	Two-compartment chamber with a semi-permeable membrane is used to evaluate active cell migration in response to specific stimuli or due to chemotaxis within a test substance gradient	Albini and Benelli, 2007
		Microfluidics assay	Creation of a diffusion-generated concentration gradient within a migration chamber, through which endothelial cells can migrate	Chung et al., 2010; Young, 2014
Morphogenesis (reviewed by Arnaoutova and Kleinman, 2010)	Tubule formation	2D-tubule formation assay	Endothelial cells are platelet on an extracellular matrix and monitored for their ability to form vessel-like tubules	Arnaoutova and Kleinman, 2010
		EC-aggregate reassembling assay	Endothelial cell spheroids or aggregates are embedded in an extracellular matrix that resembles the basement membrane environment. Upon stimulation, vessels sprout into the matrix	Li and Stuhlmann, 2011
		3D-tubule formation assay	Endothelial cells are seeded in a three-dimensional culture platform that involves extracellular matrix components and/or other cell-types. Different settings allow to study sprouting, formation, stabilization and maturation of vessel-like tubules	Hetheridge et al., 2011; Diaz-Santana et al., 2015

requiring matrix degradation, in which case it is regarded a chemoinvasion assay (Albini and Benelli, 2007), or in response to a test substance (chemotaxis). The use of microfluidic cell culture systems has overcome the difficulties of maintaining a linear gradient of the test compound by introducing microchannel compartments in which a diffusion-generated concentration gradient can be created. Furthermore, they allow live single-cell and cell-population tracking, as well as directionality and velocity estimation (Young, 2014).

Tubule Formation Assays

Tubule formation assays are used to study the assembly of capillary-like structures by adjacent endothelial cells (Arnaoutova et al., 2009). In two-dimensional assays, endothelial cells are usually seeded on extracellular matrices and the spontaneous building of capillary-like networks is analyzed. Quantitation of tubule formation is mainly addressed by immunohistochemistry and analyzed based on four main parameters: average tubule length, number of tubules, tubule area and number of branch points (Staton et al., 2009). They allow to study spontaneous tubule formation due to endothelial cell-to-cell interactions and the assembly of tight-junctions (Vailhé et al., 2001), however they do not resemble the process of sprouting angiogenesis, which is the development of new blood vessels from pre-existing major donor vessels (Ribatti and Crivellato, 2012). Also, the early formed tubules lack lumen and their length and degree of branching differ from real capillaries (Donovan et al., 2001).

On the other hand, three-dimensional culture systems of endothelial cells have been used to study the formation of more complex capillary networks inside extracellular matrix substitutes. They have helped to elucidate the role of support cells, such as fibroblasts (Bishop et al., 1999; Hetheridge et al., 2011), pericytes (Berthod et al., 2012) and adipose stromal cells (Merfeld-Clauss et al., 2010; Verseijden et al., 2010; Sarkanen et al., 2012), as well as the homo- and heterotypic cell-interactions of endothelial cells during vessel-formation, -sprouting and -anastomosis (Ayata et al., 2015; Diaz-Santana et al., 2015). Moreover, three-dimensional tubule formation assays have become an important tool to mimic *in vitro* microenvironments of tumor vascularization (reviewed by Chwalek et al., 2014; Song et al., 2014). Low standardized settings and the more challenging evaluation of the three-dimensional tubule formation are the main disadvantages of these assays.

Organ Explant Based-Assays

Also known as *ex vivo* angiogenesis models, these assays aim to analyze the angiogenic sprouting and the growth of vessel capillaries from explanted segments of vasculature. Here, isolated vasculature biopsies are placed generally over three dimensional biological matrices in the presence or absence of a test compound. Explants are then monitored for the outgrowth of vessel tubules extending from the periphery of the explant into the surrounding matrix (Rezzola et al., 2014). **Table 2** summarizes the characteristics of the most broadly used *ex vivo* assays.

Ex vivo assays have the advantage of working with native quiescent endothelial cells *in vivo* at the experimental outset (Ucuzian and Greisler, 2007; Staton et al., 2009). Further, because the tissue complexity is preserved, most of the cellular and molecular components involved in angiogenesis are present. As a result, vascular sprouts contain a lumen and a basement membrane, and are composed of a mixed population of endothelial cells, pericytes, fibroblasts, and macrophages (Nicosia et al., 2011). These assays allow the study cell proliferation, migration, tube formation, network branching, perivascular recruitment and vascular remodeling (Baker et al., 2011), in addition to other post-angiogenic mechanisms such as vessel stabilization and regression (Nicosia et al., 2011). Some of the disadvantages compared to *in vitro* assays are the more demanding technical skills, the limited number of simultaneous samples being processed, and the implicit higher experimental variability (Staton et al., 2009; Rezzola et al., 2014). On the other hand, compared to *in vivo* assays, *ex vivo* assays do not consider circulating endothelial progenitors recruited in the angiogenic process and lack the pro-angiogenic stimuli in blood flow (Irvin et al., 2014). Also, the decision over the source of the vascular material should behold that angiogenesis mainly involves the microvasculature rather than the macrovasculature, and that microvessels such as capillaries, small arterioles and venules, are composed of different tissue layers compared to large arteries and veins (Staton et al., 2009).

IN VIVO MODELS

Multiple *in vivo* models have been developed to directly study angiogenesis within an organism, and therefore evaluate the entire process of new blood vessel formation, since they allow to consider all cellular and molecular role players involved, such as supporting cells (e.g., tumor cells, pericytes, smooth muscle cells, and fibroblasts), the extracellular matrix, and the cellular and humoral components in circulating blood (Staton et al., 2004). Most *in vivo* angiogenesis assays are not designed to understand a specific process, but rather to determine the success of the outcome, with the exception being the zebrafish larva, as we discuss in the next section. Nevertheless, it is also important to point out that one of the main disadvantages of *in vivo* models is the ethical concerns they raise, and the complications they imply, due to the strict guidelines regulating animal testing in some countries. Again, the zebrafish is exempt, for the most part, from these concerns, especially during larval stages.

Corneal Angiogenesis Assays

As originally developed by Gimbrone et al. (1973), induction of angiogenesis in the cornea is among the most convincing demonstrations of neovascularization, since the cornea is richly innervated, but normally has no blood vessels (Henkind, 1978). In this assay, a stimulus induces the migration of endothelial cells from the edge of the cornea into the space between the corneal epithelium and stromal cells, forming new sprouts directed toward the source of the angiogenic signal. This method has been applied in multiple animal models including rabbit, mouse,

TABLE 2 | *Ex vivo* angiogenesis assays.

Assay	Setting	Advantage	References
Rat aortic ring assay	Thoracic aorta is dissected, cleaned and cut into rings. Upon serum-starvation, rings are embedded in extracellular matrix components in the presence or absence of the test compound. Exponential vessel outgrowth from the explant of the tubule structures is observed within 10 days	Many rings available from few animals. Supporting cells are included in the formation of vessels. Visible lumenized tubule structures develop over a time course similar to that *in vivo*	Nicosia, 2009
Mouse aortic ring assay		Cost-efficient transgenic mouse technologies and gene manipulation available. Implementable for high-quality imaging and high-throughput screening	Baker et al., 2011
Miniature ring-supported gel assay	Isolated aortae segments are placed in low volume three-dimensional collagen gel supports, which are casted by a nylon mesh ring that improves the stability of the setting	Optimized system allows better specimen handling, staining, imaging, and a more economical use of extracellular matrix reagents	Reed et al., 2011
Human arterial ring assay	Human umbilical arteries are isolated from umbilical cords, sectioned into rings, and then embedded in extracellular matrix. Tubular structures are quantified by image analysis	Provides a three-dimensional system for identification of genes and drugs that regulate human angiogenesis	Seano et al., 2013
Retinal explant assay	Explanted retina is cut and placed, over a three-dimensional gel with the photoreceptor layer facing upward. Endothelial cell sprouting is observed from day 3 and peaks at day 7	Allows the study of tip endothelial cell angiogenic responses and acute responses of retinal blood vessels at the sprouting front	Rezzola et al., 2014
Fat-tissue microfragment assay	Human subcutaneous fat tissue is fragmented and embedded in fibrin. Blood vessel growth and elongation is examined after 15 days by microscopy	Uses intact human fat tissue with quiescent vessels from which other spontaneously derive. Assay could help predict response toward a treatment	Greenway et al., 2007
Choroid sprouting assay	The choroid, a vascular bed beneath the retinal pigment epithelium, is separated from the retina, segmented, and placed over a matrix. Outgrowth of vascular sprouts can be observed within 2-6 days.	Vascular sprouts consist of endothelial cells, pericytes and macrophages. Robust, reproducible and representative model of microvascular angiogenesis Semi-automated software for quantification of sprouting area is available	Shao et al., 2013

rat and guinea pig (Ziche and Morbidelli, 2015). It has been further developed to become quantitative, by incorporation of a contrast-dye such as high molecular weight dextran and imaging analysis. Disadvantages are that it is rather expensive, and that the angiogenic process is rather atypical, since it occurs in a non-vascular environment (Norrby, 2006).

Chorioallantoic Membrane (CAM) Assay

The CAM assay allows the measurement of both inhibition and stimulation of angiogenesis over the vascularized chorioallantoic membrane of a chick embryo, which can develop normally after carefully opening the egg shell to create a window (*in ovo*), or being placed in a cup outside of the egg shell (*ex ovo* or *in vitro*), in order to get access to the CAM. From days 3.5 to 10 after fertilization, highly proliferative and immature endothelial cells rapidly grow a sprouting vascular network, which is then replaced by intussusceptive microvasculature (Ribatti et al., 2001). During early phases, the CAM assay is most suitable to study angiogenic inhibitors. In contrast, the study of pro-angiogenic factors is best accomplished from day 6 to 8, when the rapid embryonic angiogenic development has slowed down. Quantification of angiogenesis is typically based on the directionality of the blood vessels toward the graft/angiogenic stimuli, the number of sprouts, and/or the size/length of the stimulated blood vessels. The CAM assay allows repeated visualizations of the angiogenic process, and it is fast and cost effective, making it suitable for large scale screens. Its major disadvantages are the rather challenging quantification of the outcome, since it is often difficult to distinguish normal angiogenesis from the induced one, and the false positive effects that often occur from inadvertently damaging the CAM (Ribatti et al., 2001; Ribatti, 2008).

Matrigel Plug Assay

Subcutaneous injection of matrigel in mice is a common method to study angiogenesis *in vivo* in mammals. Matrigel is an extract of the Engelbreth–Holm–Swarm tumor, mostly composed by extracellular matrix proteins and growth factors (Benton et al., 2014). When cold, matrigel is liquid, but becomes solid at body temperature. This property makes simple the injection of matrigel in the midventral abdominal region of mice, where it quickly solidifies forming a "plug" (Akhtar et al., 2002). The injected matrigel can be supplemented with either angiogenic inhibitors or inducers. Then, usually about 2 weeks after injection, infiltration of new blood vessels is determined histologically. A major advantage of this method, is the simplicity to implement it. However, visualization and quantification of differences can be challenging and are mainly based in the histological analysis of explanted plugs at a final experimental point.

Hind Limb Ischemia

A common system to study angiogenesis *in vivo* from a therapeutic perspective, is the hind limb ischemia (HLI) model (Limbourg et al., 2009). In this case, the femoral artery of mice is ligated causing a strong obstruction of blood flow toward the hind limb. Since originally described (Couffinhal et al., 1998), the HLI protocol has been applied with multiple variations. A common surgical approach is the ligation of the femoral artery at distal and proximal sites, and removal of the intervening arterial fragment (Fierro et al., 2011). Another approach is a single ligation, without arterial excision, where the severity of ischemia depends on the specific site of ligation. Also a gradual arterial occlusion model has been established, by placing ameroid constrictors on the femoral artery (Yang et al., 2008). In all cases, the contralateral hind limb is left intact, as a control. Mice are usually able to recover from this injury naturally, restoring blood flow within approximately 4 weeks, by mechanisms including the formation or enlargement of collateral blood vessels (Sondergaard et al., 2010). Laser scanning Doppler imaging is the best suited method to monitor blood flow restoration upon HLI induction, because it is non-invasive, and can be performed in the same animal at multiple time points. At the end of the experiment, animals can be euthanized for further investigation including histology and gene expression analysis. A negative aspect of scanning Doppler imaging is the sensitivity of the method, since only robust differences can be noticed. Another limitation of this method is that it fails to reveal the exact mechanism underlying the blood flow restoration (e.g., angiogenesis vs. vasculogenesis).

Vascularization during Dermal Wound Repair

Our group has developed a full skin defect model that presents several advantages compared to the *in vivo* models presented above, which are intrinsic to the nature of skin. Among others: transparency, large surface, easy manipulation, external location and tissue homogeneity (Egaña et al., 2008). In this model, full skin defects are surgically created bilaterally on the back of mice, and the skin excision is replaced by biodegradable scaffolds, which can be modified to contain a specific angiogenic stimuli. Typically, after two weeks animals are euthanized, and tissue vascularization is quantified as follows: the skin, including the implanted scaffold, is removed and quickly placed over a light source. During trans-illumination, a digital picture is taken, and is later analyzed by digital segmentation (Schenck et al., 2014). This method does not affect cell integrity post mortem, allowing further analysis such as histology or protein/RNA extraction.

The Skinfold Chamber and Ear Assays

Four major types of *in vivo* models have been developed to observe the angiogenic process in two dimensions: the rat mesentery window assay (Norrby, 2011), the hamster cheek pouch assay (Monti-Hughes et al., 2015), the dorsal skinfold chamber adapted to mice, hamsters and rats (Lehr et al., 1993; Harder et al., 2014; Irvin et al., 2014), and the rabbit ear chamber assay (Clark et al., 1931; Ichioka et al., 1997). These techniques, developed as early as in the 1940s, rely on semi-transparent tissue or the implantation of a transparent chamber that allows an easy and direct visualization and quantification of the angiogenic process, including blood vessel density and blood flow velocity. In particular, the implementation of intravital microscopy along with epifluorescence, confocal and multiphoton techniques, offers the possibility of repetitive, direct, and quantitative measurements of several microcirculatory parameters, as well as microvasculature imaging at an unparalleled subcellular-resolution (Taqueti and Jaffer, 2013). However, these methods are invasive, and may cause great discomfort to animals. In addition, some methods such as the implantation of a dorsal window chamber in mice, are cumbersome (Palmer et al., 2011) and therefore difficult to implement in a number of animals sufficient for adequate technical replicates.

ZEBRAFISH AS A MODEL FOR ANGIOGENESIS RESEARCH

While the models described above have provided essential information and platforms for discovery of therapeutic targets and drugs, many questions about the biology of vascular cells and how they build the circulatory system remain unresolved. Above all, the relevance of the models is often hindered by the inaccessibility of the tissue in live animals, and much of what we know has been derived from fixed material or indirect assays. Zebrafish provides a series of advantages as a model of study due to its rapid development, optical transparency, high number of offspring and straightforward strategies for forward and reverse genetic manipulation. Furthermore, the early development of a cardiovascular system in the transparent zebrafish embryo and larva translates into a unique opportunity for direct observation of blood flow and the development of the system's related organs in both wild type and transgenic fish, without the need for complex instrumentation. Lastly, genetic studies have revealed conservation of the molecular pathways between fish and mammals making research in vascular biology in teleosts directly translatable into potentially relevant information for human health.

As the restrictions on the experimental use of mammalian models for research increase, the zebrafish emerges as a convenient alternative. Larvae can be used in massive numbers in genetic or pharmacological screens, at stages in which they lack the legal status of a "vertebrate animal" yet have all of the physiological functions of the adult, including a hematovascular system. Circulation begins 24 h after fertilization, with a simple, yet functional blood circuit. The embryos and larvae, can be kept for the first five days of life in small wells in microtiter plates, in only a few hundred microliters of water. This is the pharmacologists dream since as many replicates of the experiment as one desires can be done and dilutions of each drug can be tested *ad libitum*.

Two decades ago, the generation of the first stable transgenic zebrafish line was reported. Since then, hundreds of transgenic lines have been developed both for expression of reporter proteins or for expression of diverse proteins for functional

studies (Udvadia and Linney, 2003). At the same time, efficient mutagenesis protocols have allowed forward-genetic screening in the context of angiogenesis, generating valuable collections of mutants (Jin et al., 2007). Traditionally, gene function in zebrafish has been assessed using chemically or insertionally induced mutants that required large scale unbiased screens to identify phenotypes related with the process or organ of interest (Gaiano et al., 1996; Haffter et al., 1996). While common antisense technologies were not generally applicable to the zebrafish, the advent of oligonucleotide substitutes named morpholinos, enabled the knockdown of endogenous genes by either blocking translation of the mRNA or splicing of the pre-mRNA (Nasevicius et al., 2000). The ease of this technology spurred its widespread use, even though it presented some limitations such as the induction of undesired off-target effects or the progressive loss of the effect at late developmental stages because of diminishing activity over time. The zebrafish toolkit has been recently enriched with the introduction of gene editing technologies such as TALENs (Transcription activator-like effector nucleases, Bedell et al., 2012), and CRISPR (Clustered regularly-interspaced short palindromic repeats)-Cas based strategies (Hwang et al., 2013). As long as genomic sequence is available for the targeted locus, any gene can be mutated efficiently and permanently in the germ line; the efficiency is often high enough such that recessive phenotypes can be seen already in the injected animals. Further, the CRISPR-Cas9 system has been adapted for high throughput mutagenesis in zebrafish so that dozens of genes can be mutated in a single experiment (Varshney et al., 2015). Recently, phenotypic inconsistencies between genomic mutations induced by CRSPR-Cas9 and knockdown via morpholinos have emerged (Kok et al., 2015). It is likely that these two gene loss-of-function strategies differ in their penetrance given that genetic lesions might induce compensatory reactions in the genome obscuring the gene's function. Many authors believe that a combination of strategies is desirable when analyzing a particular gene and that it is unwise (as has been agreed by communities using other model organisms) to rely only on a gene knockdown phenotype to assign gene function (Lawson, 2016).

Despite its success and popularity, those working with the zebrafish model must consider complementing their studies with mammalian systems, if they wish to validate the knowledge gained for potential clinical applications. Gene and protein functional conservation is high, but not absolute, and obviously there are important physiological differences to be dealt with. Aquatic and terrestrial life pose unique challenges that impact on many organs, most notably the respiratory system and, thus, cardiovascular architecture. In fish, only the embryo and larva are transparent, making studies in adults just as difficult as in mammals. The small size of embryos makes some observations challenging (i.e., requiring sophisticated microscopy and imaging) and they are also developing systems, which means they are constantly in a state of change and growth. Thus, the zebrafish, with all of its attributes, should be considered a starting point for discovery and a model that can offer new hypotheses to be tested further in other models.

Vascular Development in Zebrafish

Transgenic technology has enhanced the inherent *in vivo* imaging capabilities that zebrafish larvae may offer to the investigator. Though vessels and blood flow can easily be visualized with a simple dissecting scope, it was with the introduction of tissue specific expression of fluorescent proteins that vascular and blood development could be examined in great detail. Confocal microscopy and time lapse imaging can both be carried out with live specimens which allows detailed morphogenetic movements and cell shape changes to be followed directly. Thus, vascular development has been described in great detail, both from the anatomical and cellular point of view and with a comprehensive examination of the molecular players involved (reviewed by Gore et al., 2012; Schuermann et al., 2014).

Most of the strategies which have been followed to create stable transgenic lines with vascular-specific phenotypes are based on gene-specific promoters. Both autologous and heterologous promoters have been shown to work. **Table 3** lists some of the transgenic lines, which have been designed and developed for the visualization and analysis of the vascular system. Before a complete and reliable zebrafish genome sequence was available, the promoter of a related gene from another species, most commonly a mammalian one (Baldessari and Mione, 2008), was used. However, the reporter protein expression in zebrafish did not always exactly recapitulate that of the orthologous one, because of the differences in promoter elements among species. For example, the zebrafish Tg(tie2:GFP)s849 line encoding the promoter for the murine tie2-gene (a vascular-specific tyrosine kinase receptor activated by angiopoietin ligands), successfully drove GFP expression in endothelial cells, but also showed substantial nonvascular expression in the hindbrain and the posterior neural tube, and the overall level of expression was proportionally lower compared to that in mice (Motoike et al., 2000). On the other hand, the fli1a and scl zebrafish genes, have been used as early markers of vascular and hematopoietic lateral mesoderm. While the expression of fli1a is restricted to endothelial cells, a subset of early circulating myeloid cells, and cranial neural crest derivatives (Brown et al., 2000), the expression of scl is specific for the hematopoietic lineage at later stages (Gering et al., 1998).

The development of the vascular anatomy of the zebrafish has been extensively described and has been proven to share high similarity with other vertebrates (Isogai et al., 2001; Ellertsdóttir et al., 2010; Gore et al., 2012). Many of the studies on vascular development have been achieved by using molecular tracers during the early embryonic stages of zebrafish. One of such strategies is the injection of fluorescent microspheres, and their detection after lumenization and anastomosis of the vascular network is complete (Küchler et al., 2006). This strategy has also been used to compare the development of blood and lymphatic vasculature in zebrafish (Coffindaffer-Wilson et al., 2011). Transgenic zebrafish lines have been also employed to track individual cell growth during vascular development. Using fluorescent endothelial cell markers, it is possible to observe the proliferative and migratory behaviors of single

TABLE 3 | Transgenic zebrafish lines generated for the study and visualization of the vascular system.

Line	Gene	Expression	References
Tg(5xUAS:cdh5-EGFP)	*VE-cadherin*	Pan- endothelial	Lenard et al., 2013
Tg(-7.8gata4:GFP)ae3	*Transcription factor GATA-4*	Endocardial and myocardial cells	Heicklen-Klein and Evans, 2004
Tg(dll4:EGFP)	*Notch ligand*	Endothelial cells	Sacilotto et al., 2013
Tg(efnb2a:EGFP)	*Ligand of Eph- receptor*	Artery	Swift et al., 2014
Tg(fli:eGFP)y1	*Transcription factor Fli-1*	Endothelial cells, cytoplasmic	Lawson and Weinstein, 2002a
Tg(fli1:neGFP)y7	*Transcription factor Fli-1*	Endothelial cells, nuclear	Roman et al., 2002
Tg(flt4:YFP)	*Vegfr3*	Pan-endothelial	Hogan et al., 2010
Tg(gata1:dsRed)sd2	*Transcription factor GATA-1*	Blood cells	Traver et al., 2003
Tg(gata1:GFP)	*Transcription factor GATA-1*	Erythroid lineage	Long et al., 1997
Tg(gata2:eGFP)	*Transcription factor GATA-2*	Blood cells	Traver et al., 2003
Tg(hsp70l:canotch3-EGFP)	*Notch3 intracellular domain*	Perivascular	Wang et al., 2014
Tg(kdr.eGFP)s843	*Vegfr2/flk1/kdr/Vegfr4*	Angioblast/endothelial precursors	Jin et al., 2006
Tg(kdr:G-RCFP)	*Vegfr2/flk1/kdr*	Angioblast/endothelial precursors	Cross et al., 2003
Tg(kdr:RFP)la4	*Vegfr2/flk1/kdr*	Angioblast/endothelial precursors	Huang et al., 2005
Tg(my17:eGFP)	*Cardiac myosin light chain 2*	Myocardial cells	Ho et al., 2007
Tg(nkx2.3:efnb2a,myl7:EGFP)	*Ligand of Eph- receptor*	Artery	Choe and Crump, 2015
Tg(scl-α:DsRed)	*Transcription factor Tal-1*	Endothelial cells (intermediate)	Zhen et al., 2013
Tg(scl-β:d2eGFP)	*Transcription factor Tal-1*	Endothelial cells (anterior-posterior)	Zhen et al., 2013
Tg(Tie2:eGFP)	*Tie-2 receptor tyrosine kinase*	Endothelial cells	Motoike et al., 2000
TgBAC(cdh5:Citrine)	*VE-cadherin*	Pan- endothelial	Bussmann and Schulte-Merker, 2011
TgBAC(cdh5:GAL4FF)	*VE-cadherin*	Pan- endothelial	Bussmann et al., 2011
TgBAC(dll4:GAL4FF)	*Notch ligand*	Endothelial cells	Hermkens et al., 2015
TgBAC(flt4:Citrine)	*Vegfr3*	Pan-endothelial	Gordon et al., 2013
Tg(0.8flt1:RFP)hu5333	*Flt1*	Strong expression in arterial ISV	Bussmann et al., 2011

Adapted from Baldessari and Mione (2008), Kamei et al. (2010) and Schuermann et al. (2014).

cells, and different kinds of cell types during the embryo-to-larva transition. Combining transgenic lines expressing different fluorescent proteins, it was possible to observe two cell types simultaneously. For instance, it was possible to track both endothelial progenitors and erythrocytes while following the vascular network development and the initiation of blood circulation (Lawson and Weinstein, 2002a,b; Herwig et al., 2011; Kimura et al., 2013). Moreover, combining nuclear and cell membrane specific fluorescent tags has allowed the examination of single cell morphological dynamics in living larvae during vessel formation (Yu et al., 2015). Finally, the development of stable transgenic zebrafish lines has been a valuable resource for tissue specific gene expression as well as inducible gene expression (Udvadia and Linney, 2003). The implementation of these strategies enabled the study of the sequence of events involved in the establishment of the first circulatory loop in zebrafish embryos, which consists in the connection between the heart with the dorsal aorta and the cardinal posterior vein back to the heart. Other blood vessels, which are characteristic and highly accessible in the zebrafish embryos and larvae are the intersegmental vessels, which emerge from the dorsal aorta into the embryonic trunk and tail, and later grow into the anastomosing dorsal longitudinal vessels (Strilić et al., 2009).

A remarkable feature of zebrafish compared to other vertebrates, is that they rely on passive oxygen diffusion during the early embryonic stages rather than oxygen perfusion, as the completion of the vascular development takes place after hatching. Moreover, the generation and characterization of zebrafish mutants has shown that embryos are able to sustain normal development even in absence of a functional vascular system or in the absence of blood (Stainier et al., 1995; Isogai et al., 2003). This attribute has made the analysis of late phenotypes related to circulatory system malformations possible, whereas they are lethal and hence impossible to study in living mammals (reviewed by Isogai et al., 2001; Wilkinson and van Eeden, 2014). A prime example of the power of the genetic approach was the study of the zebrafish *gridlock* mutant (Peterson et al., 2004). The *gridlock* mutation causes a syndrome similar to human aortic coarctation disrupting blood flow in the aorta. Further, mutant animals were used to design a small molecule screen that would detect reversal of the phenotype upon treatment and several compounds were found to have such an effect. **Table 4** summarizes some of the most remarkable vascular zebrafish mutant lines described thus far.

Finally, experimental analysis of blood vessels during zebrafish development has also relied on common techniques for visualizing gene and protein expression. In order to observe the expression of endogenous genes in zebrafish embryos and larvae, two methods are available: *in situ* hybridization and immunohistochemistry. While neither of these methods was specifically developed for the zebrafish vasculature studies, an increasing number of tools and protocols are becoming available that facilitate these strategies (Kamei et al., 2010; Thisse and Thisse, 2014).

TABLE 4 | Zebrafish vascular mutants.

Line	Gene	Phenotype	References
cloche	*scl, lmo2, gata1, gata2, flt1, flt*	Lack endothelial and circulating blood cells	Stainier et al., 1995
glass onion/parachute	*cdh2*	Neuronal-cadherin (N-cadherin/Cdh2)-deficient zebrafish show dysmorphic vascular network	Bagatto et al., 2006
gridlock	*hey2*	Lack trunk and tail circulation due to reduced arterial gene expression and improper assembly of the dorsal and lateral aortae	Lawson et al., 2001
heart of glass	*heg*	Morphological cardiovascular defects	Mably et al., 2003; Kleaveland et al., 2009
kurzschluss	*unc45a*	Branchial arteries fail to form properly. Arterial-venous shunts lead to loss of circulation in the trunk	Chen et al., 1996
lmo2	*lmo2*	Abnormal ocular blood vessels cause failure of optic fissure closure	Weiss et al., 2012
mindbomb	*notch5*	Mutants are defective for Notch signaling, exhibit arterial-venous shunts, defective PCV formation, and reduced arterial gene expression	Lawson et al., 2002
out-of-bounds	*plexnD1*	Display premature sprouting and mispatterned growth of the trunk intersegmental vessels due to loss of semaphorin–plexin signaling pathway	Childs et al., 2002
plcgy10	*plcg1*	Deficient in VEGF-mediated angiogenesis and arterial differentiation	Lawson et al., 2003
santa	*ccm1*	Severe dilation of major blood vessels, followed by a thinning of cell walls	Mably et al., 2006
schwentine	*flk1*	Loss of angioblasts and failure to undergo angiogenesis	Habeck et al., 2002
Segmental artery mutants	*kdrl, plcg1, plexinD1, etsrp*	Vascular mutants identified by haploid transgenic screening show defects in Vegf/Plcg1 signaling	Covassin et al., 2009
sonic you	*shh*	Defects in trunk circulation due to abnormal arterial differentiation	Lawson et al., 2002
stalactite	*mtp*	Mutant shows excessive sprouting angiogenesis due to loss of apolipoprotein-B regulation	Avraham-Davidi et al., 2012
tie2-hu1667	*tie2*	Enhancement of junctional integrity via VE-cadherin	Gjini et al., 2011
valentine	*ccm2*	Altered endothelial junctional integrity causes dilation of major vessels.	Mably et al., 2006
ve-cadherinubs8	*cdh5*	Failure to form established junctions during anastomosis	Lenard et al., 2013
VEGF-receptor mutants	*flk1*	Mutants identified in a forward genetic screen show disrupted blood vessels sprouting of normal angioblasts	Habeck et al., 2002
vhl	*vhl*	Increased VEGF-signaling induces aberrant angiogenic sprouts and retinal neovascularization	van Rooijen et al., 2009
violet beauregarde	*alk1*	Mutants develop severe edema, associated with an abnormal blood circulation and improper arterial-venous connections	Roman et al., 2002

Adapted from Lagendijk et al. (2014) and Wilkinson and van Eeden (2014).

Vascular Regeneration

The zebrafish is a broadly known model for studies on tissue regeneration. In this regard, its capacity to regenerate its organs and limbs is remarkable even in adult stages. The caudal fin, in particular, provides an ideal tissue for studies related to vascular regeneration in adult zebrafish due to its simple thin architecture and relative transparency (Poss et al., 2003). While caudal fin regeneration in zebrafish larvae takes a few days, it has been demonstrated that the adult caudal fin is capable of full regeneration after successive amputations within a couple of weeks (Azevedo et al., 2011). The caudal fin amputation model has been extensively used to study the orchestration of the mechanisms involved in regeneration, such as cell differentiation, migration and patterning, which lead to the restoration of the fin's original morphology and functionality (Pfefferli and Jaźwińska, 2015). In a landmark study, Xu et al. (2014) showed that regenerating vessels in the regenerating tail fin originate from vein-derived cells that acquire angiogenic potential. These cells migrate singly or collectively and organize into vessel in response to chemokine signaling (reviewed by Hasan and Siekmann, 2015). However, the applicability of this model to the study of vascular regeneration could be much more widely exploited. For instance, the ablation of single vessels or vessel interruption has not been addressed in the zebrafish. A new technique called electroablation (Moya-Díaz et al., 2014) has been shown to be useful for inducing small tissue lesions including blood vessel ablation in the adult tail fin.

The zebrafish larval vascular network has been subject of numerous screens over the past decade. Key to this effort was the development of the Tg(fli1:EGFP)y1 transgenic line (Lawson and Weinstein, 2002a), that fluorescently labels endothelial cells throughout life (**Figure 1**) and enables the visualization of the microvasculature in this tissue. However, most screens to date have used the larval vasculature to find molecules that disrupt (positively or negatively) the normal pattern of blood

vessels. Only a few screens have examined the role of the vasculature on tissue regeneration, even though these transgenic fish could be a remarkable tool to allow the study of the effects of test substances and genetic interference on vessel growth and restoration. As an example, Bayliss et al. examined the requirement for blood vessels in caudal fin regeneration using adult fish (Bayliss et al., 2006). In this work, the authors conclude that up to ∼1 mm avascular caudal fin tissue can be regenerated, though, for regeneration of the full limb, angiogenesis is required. Further, they showed that the model can be implemented for antiangiogenic drug screening, as it is possible to selectively inhibit highly active, abnormal vessels while leaving quiescent vessels intact.

Since angiogenesis is one of the main focuses of vascular regeneration research, models for this type of vascular development and growth have been developed. In the embryo, the intersegmental vessels form by angiogenic sprouting from the dorsal aorta and have been the target of studies using drugs or genetic perturbations (Schuermann et al., 2014). Further, since it has been shown that mammalian malignant cells can be xenotransplanted into zebrafish embryos and that they can form tumors (Haldi et al., 2006), models for tumor angiogenesis have been developed (Tobia et al., 2011). We have also shown recently (Chávez et al., 2016), that angiogenic sprouting can also be induced by xenotransplantation of cells expressing the recombinant vascular endothelial growth factor (VEGF), in this case plant cells. Thus, the factors governing angiogenic growth and inhibition are amenable to be examined *in vivo* in these contexts.

High Throughput Screens

As previously mentioned, zebrafish larvae are optically transparent until 5 days after fertilization allowing direct observation of internal tissues. This feature, coupled with the use of transgenic zebrafish lines with fluorescently labeled organs and cells, has allowed for straightforward assays to be developed to assess either positive or negative effects of chemicals or genetic perturbations on vascular integrity (Raghunath et al., 2009; Taylor et al., 2010). For instance, by using transgenic lines in a genetic screen, numerous vascular-specific mutations were identified (Covassin et al., 2009), while a chemical screen has revealed compounds that restored a normal phenotype in mutant fish (Hill et al., 2005; Asnani and Peterson, 2014).

How relevant are drug screens carried out in fish to human biology? As most human genes have a fish ortholog and sequence conservation is high, most teleost proteins targeted by drugs will predict an effect on its human counterpart (Tran et al., 2007). The relevance of this type of approach is highlighted by the fact that several small molecules identified in zebrafish are currently in clinical trial phase (MacRae and Peterson, 2015). Furthermore, these assays can be scaled into high throughput screens due to the fact that the zebrafish larvae, 2–3 mm at 3 days post-fertilization, can be arrayed into microwell plates and examined manually or automatedly by the thousands. Large chemical libraries can be screened for direct effects on the tissue of interest as compounds readily permeate the animal, and minimal amounts of each compound are required (drugs are supplied diluted in only a few ml in aqueous solution). The readout can be exceedingly simple: usually a perturbation of the normal or expected anatomical structure or cellular behavior is sought. While it is possible to visually screen hundreds of fish for a phenotype as it has been classically done (i.e., double blind scoring), there are automated and semi-automated systems for image acquisition and analysis as well as software that can quantitatively detect subtle effects (Pardo-Martin et al., 2010; Tamplin and Zon, 2010).

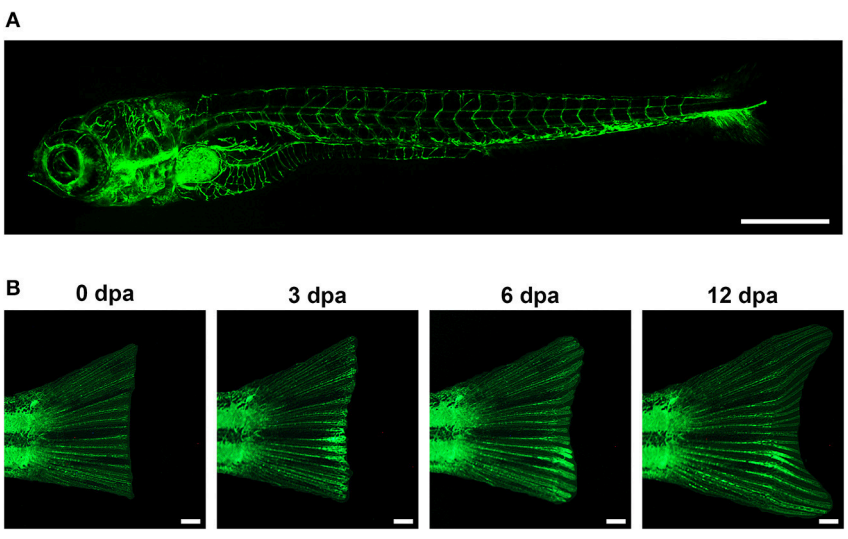

FIGURE 1 | In the transgenic zebrafish line Tg(fli1:EGFP)^{y1}, the promoter for the endothelial marker *fli1* drives the specific expression of EGFP in blood vessels. This allows the visualization, and hence the analysis of the vasculature during zebrafish embryonic development (**A**, Lawson and Weinstein, 2002a), and during adult vessel regeneration upon tail fin amputation (**B**, Huang et al., 2003). Scale bar represents 500 μm in **(A)**, and 1 mm in **(B)**.

CONCLUSIONS AND PERSPECTIVES

Since the 1990s experimentation on animals has increasingly emphasized the "three Rs": reduction (minimize the number of animals), refinement (maximize the amount of data obtained) and replacement, (substitute with *in vitro* studies, when possible; Mayer et al., 1994). Here, we have enumerated a series of alternative models for the study of vascular development and regeneration. *In vitro* studies are accessible and offer controlled conditions for manipulation, but they lack the complexity found in living tissues. As mammalian models present the closest substitutes for humans, they should be preferred as the final validation step when proposing a therapy. However, these organisms can only be used in small numbers due to the cost, cumbersomeness of the experimental designs and ethical concerns. We describe the zebrafish model as an attractive alternative because it combines the relevance of *in vivo* assays with the simplicity and versatility of *in vitro* assays. In larvae, access to the developing vasculature is straightforward thanks to fluorophore-tagged strains and the small size of the animals makes the use of high-throughput strategies possible. In adults, the tailfin is equally convenient as a model tissue as regenerating vessels are directly observable at all stages and the animals are suitable for experimental manipulation with compounds, for instance. The advent of new genome modification techniques opens up even more tools for the vascular biologist as new therapeutic targets can be identified through mutational analysis.

AUTHOR CONTRIBUTIONS

MC, GA, FF, MA, and TE all contributed to the conception of this manuscript, as well as to the acquisition and critical analysis of the intellectual content reviewed in this work. MC and GA were responsible for generating the images shown in **Figure 1**. The authors declare no potential conflict of interest, and approve this manuscript as the final version to be published.

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How Necessary is the Vasculature in the Life of Neural Stem and Progenitor Cells? Evidence from Evolution, Development and the Adult Nervous System

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Augmenting evidence suggests that such is the functional dependance of neural stem cells (NSCs) on the vasculature that they normally reside in "perivascular niches". Two examples are the "neurovascular" and the "oligovascular" niches of the adult brain, which comprise specialized microenvironments where NSCs or oligodendrocyte progenitor cells survive and remain mitotically active in close proximity to blood vessels (BVs). The often observed co-ordination of angiogenesis and neurogenesis led to these processes being described as "coupled". Here, we adopt an evo-devo approach to argue that some stages in the life of a NSC, such as specification and commitment, are independent of the vasculature, while stages such as proliferation and migration are largely dependent on BVs. We also explore available evidence on the possible involvement of the vasculature in other phenomena such as the diversification of NSCs during evolution and we provide original data on the senescence of NSCs in the subependymal zone stem cell niche. Finally, we will comment on the other side of the story; that is, on how much the vasculature is dependent on NSCs and their progeny.

Keywords: neural stem cells, vasculature, blood vessels, neurogenesis, proliferation, differentiation, migration, stem cell niche

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INTRODUCTION

The observation that neurogenesis and angiogenesis are seasonally coordinated in the brain of songbirds (Louissaint et al., 2002) produced the first evidence on the existence of a cross-talk between neural stem cells (NSCs) and blood vessels (BVs). More recently, it was shown that endothelial cells control the function of adult brain NSCs via direct cell contact and diffusible signals (Ottone and Parrinello, 2015). But is this the truth and nothing but the truth? The first neurons and glia appeared in animals that had no vasculature (Satterlie, 2015) and in early neurodevelopmental stages of mammals NSCs emerge and form the neural tube in the absence of vascularization. This strongly suggests that NSCs can exist and function in the absence of BVs and raises the challenging question: how much does the existence and the function of NSCs depend on the vasculature?

To address this question in a systematic and comprehensive way we defined the major functional stages in the life of a NSC, informed both by evolution and development

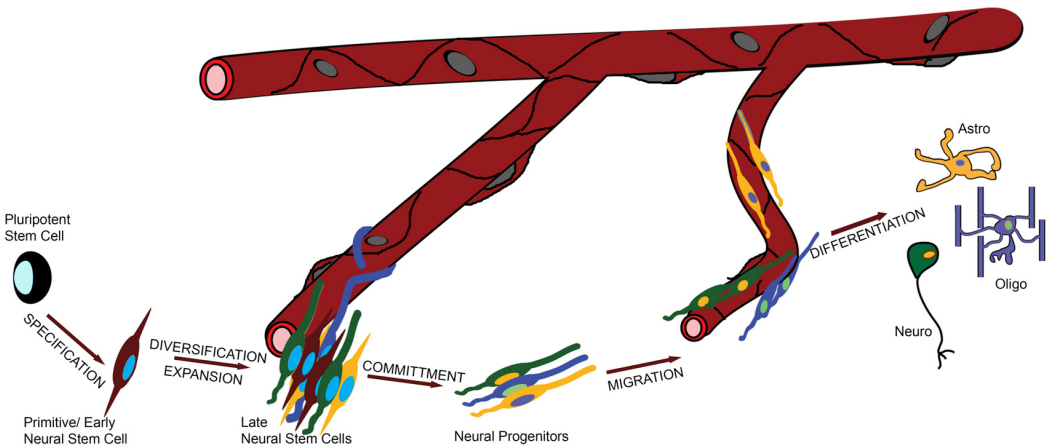

FIGURE 1 | The role of the vasculature in the life of a neural stem cell (NSC). In this graphic illustration, the different stages in the life of a NSC are shown and the involvement of the vasculature is depicted by the distance of the cells from the blood vessels (BVs). For example, specification and commitment of NSCs appear to happen away from the vessels, whilst proliferation and migration in close proximity.

(**Figure 1**): (i) specification of NSC identity from earlier pluripotent—e.g., embryonic or ancestral stem cells; (ii) proliferation/diversification, during which the pool of NSCs is expanded and becomes heterogeneous; (iii) commitment to a specific fate (such as neuronal or glial); (iv) migration and finally; (v) differentiation. During stage (iii) NSCs become, or give rise to, neural progenitors, and overall migration can be minimal or absent.

GENERALIZATIONS

The *In Vitro* Life of NSCs

Neural stem cell culture protocols (iPSCs, primary cells, cell lines) have proven that all stages in the life of a NSC can be recapitulated *in vitro*. Embryonic or induced stem cells can be programmed to adopt NSC fate and can be differentiated into a range of neuronal and glial cell types. This, obviously, does not exclude the possibility that BVs or their ancestral systems are necessary *in vivo*, especially as cell culture media are rich in components that are provided by BVs in the tissue. Endothelial cells have been found to enhance neurogenesis in many cell culture assays, but few studies have gone the extra mile to directly link these *in vitro* results to the role of endothelial cells in the live organism (Shen et al., 2004; Androutsellis-Theotokis et al., 2010).

Main Mechanisms of NSC-BV Interaction

NSC-BV interaction can be achieved through three different, but possibly co-operating, mechanisms. First, via direct contact between NSCs and BV components, such as endothelial cells and perivascular extracellular matrix (Javaherian and Kriegstein, 2009; Ottone et al., 2014). Second, via diffusible signals generated by vascular and perivascular cells, such as in the case of endothelium-derived neurotrophin-3 (Delgado et al., 2014). Third, via diffusible signals that BVs transport but don't generate themselves. In small organisms, such as planarians, nutrients can be diffused directly from the environment, and in insects NSCs are directly bathed in the hemeolymph (Limmer et al., 2014; Spéder and Brand, 2014). In larger animals blood circulation is required for necessary factors to reach their target areas. One such example, crucial for NSCs, is insulin (Masjkur et al., 2012). Recently we have identified a possible fourth mechanism, in which the function of NSCs is controlled by platelets (a circulatory element), possibly via active mediation by endothelial cells (elements of the BV structure; Kazanis et al., 2015).

What is the Nature of a NSC?

It remains challenging to define what a NSC is and when it is reduced to neural progenitor status (exhibiting a more restricted potential). Here, we adopt an extended version of the unified hypothesis (Alvarez-Buylla et al., 2001), according to which the cardinal NSC properties are found equally in primitive/early NSCs with a neuroepithelial-like phenotype, in more developed cells with a radial glial phenotype and in some species (mostly in mammals), in mature cells with an astroglial phenotype (**Figure 1**). A surprising deviation was recently reported in the adult crayfish brain, in which the neurogenic stem cell pool does not contain *bona fide* NSCs but is constantly replenished from the hematopoietic system. Vascular extensions of the cerebral artery facilitate this process and this is an intriguing example of vessel-dependent support of neurogenesis (Chaves da Silva et al., 2013; Benton et al., 2014).

THE ROLE OF THE VASCULATURE

Specification

The *in vitro* (e.g., from iPSC differentiation or trans-differentiation experiments) and *in vivo* (e.g., from early embryonic developmental stages, or from evolutionary evidence based on zoological observations) data currently available indicate that the specification of pluripotent stem cells towards

a neural identity does not depend on any form of interaction with some type of vasculature. The first neuron-like sensory cells and primitive nervous systems appeared in species that lacked BVs (Jékely et al., 2015) and the specification of neuroectoderm and of neuroepithelial cells in mammals marginally precedes the angiogenic specification of mesoderm in the forebrain (Vasudevan et al., 2008; Javaherian and Kriegstein, 2009). Nevertheless, after the closure of the neural tube, the primitive neuroepithelium remains in contact with a CSF-like fluid that is partly constituted by the early BV network of deeper layers (Lun et al., 2015).

Proliferation/Diversification

As soon as NSCs become specified the processes of self-renewal and expansion are initiated. Low levels of proliferation, adequate for the generation of single neurons and glia and for the construction of primitive neuronal networks and of the early neural tube, can occur in the absence of vascularization (Rodriguez Celin et al., 2015). The emergence of larger and more complicated nervous systems required higher levels of proliferation in embryonic neural stem and progenitor cells, and this was partly achieved through their diversification. The evolution of the neocortex was facilitated by the appearance of radial glial-type NSCs (see "What is the Nature of a NSC?" Section) that exhibit high self-renewing potential and generate transit amplifying progenitors that significantly increase the cell-generation capacity per initial NSC. Gyrenecephalia—mainly observed in primates—is also correlated with the addition of outer subventricular zone progenitors (Florio and Huttner, 2014). This evolutionary process of expansion of the embryonic NSC pool through diversification has not been cell-autonomous, with the embryonic microenvironment (the extracellular matrix, for example) playing a crucial role (Garcion et al., 2004; Loulier et al., 2009; Fietz et al., 2012; Pollen et al., 2015). However, only limited evidence exists to suggest a contribution of the vasculature. The chicken germinal cortical zones remain largely a-vascular (Rodriguez Celin et al., 2015) and in mice, even though BVs appear at the time of expansion of the neuroepithelial cell pool, only transit amplifying neural progenitors proliferate in close proximity to them (Vasudevan et al., 2008; Javaherian and Kriegstein, 2009). Nevertheless, in both examples the long basal processes of radial glia remain in constant contact with BVs positioned deeper in the tissue (Vasudevan et al., 2008; Rodriguez Celin et al., 2015) a feature shared by adult NSCs (Mirzadeh et al., 2008).

In the postnatal mammalian brain active NSCs survive in specialized NSC niches (Kazanis, 2013) and accumulating evidence points to the vasculature as an important element of these microenvironments (Goldman and Chen, 2011). The BV bed in the NSC niche of the subependymal zone (SEZ-located at the lateral walls of the lateral ventricles) is different from all neighboring areas: the density of BVs is higher and vessels are positioned differently in respect to the plane of the ventricle (**Figures 2C,E**; Kazanis et al., 2010; Culver et al., 2013). Furthermore, BVs are more leaky (Tavazoie et al., 2008) and blood flow is slower, suggesting the existence of hypoxic conditions (Culver et al., 2013). This finding is consistent with reports revealing enhanced efficiency in culturing neural progenitors under hypoxia (Stacpoole et al., 2013). On the other hand, we have shown that in evolution—for example, when comparing rodent brains of different sizes—the number of adult NSCs that populate the niche correlates strictly with the number of ependymal cells and not with the volume of the niche that would reflect the volume of the vasculature (Kazanis and ffrench-Constant, 2012). This strengthens the hypothesis that during evolution/development the role of the vasculature becomes crucial for NSCs after they have been established in the system. Adult NSCs remain in a stage of quiescence (Doetsch et al., 1999). We have shown that NSCs are preferentially positioned next to the ependyma (Kazanis et al., 2010; Kazanis and ffrench-Constant, 2012), which produces pro-neurogenic signals such as noggin (Lim et al., 2000). However, recent experimental work revealed that NSC quiescence is controlled via direct cell-to-cell contacts with endothelial cells (Ottone et al., 2014) and via the activity of diffusible endothelium-derived factors such as neurotrophin-3, angiopoietins 1 and 2 and placental growth factor 2 (PlGF-2; Masjkur et al., 2012; Delgado et al., 2014; Crouch et al., 2015; see also reviews of Goldman and Chen, 2011; Ottone and Parrinello, 2015). In contrast, the mitotically active transit amplifying progenitors are physically located in close proximity to BVs and specifically in domains void of astrocytic endfeet and pericytes (Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). However, our work also indicates that the proximity to BVs cannot be the only factor controlling adult neural progenitor activity because within the narrow architecture of the niche mitotic cells are often positioned only at the side of BVs facing the lateral ventricle (Kazanis et al., 2010) while numerous proliferating progenitors can be also found away from BVs (**Figures 2A,B**).

In homeostatic conditions the majority of cells generated in adult niches die via apoptosis (Morshead and van der Kooy, 1992; Morshead et al., 1998). An alternative pathway is senescence: the exit from the cell cycle without differentiation. Senescence has not been properly investigated in adult NSCs, with the exception of one report on oligodendrocyte progenitor cells entering senescence during ageing (Kujuro et al., 2010). We and others have observed that a small fraction of adult NSCs show signs of senescence when cultured *in vitro* (**Figure 2**; Ross et al., 2008). We have also reported that senescent cells can be detected in the ventral domain of the SEZ even in young adult rats (Kazanis et al., 2013) and that in the same area normal mitotic activity and response of NSCs to injury are significantly weaker when compared to dorsal domains (Kazanis et al., 2013). Furthermore, the occurrence of senescent cells spreads dorsally over time (**Figure 2**), a phenomenon that seems to correlate with the gradual age-related shrinkage of the SEZ (Shook et al., 2012). So far there is no evidence that the BV network shows significant structural or functional variation among different domains of the niche (for example, in the ventral areas) or that this might be crucial in the occurrence of senescence. However, the observation that mitotically active progenitors are located

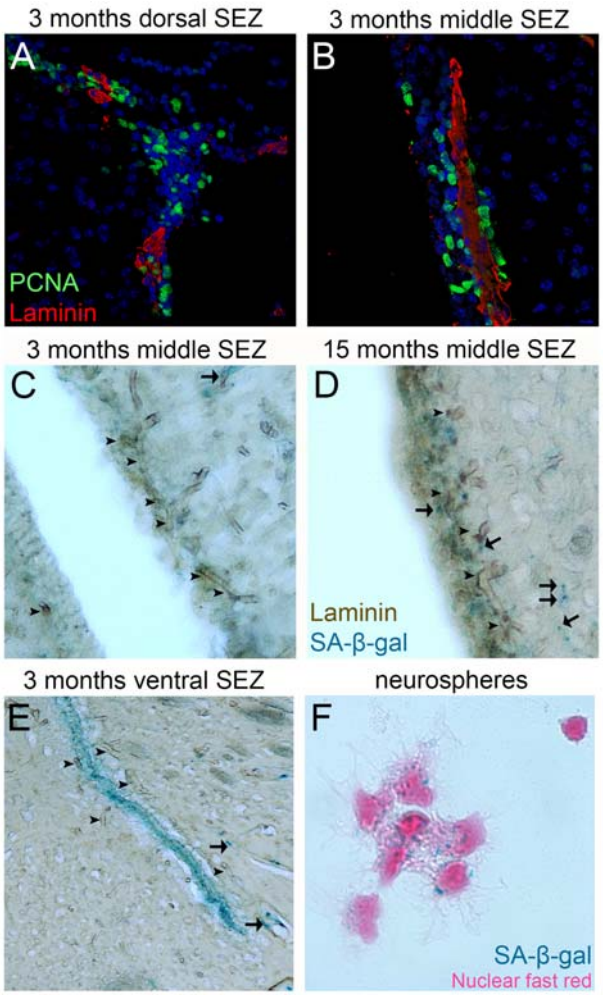

FIGURE 2 | Proliferation and senescence in the subependymal zone (SEZ). (A,B) High magnification photographs of domains of the SEZ (dorsal in **A**, middle in **B**) taken from young adult mouse brain tissue immunostained for PCNA (to mark proliferating cells) and laminin (to mark blood vessels, BVs). Note the existence of multiple proliferating cells around the long BV running in parallel to the lateral ventricle in **(B)**, but also the existence of high proliferative activity in areas distant from BVs in **(A)**. **(C–E)** High magnification photographs of domains of the SEZ (middle in **C,D** and ventral in **E**) taken from young (in **C,E**) and aged (in **D**) rat brain tissue immunostained for laminin and chemically stained for senescence-associated β gal (in blue). Arrowheads indicate BVs and arrows senescent cells. Note the significantly lower density of BVs at the non-neurogenic side of the lateral ventricle (at the left of **C**), the existence of senescent cells along BVs outside the SEZ and the existence of high numbers of senescent cells in the ventral domain of the young-adult rat SEZ (in **E**). **(F)** High magnification of adult mouse NSCs isolated from the SEZ and kept in culture. Note the existence of senescent cells (nuclei are counterstained with nuclear fast red). [Antibodies used: rabbit anti-laminin: 1/500 (Abcam), mouse anti-PCNA: 1/500 (Abcam). Alexa goat anti-rabbit 568 and goat anti-mouse 488 (Invitrogen). Biotinylated goat anti-rabbit and DAB staining kit (Vector laboratories). Senescence-associated β gal staining kit (Millipore). Adult NSCs cultured in DMEM/F12 supplemented with B27 (Gibco), FGF2 (20 ng/ml) and EGF (20 ng/ml). All animal work was performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and was approved by the University of Cambridge Animal Welfare and Ethical Review Body].

proximal to BVs can lead to the hypothesis that remoteness from BVs might be correlated with senescence, or even cell death of NSCs.

Commitment and Differentiation

It could be hypothesized that as the brain became larger and more complicated during evolution, the contribution of BVs in controlling the commitment and differentiation of NSC increased. Interestingly, similarly to diversification, there is no strong evidence on the existence of such dependance. The single example of a vessel-derived factor regulating cell fate decisions is pigment epithelium-derived factor (PEDF) that acts to instruct adult NSCs of the SEZ to switch mode of division from asymmetric differentiating to self-renewing (Ramírez-Castillejo et al., 2006). More specific to differentiation is the role of PlGF-2, diffused from endothelial cells and pericytes, that was recently shown to bias cell fate of adult NSCs and transit amplifying progenitors towards neurogenesis, at the expense of astrogliogenesis (Crouch et al., 2015). Enhanced neurogenesis was also observed in co-cultures of human NSCs and endothelial cells; albeit via unknown mechanisms (Chou et al., 2014). The available evidence suggests that cell fate choices are primarily controlled in a cell-autonomous manner, as has been shown from *in vitro* cultures of isolated embryonic and adult NSCs (Okano and Temple, 2009; Ortega et al., 2013). It should be noted that BV-derived signals might not be essential for instructing cell-fate of neural stem and progenitor cells, but for the survival of certain types of newborn neurons (Kirschenbaum and Goldman, 1995; Leventhal et al., 1999), a "selection" role that can give the illusion of an effect on cell-fate instruction.

Migration

During embryonic development neural stem and progenitor cells migrate using radial glial processes, while adult SEZ-derived neuroblasts use chain-migration to exit the niche and reach their target area via an extracellular matrix-rich corridor, the rostral migratory stream. The first solid evidence that BVs also play a role in migration of neural progenitors came from animal models of cerebral ischemia, in which neuroblasts were shown to migrate towards the area of infarction along BVs (Yamashita et al., 2006; Thored et al., 2007; Kojima et al., 2010). Subsequently, vessel-supported migration was also found to be part of the homeostatic movement of neural progenitors, either in the granular cell layer in the hippocampus (Sun et al., 2015), or along the rostral migratory stream (Bovetti et al., 2007) and within the olfactory bulbs (Bovetti et al., 2007). Even more recently, BVs were shown to facilitate migration of oligodendroglial progenitors from the SEZ to the corpus callosum (Cayre et al., 2013) and the invasion of glioblastoma tumour cells into neighboring areas of the brain (Dubois et al., 2014). In the SEZ, SDF1/CXCL12 acts to attract neuroblasts expressing the CXCR4 receptor toward BVs (Kokovay et al., 2010), while endothelial-derived BDNF has been implicated to the attraction of neuroblasts to ischemic areas (Grade et al., 2013) and netrin-1 is necessary for the migration of oligodendroglial progenitors to the corpus callosum (Cayre et al., 2013). Overall, accumulating evidence indicates that migration

is one phase in the life of NSCs that is highly dependent on the vasculature.

COUPLING OF NEUROGENESIS AND ANGIOGENESIS; WHO NEEDS WHOM?

A strong correlation between angiogenic and neurogenic events has been observed but the functional substrate remains elusive; hence, the term "coupling" has been adopted. Such coupling events have been described in the developing rodent nervous system, in which endothelial cells share expression of transcription factors with surrounding NSCs according to the anatomical location (Vasudevan et al., 2008) and in the plastic areas of the adult song-bird. Moreover, in the post-stroke recovery in the adult rodent brain, induction of angiogenesis and of NSC-driven cytogenesis seem to be co-ordinated (Thored et al., 2007; Plane et al., 2010; Zhang et al., 2014), while pulses of synchronous NSC proliferation in the SEZ induce increased blood flow (Lacar et al., 2012a). Although some architectural and structural specializations of the adult NSC niche vasculature have been described, leading to the use of the term "neurovascular" niche, the absence of a functional specialization in BVs outside the niche has not yet been proven. Recent experimental work demonstrated that isolated endothelial cells from non-neurogenic areas of the adult brain exhibit equal, if not superior, potential in promoting NSC proliferation and differentiation when compared to endothelial cells from neurogenic areas (Crouch et al., 2015). In addition, mitotically active oligodendrocyte progenitors in the brain parenchyma have been reported to cross-talk with endothelial cells within "oligovascular niches" (Arai and Lo, 2009; Pham et al., 2012). Notably, in experimental animal models of stroke or multiple sclerosis, transplanted NSCs form "atypical neurovascular niches" using BVs outside the established stem cell areas (Pluchino et al., 2010). Finally, accumulating evidence suggests that dormant NSCs exist in the non-neurogenic brain parenchyma of rodents (Sirko et al., 2013), possibly next to BVs (Bardehle et al., 2013), and that in the human brain such progenitors might not be dormant at all (Ernst et al., 2014). On the other hand, we have observed that, in response to a demyelinating lesion in the corpus callosum, platelets accumulate specifically in the vasculature of the SEZ, suggesting an underlying specialization of BVs (Kazanis et al., 2015). Furthermore, by staining for senescence-associated markers we have also observed that although a high number of endothelial cells in BVs spread throughout the adult rat brain are senescent, the niche vasculature remains senescence-free (**Figure 2**), a possible indication of higher vascular plasticity potential in the specific area.

Another significant aspect of the cross-talk between BVs and NSCs is the transport of factors that might be important to NSCs but are not produced by vascular and perivascular cells. Two examples have already been mentioned: oxygen (Lacar et al., 2012a; Culver et al., 2013) and insulin (Masjkur et al., 2012). Recent experimental work has also revealed that blood-derived factors, such as GDF11, can act to rejuvenate aged neural stem and progenitor cells (Ruckh et al., 2012; Katsimpardi et al., 2014). In other words, by feeding the aged brain with young blood, scientists were able to reverse some of the effects of ageing on NSCs. However, it still remains unknown if the effect was direct to NSCs or if it was dependent on rejuvenating the vasculature or macrophages. A final and intriguing aspect is the possible instructive role of NSCs on the vasculature. The dominant hypothesis is that the vasculature directs NSCs, exemplified by the observation that grafted NSCs are ectopically homed perivascularly (Pluchino et al., 2010). However, SEZ NSCs can influence the function of BVs, for example, the blood flow (Lacar et al., 2012b), and oligodendrocyte progenitors can control angiogenesis through hypoxia-inducible factors (Yuen et al., 2014). These are in concert with evidence that embryonic cortical NSCs are important for the establishment of the developing vasculature (Gerhardt et al., 2004; Ma et al., 2013) and that in many cases vascularization tightly follows the maturation of the nervous system (Rodriguez Celin et al., 2015). Recently published work with human NSCs also showed that they provide the necessary juxtacrine and paracrine signals to drive human endothelial cells to form "vasculature-like structures" (Chou et al., 2014) and promote angiogenesis in the rodent brain (Hicks et al., 2013). Therefore, the bidirectional cross-talk between NSCs and BVs is a line of research that needs to be developed further.

AUTHOR CONTRIBUTIONS

CK contributed original data wrote and approved the manuscript. IK contributed original data, developed the concept, wrote and approved the manuscript.

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REVIEW
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Brain and Retinal Pericytes: Origin, Function and Role

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Pericytes are specialized mural cells located at the abluminal surface of capillary blood vessels, embedded within the basement membrane. In the vascular network these multifunctional cells fulfil diverse functions, which are indispensable for proper homoeostasis. They serve as microvascular stabilizers, are potential regulators of microvascular blood flow and have a central role in angiogenesis, as they for example regulate endothelial cell proliferation. Furthermore, pericytes, as part of the neurovascular unit, are a major component of the blood-retina/brain barrier. CNS pericytes are a heterogenic cell population derived from mesodermal and neuro-ectodermal germ layers acting as modulators of stromal and niche environmental properties. In addition, they display multipotent differentiation potential making them an intriguing target for regenerative therapies. Pericyte-deficiencies can be cause or consequence of many kinds of diseases. In diabetes, for instance, pericyte-loss is a severe pathological process in diabetic retinopathy (DR) with detrimental consequences for eye sight in millions of patients. In this review, we provide an overview of our current understanding of CNS pericyte origin and function, with a special focus on the retina in the healthy and diseased. Finally, we highlight the role of pericytes in de- and regenerative processes.

Keywords: pericytes, blood flow regulation, blood retina barrier, retinal diseases, tissue regeneration

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INTRODUCTION

Pericytes are specialized cells located at the abluminal surface of capillary blood vessels with key functions in vascular homoeostasis (Díaz-Flores et al., 2009). They were first described in the 1870's by Eberth (1871) and Rouget (1879) and at that time named after the investigator "Rouget cells". Due to their location in close proximity to endothelial cells, and their perivascular association to the microvasculature, Zimmermann (1923) later renamed them to "pericytes". However, the definition and also proper identification of pericytes remains challenging. Depending on the vascular bed and their differentiation state, pericytes exhibit varying morphologies and express different marker profiles. In the last decades functional roles of pericytes in blood vessel stabilization (von Tell et al., 2006), in blood flow regulation

Abbreviations: CNS, central nervous system; vSMC, vascular smooth muscle cells; GFP, green fluorescent protein; BM, bone marrow; BRB, blood retina barrier; BBB, blood brain barrier.

(Hamilton et al., 2010), and in the formation of the blood-brain/retina barrier (BBB/BRB; Zlokovic, 2008; Bell et al., 2010; Winkler et al., 2011; Pfister et al., 2013) have been demonstrated. Further, pericytes possess a multipotent differentiation potential, which allows for generation of various different cell types (Crisan et al., 2009; Díaz-Flores et al., 2009). Due to this multipotency, these cells are a potential target for tissue repair and therapeutic approaches in regenerative medicine (Ozen et al., 2012).

This review covers pericytes within the central nervous system (CNS), focusing on retinal pericytes and their vascular functions as well as their contribution to retinal pathology progression. Because of its identical embryological origin, the mammalian retina is considered a part of the CNS. The nutrition of the retina is provided by two different vascular beds: the outer retina (photoreceptors) is passively supplied by the choroidal vasculature, whereas the inner retina is supplied by the retinal vasculature. The latter is comparable to CNS vasculature, constituting a tightly regulated cellular barrier. Retinal pericytes are essential constituents of the BRB and fulfill important functions to maintain vessel homeostasis. To provide a more comprehensive vision, this review will also include findings of other, non-retinal CNS pericytes. Finally, next to describing the vascular function of CNS pericytes the emerging hypothesis arguing in favor of a regenerative function and hence a therapeutic use of pericytes in tissue repair will be discussed.

ORIGIN OF PERICYTES

Pericytes are generated during embryonic and postnatal life (Armulik et al., 2011; Winkler et al., 2011). During developmental stages, CNS pericytes originate from neuroectodermal neural crest cells as demonstrated by quail-chick transplantation experiments of forebrain pericytes (Etchevers et al., 2001). These findings were supported by the possibility of neuroectodermal cells to differentiate into pericytes and vascular smooth muscle cells (vSMCs) of embryonic cerebral vessels (Korn et al., 2002). The neural crest origin of retinal pericytes was demonstrated using a Wnt-1 Cre-recombinase fate mapping mouse model, which specifically labels neural crest and neural crest–derived cells (Danielian et al., 1998; Gage et al., 2005). Within the retina, choroid and optic nerve, a neural crest origin was further demonstrated using a Sox10-Cre neural crest fate mapping mouse model (Trost et al., 2013). Along these lines, Simon et al. (2012) reported GFP-positive pericytes in the cortical gray matter using an inducible Sox10-Cre eGFP mouse model. Finally, neural crest origin of pericytes has also been described in thymic vessels of Wnt-1-Cre (Müller et al., 2008) and Sox10-Cre (Foster et al., 2008) mouse models.

In addition to the neural crest origin, pericytes potentially also derive from mesodermal cells (Etchevers et al., 2001). Using a XlacZ4 reporter under the control of an adipose tissue specific promoter (aP2), Tidhar et al. (2001) demonstrated reporter gene expression in vSMCs and pericytes throughout the vascular bed, including retinal microvessels. Also, bone marrow (BM) cells can be recruited during tumor- and cytokine-induced neoangiogenesis, giving rise to cells morphologically resembling pericytes and expressing the pericyte marker chondroitin sulfate proteoglycan 4/neural glial antigen 2 (NG2; Rajantie et al., 2004; Ziegelhoeffer et al., 2004; Song et al., 2005; Lamagna and Bergers, 2006). Finally, in corneal neovascularization almost half of the neovascular pericytes are BM-derived (Ozerdem et al., 2005). Also, fate mapping of GFP labeled BM using the stem cell antigen 1 promoter (sca-1), illustrated a BM origin of pericytes and a contribution of these cells to vascular remodeling during postnatal retinal angiogenesis as well as pathological angiogenesis in the retina (Pfister et al., 2013). Taken together, these data strongly suggest a mesodermal origin of pericytes resembling a pericyte reservoir for postnatal pathological neoangiogenesis.

IDENTITY OF PERICYTES

Under physiological conditions pericytes are located at the abluminal surface of microvessels, embedded in a common basement membrane with endothelial cells (**Figure 1**). The cytoplasmic processes of pericytes can span several endothelial cells and can have different morphologies (Dore-Duffy and Cleary, 2011), depending on the vascular bed and their differentiation/developmental state. The pericyte density and microvascular coverage varies according to the vascular bed, revealing the highest density in the CNS (Tilton et al., 1985; Frank et al., 1990).

Although the identification of pericytes by ultrastructural analysis may represent the "gold standard", this method is not applicable in every experimental setting. Therefore a variety of potential pericyte-specific histological markers have been proposed (reviewed in Armulik et al., 2011), including platelet-derived growth factor receptor β (PDGFRb; Lindahl et al., 1997; Winkler et al., 2010), NG2 (Ozerdem et al., 2001; Trost et al., 2013), CD13 (Kunz et al., 1994), desmin (Nehls et al., 1992), vimentin (Bandopadhyay et al., 2001). In addition, the potassium channel complex Kir 6.1 has been used as a marker particular for CNS pericytes (Bondjers et al., 2006; **Table 1**). On the other hand, pericytes are negative for endothelial cell markers such as CD31 and von Willebrand factor and markers of other perivascular cell types, such as glial cells (GFAP, Olig2), microglial cells (Iba1) and neuronal cells. For the identification of retinal pericytes a combination of NG2 and PDGFRb can be recommended based on recently published data (Trost et al., 2013). However, it is important to note that the expression of antigens may differ for *in vitro* and *in vivo* conditions. For example, alpha smooth muscle actin (aSMA) has widely been used to identify pericytes and vSMCs *in vitro* and *in vivo*, however capillary pericytes do not express aSMA *in vivo* (Nehls and Drenckhahn, 1991; Trost et al., 2013; Hill et al., 2015). Therefore, aSMA labels vSMCs located on larger vessels, but not capillary pericytes. As several other markers such as NG2, PDGFRb or desmin are expressed by pericytes and vSMCS the identification and discrimination of these two cell types cannot be based solely on marker expression, but vascular localization must also be considered. As demonstrated in **Figure 2**, vSMCs

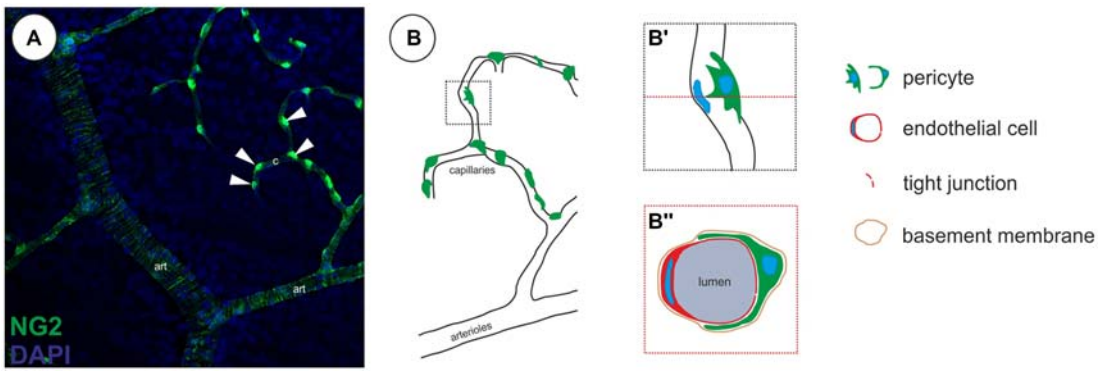

FIGURE 1 | Localization of pericytes within the retinal vasculature. (A) Retinal vasculature, showing a single neural glial antigen 2 (NG2)-positive (green) vSMCs layer on arterioles (art) and single NG2-positive pericytes on capillaries (c, arrowheads; in accordance with results in Trost et al., 2015). **(B)** Illustration of the localization of pericytes on capillaries. **(B′)** Pericytes are located abluminal of endothelial cells, **(B″)** wrapping around the capillary, embedded within the same basement membrane as demonstrated in a capillary cross-section.

TABLE 1 | Summary of pericyte markers.

Marker		Reference
PDGFRb	Platelet-derived growth factor receptor β	Lindahl et al. (1997) and Winkler et al. (2010)
NG2	Chondroitin sulfate proteoglycan 4/neural glial antigen 2	Ozerdem et al. (2001) and Trost et al. (2013)
CD13	Alanyl membrane aminopeptidase	Kunz et al. (1994)
Vimentin	Intermediate filament protein	Bandopadhyay et al. (2001)
Desmin	Desmin, structural protein	Nehls et al. (1992)
Kir6.1	Potassium inwardly rectifiying channel, subfamily J, member 8	Bondjers et al. (2006)

on arterioles as well as pericytes on capillaries are labeled by NG2, however aSMA identifies only vSMCs on arterioles and is absent in retinal pericytes (**Figure 2**). This marker expression profile indicates a morphological and biochemical continuum from vSMC to pericytes, which is further supported by the common expression of the neural crest specific marker Sox10 (Trost et al., 2013). However, under pathological conditions pericytes may alter their expression. Therefore, the use of genetic models permanently labeling pericytes and their progenies (e.g., NG2-dsRed (Schallek et al., 2013) or NG2-CreERT2-eGFP mouse model (Hill et al., 2015) is essential and will enable to study the fate of pericytes also under pathological conditions). In summary, the specific identification of pericytes currently requires a combination of at least two markers as well as the consideration of the morphology and vascular localization and some circumstances will require appropriate transgenic animal models.

PERICYTE FUNCTIONS

Over the last decades different functions have been assigned to pericytes: they fulfill important functions during (a) angiogenesis and vessel stabilization; (b) participate in blood flow regulation and neurovascular coupling; and (c) are an essential constituent of the BBB/BRB. As mentioned above, next to their vascular functions they possess a multipotent differentiation potential (**Figure 3**).

Angiogenesis and Pericyte-Endothelial Cell Interaction—The PDGF-B/PDGFRb Signaling Pathway

Pericytes play a major role in angiogenesis, participating in vessel formation, remodeling and stabilization (Gerhardt and Betsholtz, 2003). A number of signaling pathways and factors have been reported to be important for the intercellular communication between endothelial cells and pericytes, including transforming growth factor β (TGF β), angiopoietins, platelet-derived growth factor B (PDGF-B), spingosine-1-phosphate and Notch (reviewed in Armulik et al., 2005, 2011; Winkler et al., 2011).

Here, we will focus on PDGF-B/PDGFRb signaling, since this pathway is involved in pericyte proliferation, migration, survival and attachment. The importance of this signaling pathway was highlighted using genetically modified depletion and knockout models (reviewed in Betsholtz, 2004). During angiogenesis, sprouting endothelial cells secrete PDGF-B, which binds with high affinity to the pericyte-specific receptor PDGFRb, leading to the recruitment and attachment of pericytes. In general, impaired PDGF-B/PDGFRb signaling results in a failure of pericyte recruitment and in reduced microvascular pericyte coverage ultimately leading to endothelial hyperplasia, abnormal vascular morphogenesis and formation of microaneurysms (e.g., Lindahl et al., 1997; Hellström et al., 2001). Further, genetic deletion of either *pdgfrb* (Soriano, 1994) or *pdgfb* (Levéen et al., 1994) results in perinatal

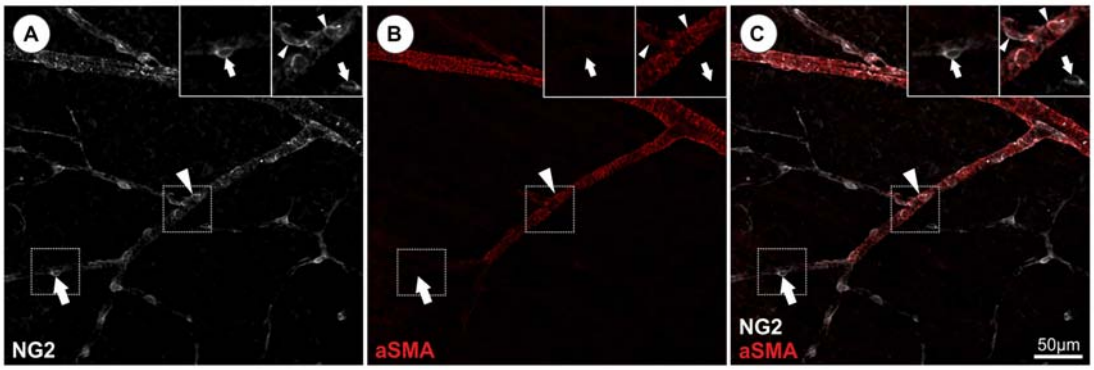

FIGURE 2 | Identification and discrimination of pericytes from vascular smooth muscle cells (vSMCs) in the retina visualizing NG2 and alpha smooth muscle actin (aSMA) protein expression. (A) NG2 immunopositivity can be detected in both, retinal pericytes (representative arrow) and aSMA positive arteriolar SMCs (representative arrowhead). **(B)** Retinal capillary pericytes lack the aSMA signal (arrows) and **(C)** can be therefore discriminated from vSMCs as illustrated in the merged picture (in accordance with results in Trost et al., 2013).

death due to vascular dysfunction. The mutation of particular downstream signal transduction molecule binding sites of PDGFRb resulted in a reduction of pericyte coverage in the heart and kidney vasculature, which was further decreased by crossing these mutants with PDGFRb null allele mice (Soriano, 1994). Investigating pericyte coverage in the retina in mice with multiple mutations, a reduced number of pericytes was detected and resulted in severe hemorrhage (Tallquist et al., 2003). The importance of PDGF-B is further underscored by studies demonstrating that ablation of endothelial derived PDGF-B resulted in reduced retinal pericyte coverage, leading to variable capillary and venous diameter, regressing capillary branches and the presence of microaneurysms. Furthermore, all mice showing <52% of normal pericyte density developed signs of proliferative retinopathy (Enge et al., 2002).

Another approach to investigate PDGF-B/PDGFRb signaling is the deletion of the retention motif in PDGF-B. The localization of the endothelial secreted PDGF-B in the vicinity of the developing vessel is guaranteed by binding of the PDGF-B retention motif to heparin sulfate proteoglycans (HSPG), resulting in subsequent binding of PDGFRb expressing pericytes to the endothelial tip cells (Lindblom et al., 2003; Armulik et al., 2005). Deleting this motif leads to impaired recruitment of pericytes, resulting in a severely disorganized retinal vasculature with partially detached pericytes and processes extending away from the vessel (Lindblom et al., 2003; Genové et al., 2014). As the truncated PDGF-B protein reveals full biological activity (Ostman et al., 1989), the endothelial localization of PDGF-B by the retention motif is suggested to be responsible for proper pericyte embedment in the microvessel wall. Besides the retention of PDGF-B at

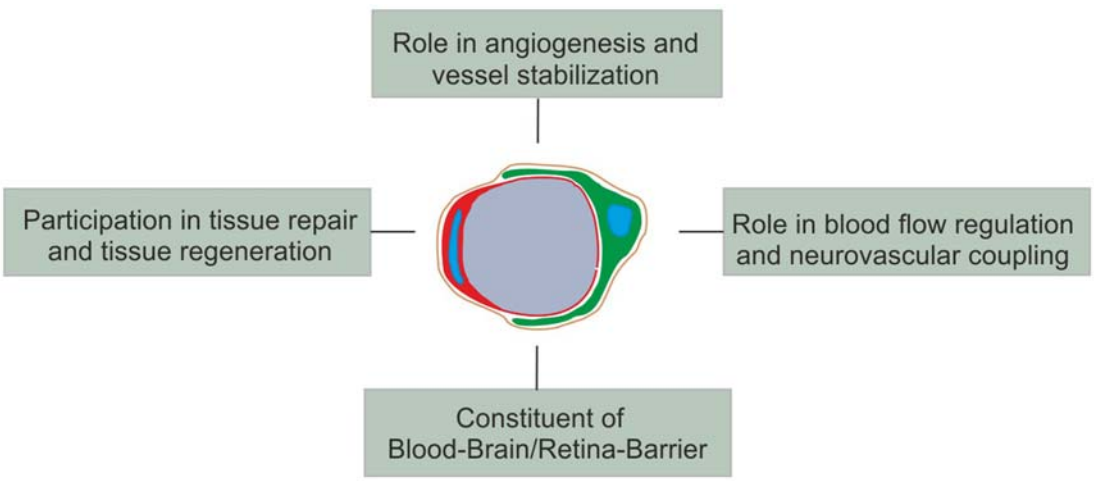

FIGURE 3 | Schematic overview of (the main) pericyte functions.

the developing vessel, the tissue concentration of PDGF-B is important for determining retinal pericyte density, as revealed by a reduction in pericyte coverage in mice with a monoallelic deficiency of PDGF-B (PDBF-B$^{+/-}$; Hammes et al., 2002).

The impact of transgenic overexpression of PDGF ligands has also been investigated. Specific overexpression of PDGF-B in photoreceptor cells resulted in increased proliferation of pericytes, but also astrocytes and endothelial cells. These cells formed disorganized sheets and cords migrating into the inner retina, causing retinal tractions which ultimately resulted in a phenotype resembling retinal detachment in proliferative retinopathies. Furthermore, the formation of the deep capillary bed was inhibited in PDGF-B overexpressing mice (Seo et al., 2000; Mori et al., 2002; Vinores et al., 2003). The pathogenic effect of ectopic PDGF-B expression on retinal architecture and development was also demonstrated by overexpressing PDGF-B under the control of the myelin basic protein promoter (myelinating tracts), showing capillary and retinal disorganization (Forsberg-Nilsson et al., 2003). In line with this, expression of PDGF-B under the control of the nestin enhancer element (active in progenitor cells during development) resulted in severe retinal developmental defects, such as retinal folding, disorganized retinal lamination, delayed and abnormal vascular development, and progressive retinal degeneration (Edqvist et al., 2012).

It can be concluded that the balanced and well controlled secretion of endothelial PDGF-B, proper binding in close vicinity to the sprouting vessel and the function of the pericyte's PDGFRb are essential for the proper development and maintenance of the CNS vasculature in general, and the retinal vasculature in particular.

Blood Flow Regulation and Neurovascular Coupling

As pericytes express contractile proteins (Bandopadhyay et al., 2001) and are located on capillaries abluminal of endothelial cells, where vSMCs are absent, they are proposed to participate in microvascular blood flow regulation. Although the contractility and responsiveness of pericytes to vasoactive peptides has been demonstrated *in vitro* (Markhotina et al., 2007), concisive *in vivo* data remain limited. Evidence for pericyte contractility in response to vasoactive molecules/neurotransmitters was demonstrated using isolated retinal vessels (Kawamura et al., 2003; Wu et al., 2003) and by *in situ* studies using isolated rat retina (Schönfelder et al., 1998; Peppiatt et al., 2006) and cerebellar slices (Peppiatt et al., 2006). In line with these findings, Fernández-Klett et al. (2010) demonstrated the ability of pericytes to modulate local cerebral capillary blood flow in brain slice preparations *in situ* and cortex *in vivo*. In contrast, Hill et al. (2015) excluded a contribution of CNS pericytes to regulate cerebral blood flow on the capillary level. Focusing on aSMA-negative pericytes located on capillaries with a diameter of >10 μm, the authors detected a highly variable spontaneous vasomotion and calcium fluctuations, not correlating with changes in vessel diameter.

In vivo contractility of CNS pericytes and their influence on neurovascular coupling has been investigated in brain and to a lesser extent in retinal microvessels. The response of regional blood flow to neuronal activity (functional hyperemia) involves the interaction between neurons, glia and vascular cells (neurovascular coupling). Although Fernández-Klett et al. (2010) demonstrated active pericyte contraction after vasoconstrictor addition, they were unable to provide evidence that pericytes participate in neurovascular coupling after neuronal activity-induced hyperemia. In contrast, Hall et al. (2014) showed that cerebral pericytes actively dilate the capillaries after electrical whisker pad stimulation, where capillary dilation precedes arteriolar dilation. Although in both studies a similar experimental set-up was applied to monitor dilations of cerebral capillaries in response to neuronal activation, the mode of electrical stimulation to increase capillary blood flow was different and may account for the conflicting findings on the impact/ability of pericytes to increase cerebral blood flow.

Studying the impact of retinal pericytes on capillary blood flow regulation, the dilation of intermediate layer capillaries upon flicker stimuli was proposed to be mediated by active relaxation of pericytes, however without presenting direct evidence that pericytes actively dilated. Nevertheless, the authors concluded that functional hyperemia is driven primarily by active dilation of retinal arterioles, covered by vSMCs (Kornfield and Newman, 2014).

Although single studies propose a lack of pericyte contractility (Hill et al., 2015), currently the majority of studies suggests that CNS pericyte contractility is important to regulate local blood flow under pathological conditions, such as traumatic brain injury (Dore-Duffy et al., 2011) or ischemia (Peppiatt et al., 2006; Yemisci et al., 2009; Hall et al., 2014). Nevertheless, the active regulation of the capillary blood flow by pericytes remains a controversial issue, most likely due to the poor definition and identification of pericytes. Although several studies suggest pericytes to possess an important role in neurovascular coupling, more experimental *in vivo* evidence and a standardized definition of pericytes is necessary to compare and combine findings on the role of pericytes in blood flow regulation. Certainly, the use of genetically modified mice, labeling pericytes as well as vSMC in combination with pericytic markers will provide a more in depth and standardized tool to study the contribution of pericytes in blood flow regulation. Investigating pericytes in the retina for example, Schallek et al. (2013) imaged retinal pericytes non-invasively in the living eye using the NG2-dsRed mouse model. This model, also used to investigate cerebral blood flow (Hall et al., 2014), represents a great tool to study the impact of retinal pericytes on the regulation of capillary diameter.

Blood-Brain-Barrier/Blood-Retina-Barrier and Associated Diseases

In the last 20 years, pericytes have been proven to be an essential constituent of the BBB and BRB. The BBB/BRB is a highly regulated barrier, controling paracellular flow between cells and transendothelial fluid transport, ensuring optimal chemical composition of the neuronal microenvironment and at the same

time protecting from potential harmful substances. Although the main role in BBB/BRB tightness is mediated by tight and adherens junctions between endothelial cells, several studies have demonstrated that pericytes form and maintain together with endothelial, neuronal and glial cells the BBB/BRB and guarantee barrier function and tissue homeostasis. The BRB is composed of the inner BRB (retinal capillary endothelial cells) and the outer BRB (retinal pigment epithelial cells). Although the BBB is structurally similar to the inner BRB and both express several common transporters/receptors (Mori et al., 2003; Hosoya et al., 2009), some reports describe heterogeneous transport properties of the brain and retina neurovascular unit (André et al., 2012). The impact of pericyte loss to BBB and BRB breakdown and subsequent increased permeability has been reported in a multitude of studies: using PDGFRb signaling deficient mice (*Pdgfrb*$^{+/+}$, *Pdgfrb*$^{+/-}$, *Pdgfrb-F7*), Bell et al. (2010) demonstrated that reduced pericyte coverage in the brain results in BBB breakdown and an accumulation of plasma derived proteins, ultimately resulting in secondary neuronal degenerative alterations (e.g., impairments in learning and memory). Increased BBB permeability through pericyte deficiency was further demonstrated in studies using pericyte deficient mouse mutants (*Pdgfb*$^{ret/ret}$ and R26P$^{+/-}$, R26P$^{+/0}$) indicating a regulatory role of pericytes on the gene expression profile of endothelial cells and astrocytes (Armulik et al., 2010). The crucial role of pericytes in BBB formation was also confirmed during embryogenesis. Using different PDGFRb mouse mutants with reduced pericyte coverage (*Pdgfrb*$^{-/-}$, *Pdgfrb*$^{F7/F7}$, *Pdgfb*$^{F7/-}$), Daneman et al. (2010) demonstrated a correlation of BBB permeability with pericyte loss, concluding that the pericyte amount determines the relative permeability of CNS vessels during development. The impact of pericytes on BBB mechanisms and subsequent neuronal damage has been investigated extensively using the aforementioned mouse models, however only few studies also investigated the impact of reduced pericyte coverage on BRB permeability and neuronal damage in the retina. Using endothelial specific PDGF-B transgenic mice (*Pdgf-b*$^{ret/ret}$, *Pdgf-b*$^{lox/-}$, *Pdgf-b*$^{-/-}$), a loss of neuronal layers and folding of the photoreceptor layer in the retina was associated with reduced pericyte coverage, resembling signs of diabetic retinopathy (DR; Enge et al., 2002; Lindblom et al., 2003). Further, investigating tight junction formation in the developing retinal vasculature within the first three postnatal weeks, the contribution of pericytes to tight junction and BRB formation was demonstrated by the increased expression of the tight junction protein ZO-1 during maturation and enhanced pericyte coverage (Kim et al., 2009). However, a limitation of this study is the identification of pericytes using aSMA. As mentioned above, the development of tight junctions interconnecting endothelial cells is crucial for an intact BBB and the contribution of pericytes to tight junction formation at the BRB has been confirmed by electron microscopy *in vivo* as well as in endothelial-pericyte co-culture models (Daneman et al., 2010).

Taken together, as a disrupted BBB/BRB is associated with a variety of neuropathological processes, *in vitro* (Wisniewska-Kruk et al., 2012) as well as *in vivo* models are important to study the underlying mechanisms.

PERICYTES IN RETINAL DISEASE AND NEUROPATHOLOGIES

Altered pericyte function and coverage have been described in diverse CNS diseases (reviewed in Lange et al., 2013). Diseases with a clear pericyte participation include DR (Beltramo and Porta, 2013), neonatal intraventricular hemorrhage (Braun et al., 2007), Alzheimer's disease (AD; Winkler et al., 2014) or amyotrophic lateral sclerosis (ALS; Winkler et al., 2013) as well as diverse rare diseases like Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL; **Table 2**). One of the best studied pathological conditions associated with reduced pericyte coverage in the retina is DR. DR is a major complication of diabetes mellitus, characterized by increased vascular permeability (BRB breakdown), progressive vascular occlusion, microaneurysms and neuronal changes ultimately resulting in vision threatening diabetic macular edema and proliferative DR (Hammes et al., 2002, 2011). Although the pericyte loss is a well established fact of DR pathology, the underlying mechanisms for the development and progression of DR remain unclear (Frank, 2004; Qian and Ripps, 2011; Klaassen et al., 2013). Pericyte loss through apoptosis and destructive pathways under hyperglycemic conditions has been suggested (Behl et al., 2008, 2009). Altered glutamate excitation, reduced trophic factor signaling, oxidative stress, and neuroinflammation have also been associated with increased pericyte apoptosis (reviewed in Barber et al., 2011).

TABLE 2 | Excerpt of diseases with associated pericyte dysfunction.

Disorders	Findings	Reference
Diabetic retinopathy	Reduced retinal pericyte coverage	Hammes et al. (2002, 2011), Beltramo and Porta (2013), and Klaassen et al. (2013)
Neonatal intraventricular hemorrhage	Decreased brain pericyte coverage	Braun et al. (2007)
Alzheimers disease	Degeneration of brain pericytes	Farkas et al. (2000), Sengillo et al. (2013), Sagare et al. (2013), Winkler et al. (2014), and Halliday et al. (2015)
Amyotrophic lateral sclerosis	Reduced pericyte coverage	Winkler et al. (2013)
CADASIL		Robinson et al. (2001), Haritoglou et al. (2004), and Roine et al. (2006)
Adams oliver syndrome	Reduced pericyte coverage	Patel et al. (2004)
Multiple sclerosis	Reduced brain pericyte amount	Kunz et al. (1995)
Stroke	Loss of capillary pericytes	Fernández-Klett et al. (2013)

Next to these alterations, growth factor-mediated pericyte depletion via angiopoietin-2/Tie-2 has been suggested (Hammes et al., 2004; Pfister et al., 2008; Park et al., 2014).

Different genetically modified mouse models as well as pharmacologically induced diabetic rodent models (Enge et al., 2002; Huang et al., 2011; Lai and Lo, 2013; Qiu et al., 2015) have been used to study the molecular mechanisms of pericyte loss and vascular leakage in DR, but despite these available models, BRB breakdown remains poorly understood. Recently, Jadeja et al. (2013) characterized the causative mutation in the "redeye" mouse, identifying a mutation in the *PDGFRb* gene resulting in a reduction of normal/wt *PDGFRb* transcript. They verified this mouse being a useful model to study non-proliferative DR, demonstrating CNS-restricted, reduced pericyte coverage and BRB leakage followed by retinal neurodegeneration (i.e., reduced number of RGCs; Jadeja et al., 2013). To study the underlying molecular mechanisms at later stages of DR, the recently developed Akimba ($Ins2^{Akita}VEGF^{+/-}$) mouse, developing retinal leakage, neovascularization with hyperglycemia and signs of advanced clinical DR (diabetic macular edema, proliferative DR) might represent a suitable model (Wisniewska-Kruk et al., 2014).

The impact of pericytes as well as reduced pericyte coverage on microvessels is also discussed to contribute to glaucoma. While single studies report increased leakage of the BRB in the optic nerve head (ONH) in glaucomatous patients (Arend et al., 2005; Grieshaber and Flammer, 2007), the tightness of the BRB in this disease remains unclear. The density or degeneration of capillaries has been analyzed in several studies (May and Mittag, 2006; Mi et al., 2012; Almasieh et al., 2013), and vascular dysregulation has been suggested to be an important factor in the pathogenesis of glaucoma (Venkataraman et al., 2010). However, investigating pericyte coverage in an acute short-term glaucoma model revealed no alteration in pericyte coverage (Trost et al., 2015) and further studies are needed to understand the contribution of pericytes in the glaucomatous diseases.

Similarly to the retina, pericyte loss and dysfunction has also been reported to be associated with neurodegenerative disorders in other CNS regions. The degeneration of brain pericytes by specific amyloid-beta has been demonstrated by several studies *in vitro* (Verbeek et al., 2000; Rensink et al., 2002). In cerebral microvessels of patients suffering from AD, a degeneration of pericytes has been observed *in vivo* (Farkas et al., 2000) and this phenomenon was correlated with the severity of BBB breakdown (Sengillo et al., 2013). This finding was also confirmed in AD mouse models (Sagare et al., 2013; Halliday et al., 2015) further suggesting a contribution of pericytes to the pathogenesis of AD. Therefore, pericytes may represent a potential therapeutic target for AD (reviewed in Winkler et al., 2014).

Further, vSMC and pericyte degeneration has been described for the CADASIL syndrome caused by NOTCH 3 gene mutations (Dziewulska and Lewandowska, 2012; Gu et al., 2012; Craggs et al., 2015). It has been speculated that ischemic events and a consequently increased BBB permeability may account for the observed severe injury to the cerebral white matter in these patients. In addition, pericyte and vSMC degeneration has been confirmed in retinal vessels of CADASIL patients (Haritoglou et al., 2004), which is in accordance with observed alterations of the retinal vasculature (Robinson et al., 2001; Roine et al., 2006). In line with the above described findings, recently the aggregation of mutated Notch3 receptors on pericytes has been associated with the reduction of pericyte number and coverage of cerebral microvessels, resulting in the loss of BBB integrity and subsequent leakage of plasma proteins in a CADASIL mouse model ($TgNotch3^{R169C}$; Ghosh et al., 2015). Another disease characterized by reduced pericyte coverage is the Adams-Oliver-Syndrome (AOS), associated with severe scalp and limb defects as well as impaired cardiovascular function (Patel et al., 2004). Furthermore, in an experimental mouse model for multiple sclerosis (MS), a reduction of pericytes has been detected and correlated to the functional state of the BBB (Kunz et al., 1995). As BBB dysregulation and transendothelial migration of activated leukocytes are among the earliest cerebrovascular abnormalities in MS (Ortiz et al., 2014), the contribution of pericytes to MS pathology is very likely. Finally, inducing cerebral ischemia in wild-type and pericyte specific reporter mice ($rgs5^{GFP}$), as well as investigating histological samples of human stroke brains, Fernández-Klett et al. (2013) reported a progressive loss of capillary pericytes at the lesioned region.

Considering the fundamental role of pericytes in the formation and maintenance of the BRB/BBB and the impact of pericyte dysfunction pericytes represent a potential target for therapeutic therapies.

PERICYTES AND TISSUE REGENERATION

Pericytes represent a very heterogeneous cell type displaying different embryonic origins ranging from mesodermal to neuroectodermal germ layers. Their capacity to differentiate into mesenchymal cell types (e.g., adipocytes, chondrocytes, osteoblasts, fibroblasts, vSMCs) has been proven in a multitude of studies (reviewed in Díaz-Flores et al., 2009), including CNS and especially retinal pericytes (Canfield et al., 1996; Doherty et al., 1998; Farrington-Rock et al., 2004). Furthermore, pericytes have the ability to differentiate also into non-mesenchymal cell types, such as neural cells. For example, a recent study has shown the differentiation of a skeletal muscle pericyte subtype into Tuj1-expressing neurons (Birbrair et al., 2013). Moreover, the ability of CNS pericytes to differentiate into neurons and glial cells has been demonstrated (Dore-Duffy et al., 2006; Paul et al., 2012) and CNS pericytes can be reprogrammed into neuronal cells by ectopic expression of the pro-neurogenic fate determinants Sox2 and Mash1 (Karow et al., 2012). A recent study demonstrated that CNS pericytes, isolated from ischemic brain regions, are able to differentiate into neural and vascular lineage cells, suggesting their contribution to neurogenesis and vasculogenesis at sites of brain injury (Nakagomi et al., 2015). Besides the neural differentiation potential of CNS pericytes, evidence for their ability to differentiate into stromal cells has also been provided in spinal cord injury (SCI) models *in vivo*. A specific pericyte subtype was demonstrated to give rise to cells forming fibrotic scar tissue after SCI and was further

found to be crucial for wound closure as shown by specific depletion of this type A pericyte population (Göritz et al., 2011). Consistent with these findings, a recent study proposed an increase of PDGFRb+ pericytes in the injured spinal cord region, intermingled with GFAP+ astrocytes (Matsushita et al., 2015). Using a Nestin-GFP/NG2-dsRed fate mapping model, the type I pericytes (Nestin−/NG2+) have been shown to participate in scar formation after SCI (Birbrair et al., 2014), however as these cells lack PDGFRb expression in the fibrous scar they probably represent an additional pericyte subtype. Taken together, the proliferation of CNS derived PDGFRb+ stromal cells, identified as pericytes, has been described in SCI (Göritz et al., 2011; Matsushita et al., 2015) and cerebral ischemia (Fernández-Klett et al., 2013), indicating a crucial role of CNS pericytes in pathological fibrotic processes, being a potential target for future therapeutic strategies. Further the potential of CNS pericytes to differentiate into mesodermal as well as neuroectodermal cell types highlights their potential role in regenerative processes.

Due to their similarity to mesenchymal stem cells (MSCs), in terms of their expression profile and their differentiation potential as well as of their stromal capability, CNS pericytes have been considered to be the "CNS-resident MSCs" (Lange et al., 2013). Indeed, similarly to MSCs, CNS pericytes- release a plethora of bioactive molecules able to regulate proliferation and migration of CNS progenitor cells (Choe et al., 2014; Maki et al., 2015). This last property provides pericytes with the ability to regulate CNS endogenous progenitor/stem cell function during development and regeneration. Therefore, on the one hand via their stromal features pericytes could create a regenerative milieu by modulating/enhancing CNS-resident progenitor cells function and on the other hand a direct differentiation of pericytes into CNS reparative cells would be conceivable due to their enormous cellular plasticity.

Until now there are several studies showing a contribution of peripheral and CNS pericytes in peripheral tissue regeneration, but there is a lack of studies describing a regenerative capacity of CNS and especially, retinal pericytes in CNS tissue *in vivo*. In contrast, a multitude of studies report the capacity of pericytes from various peripheral tissues to improve and regenerate injured tissue: Crisan et al. (2008) isolated perivascular cells (CD146+/CD34−/CD45−/CD56−) from human skeletal muscle, pancreas, white adipose tissue and placenta, to demonstrate their myogenic potential and moreover the formation of myofibers after injection into the skeletal muscle of injured mice. The myogenic potential of human skeletal muscle pericytes was also confirmed in studies using a muscular dystrophy mouse model, showing host-muscle colonization and the generation of fibers expressing dystrophin (Dellavalle et al., 2007). As these cells, next to pericyte markers (NG2+, CD146+, aSMA+), also expressed markers typical for MSCs, the authors suggested that MSCs may have developed from perivascular cells/pericytes (Caplan, 2008; Crisan et al., 2008). The potential of MSCs to differentiate into distinct cell types such as bone, cartilage, tendon, fat or dermis and their participation in tissue regeneration has been described in a multitude of studies (reviewed in Caplan, 2007; Lange et al., 2013). The similarities between pericytes and MSCs highlight the relevance of pericytes in tissue regeneration. In addition to pericyte participation in myogenic regeneration, the potential to repair ischemic heart muscle has been provided in several recent studies: transplantation of skeletal muscle derived pericytes diminished ventricular dilatation and improved cardiac contractility in acutely infarcted mouse hearts (Chen et al., 2013). Avolio et al. (2015) provided evidence that cardiac pericytes possess the ability to penetrate and colonize xenograft tissues, which represents a potential future application in reconstructive surgery of congenital heart diseases. However, these cardiac "pericytes" were isolated by positive selection for CD34 and negative selection for CD146, which is in contrast used as a negative and positive selection marker respectively in the studies of Chen et al. (2013) and Avolio et al. (2015). This CD34+ population, located around the vasa vasorum in the adventitia of arteries and veins, expressed pericytic (NG2, PDGFRb, RGS5) as well as mesenchymal markers (CD44, CD90, CD73, CD29), probably representing a further pericyte subpopulation. Several studies report the regenerative capacity of this CD34+ pericyte population, as demonstrated in a mouse myocardial infarction model showing long term improvements of cardiac function after pericyte transplantation (Katare et al., 2011). These differences in pericyte isolation methods clearly indicate the necessity of standardized protocols to obtain homogenous and comparable pericyte preparations for use in translatable, regenerative therapeutic approaches. Furthermore, growing evidence suggests the existence of diverse pericyte subpopulations, fulfilling different functions, most likely also due to their varying anatomical locations. Two different pericyte subtypes have been identified by Birbrair et al. (2013), using a Nestin-GFP/NG2-dsRed transgenic mouse model. They investigated the contribution of pericyte subtypes to fibrotic tissue formation in models for diverse peripheral as well as CNS injuries and described a particular subtype, the "type I pericyte" (Nestin-GFP−/NG2-dsRed+), to accumulate near the site of injury (Birbrair et al., 2014). Furthermore, in an animal model with lung lesion, a fraction of pericytes was additionally shown to be involved in collagen production (Birbrair et al., 2014). The perivascular origin of collagen producing cells/myofibroblast has been further proposed for dermal scarring (Sundberg et al., 1996) and kidney fibrosis models (Humphreys et al., 2010).

Although, until now there is no study showing a positive effect of transplanted pericytes for any eye disease, there are some studies investigating regenerative properties of pericyte-related cells in eye pathologies. The first indication for a potential role of pericyte-like cells in regenerative processes in the retinal vasculopathies has been demonstrated by the use of adipose derived stem cells (ASCs). These cells represent an alternative type of adult MSCs expressing pericyte specific markers *in vitro* and further are able to differentiate into pericytes (NG2+/PDGFRb+/SMA+). The injection of ASCs after oxygen-induced retinopathy improved microvascular regrowth, and further prevented retinal capillary dropout in case of pre-injury injection, therefore providing proof for functional vascular protection (Mendel et al., 2013).

Although the differentiation potential of CNS pericytes, including retinal pericytes, has been demonstrated (Farrington-Rock et al., 2004; Dore-Duffy et al., 2006; Karow et al., 2012; Paul et al., 2012), to our knowledge no regenerative approaches have been conducted applying CNS pericytes in any kind of *in vivo* CNS (injury) models. As pericyte dysfunction and reduced pericyte coverage plays a crucial role in disease progression and as pericytes exhibit similar characteristics as MSCs, they seem to be highly suitable for cell-based therapies. Moreover, with increasing knowledge concerning CNS pericyte modulation and differentiation, future attempts may involve molecular therapies to modulate these cells *in vivo*.

CONCLUSION

Pericytes play a crucial role in maintaining tissue homeostasis, and pericyte dysfunctions as well as a decrease in number underlie distinct pathologies. Their involvement in numerous diseases and their multipotency qualifies them as promising targets for future therapeutic regenerative approaches. CNS degenerative diseases and especially retinal degenerations often involve pathologies of the vasculature resulting in impaired vascular functions. As pericytes are crucially involved in vascular physiology as well as degenerative/regenerative processes, they are possible targets for therapeutic interventions. However, to fully harness pericytes as molecular "drug stores", we need to increase our understanding of pericyte function in health and disease, leaving ample room for future studies.

AUTHOR CONTRIBUTIONS

AT, SL and FJR wrote the manuscript. FS, DB, KAM, BB, AK-E, CS, CR, LA and HAR critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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Patient-Specific Age: The Other Side of the Coin in Advanced Mesenchymal Stem Cell Therapy

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Multipotential mesenchymal stromal cells (MSC) are present as a rare subpopulation within any type of stroma in the body of higher animals. Prominently, MSC have been recognized to reside in perivascular locations, supposedly maintaining blood vessel integrity. During tissue damage and injury, MSC/pericytes become activated, evade from their perivascular niche and are thus assumed to support wound healing and tissue regeneration. *In vitro* MSC exhibit demonstrated capabilities to differentiate into a wide variety of tissue cell types. Hence, many MSC-based therapeutic approaches have been performed to address bone, cartilage, or heart regeneration. Furthermore, prominent studies showed efficacy of *ex vivo* expanded MSC to countervail graft-vs.-host-disease. Therefore, additional fields of application are presently conceived, in which MSC-based therapies potentially unfold beneficial effects, such as amelioration of non-healing conditions after tendon or spinal cord injury, as well as neuropathies. Working along these lines, MSC-based scientific research has been forged ahead to prominently occupy the clinical stage. Aging is to a great deal stochastic by nature bringing forth changes in an individual fashion. Yet, is aging of stem cells or/and their corresponding niche considered a determining factor for outcome and success of clinical therapies?

Keywords: vascular niche, cell-based therapy, aging biology, cellular dysfunction, age-associated pathology, regenerative medicine

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BACKGROUND

Mesenchymal stem cells (MSC) are multipotential precursor cells that maintain and repair tissues in the body of adult individuals. MSC have been isolated from embryonic tissues as well as from adult up to advanced ages (Landgraf et al., 2011; Batsali et al., 2013; Beane et al., 2014a; Todeschi et al., 2015). Yet experimental knowledge about *in vivo* activities in regenerating models is still scarce (Wang et al., 2013a; Zhao et al., 2015). Besides replenishing mesenchymal tissues, MSC also modulate haematopoiesis as well as immune response (Pontikoglou et al., 2011; Hao et al., 2012; Law and Chaudhuri, 2013; Bianco, 2014). Conceivably residing in perivascular locations, MSC are identified with cells better known as pericytes. This cell type is involved in maintaining blood vessel integrity under normal conditions. During tissue damage and injury, MSC are thought to become instantaneously activated and by evading from their perivascular niche to support wound healing and tissue regeneration (Murray et al., 2014; Wong et al., 2015).

MSC are acknowledged for their potential to regenerate damaged tissue due to their ability to terminally differentiate into a broad variety of cell types. Deliberately, stem cells are perceived being ageless by nature. Yet, it is by now generally accepted that, with advancing age, a decline of stem cell

function and activity has its share in delaying the replacement and the turnover of damaged cells in compromised renewable tissues (Bajek et al., 2012; Bethel et al., 2013). Also, stem cells in their niches are exposed to threads such as reactive oxygen species, harmful chemical agents or physical stresses, which trigger premature senescence, provoke accelerated cell death or cellular transformation (Li et al., 2014a). In osseous tissues at an advanced age, both mass and mineral density of cortical and cancellous bone steadily decreases. At the same time, fat cells emerge within the bone marrow and muscles. Fat cell-specific expedition of systemically deteriorating adipokines and pro-inflammatory cytokines primes the emergence of age-associated diseases. Hence, aged or senescent circumstances call for advanced therapies (Reitinger et al., 2015). Scientific approaches aiming at standardized medical treatment often neglect these biological and patho-physiological constraints. Nevertheless, these should be distinctly considered. Otherwise rightly conceived and diligently established strategies are bound to fail.

UNRESOLVED QUESTIONS REGARDING PHENOTYPIC APPEARANCE AND *IN VITRO* TECHNIQUES

Biological Properties

Stromal cell types exhibit characteristic features. The rather large spindle-shaped cells present microvilli on their surface and produce extracellular matrix, which together facilitates MSC to firmly adhere to cell culture plastic (Friedenstein, 1976; Castro-Malaspina et al., 1980). This property is often exploited to isolate and culture-purify MSC from biopsies (Owen and Friedenstein, 1988). Variant culture conditions significantly impact on cell adhesion and consequently isolation outcome and MSC expansion. Therefore, inconsistencies often arise when employing inappropriate brands of cell culture plastic and media supplements.

MSC Immunophenotype

Another selection criterion for MSC is a tri-lineage differentiation potential forming osseous, adipose, and cartilaginous progenitors (Mark et al., 2013; Patrikoski et al., 2014), and a distinguished immune phenotype positive for CD105, CD73, and CD90, and negative for CD45, CD34, HLA-DR, and other markers (Dominici et al., 2006; Al-Nbaheen et al., 2013). This marker canon is not always unequivocal, as other cell types may also fulfill these criteria. MSC-like cells often exhibit differential marker expression depending on tissue origin and period of culture expansion (Gronthos et al., 1999; Wagner et al., 2005; Kaiser et al., 2007; Riekstina et al., 2008). A prominent example is the surface marker STRO-1. Due to the availability of a highly affine monoclonal antibody, STRO-1 has not only gained popularity as a marker but also for use in cell enrichment (Stewart et al., 1999). Endothelial cells may however also express STRO-1 thus questioning the specificity of this marker (Lin et al., 2011; Ning et al., 2011). Though, the likely equivalence of MSC to vascular pericytes reconciles STRO-1 being a good marker for true MSC (Feng et al., 2011; Chen et al., 2012; da Silva Meirelles et al., 2015). Another currently debated marker is CD34. Previously, MSC were considered CD34 negative, yet adipose-derived MSC express CD34 (Lin et al., 2008; Baer, 2014). Likewise, CD271 and CD146 markers have also been described (Rasini et al., 2013; Busser et al., 2015; Cuthbert et al., 2015). During culture expansion marker expression can change. Whether, these changes reflect the biological age of MSC has not been thoroughly studied. Also specific markers amenable for the quantification of MSC age are so far not available. In appreciation of this experience, further standards in MSC validation are needed, in particular when surface antigens expression differs in primary vs. culture-expanded MSC. Together with the restricting biological constraint of biological and replicative age, also tissue origin has to be accounted.

Isolation Techniques

To enhance isolation yields, often cell purification and fractionation by density gradient centrifugation is performed. This procedure bears the enhanced risk of contamination and therefore requests skilled operators. To reduce variability, closed, semi-automated separation devices, granting higher recovery rates, have been engineered. Their operational speed and efficiency warrant reproducible processing thereby, easing standardization for fulfilling compliance criteria in "Good Manufacturers Practice" (GMP) (Ito et al., 2010; Otsuru et al., 2013, 2015). Production of clinical-grade MSC must be performed in accordance to GMP standards and compels not only reproducibility, but also scalability. Working along the same line, novel automated cell platforms have been introduced for production of higher cell numbers in reduced times and passages (Roberts et al., 2012; Nold et al., 2013; Rojewski et al., 2013; Hanley et al., 2014). Gaining a pure MSC culture is difficult in particular when attempting to erase single-lineage committed progenitors or contaminating hematopoietic cells (Kerk et al., 1985; Kuznetsov et al., 1997). Therefore, selection enrichment using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) has been introduced. Although FACS was initially favored because it provides higher purities, shear stress within the fluids compromised cell viability, and chemotaxis (Deschaseaux et al., 2003; Rada et al., 2011; Li et al., 2013).

Culture Conditions

Further open issues are (i) seeding densities in a defined growth medium (Ben Azouna et al., 2012; Hagmann et al., 2013), (ii) specification of media supplements (Aldahmash et al., 2011; Bieback et al., 2012; Chimenti et al., 2014; Stern-Straeter et al., 2014), and (iii) atmospheric oxygen conditions during culture and handling (Grayson et al., 2006; Klepsch et al., 2013; Ito et al., 2015). Not solely in culture expansion, serum is also often used in cryopreservation. The use of animal-derived serum bears contamination risks and lot-to-lot variability. For clinical translation, it is therefore reasoned to replace animal-derived supplements (Tekkatte et al., 2011). Using autologous human serum has been proposed (Stute et al., 2004). Arguments such as high costs and the high likelihood of factors in the blood of

donors, which may dominantly impact on MSC growth, greatly promoted the development of serum-free, chemically defined media (Mimura et al., 2011; Chase et al., 2012; Li et al., 2015). Alternatively, standardized human blood-derived products have been proposed (Díez et al., 2015; Riordan et al., 2015). Still unresolved and seemingly important in establishing MSC for therapeutic application is the definition of potency assays, that address patient's age.

MOVING INTO CLINICS

In recent years, more than 500 clinical trials employing MSC for the treatment of various diseases have been registered worldwide (http://www.clinicaltrials.gov, 60 thereof in Europe www.clinicaltrialsregister.eu). Besides applications in musculoskeletal defects and trauma MSC are also widely tested in pathologies, which are of immunological etiology such as graft-vs.-host disease or multiple sclerosis, lupus, diabetes type I, and Crone's disease. The reason is, MSC exhibit dominant immune-modulatory properties (Castro-Manrreza and Montesinos, 2015). More and more details regarding the underlying molecular mechanisms and cellular interactions how MSC control immune competent cells are being unraveled (Glenn and Whartenby, 2014). MSCs are also tested for liver and heart pathologies as well as ocular diseases (Li et al., 2014b).

Clinical Trial Cohort Variability

Many approaches are still exploratory and many ongoing clinical trials are still in Phase I Most strategies were carefully validated in diligently designed preclinical tests. However, commencing clinical trials firstly address safety issues thus rarely corroborating results cannot be expected. Early phase clinical studies also often comprise small heterogeneous groups exhibiting variant health status, age and ethnicity. Also sex-specific differences appear to be of relevance (Tajiri et al., 2014). For example, MSC from female bone marrow could be smaller and lower in number (Zanotti et al., 2014) but divided more rapidly than found for most male MSC. They had higher clonogenic activity and exhibited enhanced expression of the surface antigens, CD119 and CD130 (Siegel et al., 2013). Furthermore, functional differences were reported to be sexually dimorphic. Suppression of T-lymphocyte proliferation is more marked in females, while male-derived MSC possess a more robust osteogenic activity (Siegel et al., 2013; Ranganathan et al., 2014; Park et al., 2015). Female MSC showed a higher resistance in endotoxic and hypoxic injury models, inferring that improved survival in adverse micro-environments may be dependent on sex steroids. Indeed estrogen and estradiol exert a protective activity against apoptosis, favor proliferation, and delay the onset of senescence (Huang et al., 2013; Li et al., 2014c; Sung et al., 2015).

Most trials refrain from stratifying into age groups for obvious reasons of addressing a specific pathology rather than a gerontological principle. Animal trials are mostly done on young animals; clinical trials are rarely concerned with young or middle aged adults. MSC for animal experimentation are most often isolated from young animals, instead in humans autologous approaches are performed mostly in aged individuals. Further concerns are the greatly varying cell isolation and expansion procedures, as the outcome of a clinical trial is pertinently influenced by the quality of a cellular product. In fact, the population of cells harvested from bone marrow aspirates is very heterogeneous, consisting only of 0.001–0.01% mesenchymal precursor cells. To gain sufficient numbers of potential stem cells, the isolates are purified for subsequent *in vitro* expansion. At that point the uncertainty arises whether replicating cells accumulate damage which turns them senescent (Bonab et al., 2006). Suffice it to say that the recommendation is to restrict applications to replicating young MSC.

MSC Aging: Cellular Changes and Determinants

First animal studies showed, already 10 years ago, that the transplantation of aged rat MSC was less effective (Zhang et al., 2005). Hence, the vexed question arose whether donor age influences the therapeutic efficacy in clinical trials (Wang et al., 2013b). MSC derived from old patients exhibit reduced proliferative capacity and in some cases show skewed multi-lineage differentiation potentials, telomere shortening, or DNA damage accumulation (Behrens et al., 2014; Efimenko et al., 2014; Kizilay Mancini et al., 2015; Reitinger et al., 2015). They also exhibit increased levels of reactive oxygen species and nitric oxide, lower superoxydismutase activity senescence-associated β-galactosidase activity, enlarged morphology, and p53 protein upregulation. These observations confirm the doubts that aged cells may only be acceptable for transplantation if specially treated or selected (Kornicka et al., 2015). In cases of autologous cell-based applications, constitution, and health status of the patient may pertinently impact on the therapeutic outcome. Changes in MSC morphology, proliferation capacity, senescence, and multi-lineage potential are linked to advanced age but could be also induced by several disease conditions, thus compromising their therapeutic potencies (Sethe et al., 2006; Choudhery et al., 2014; Escacena et al., 2015).

It is aged people, who are the primary targets for stem cell therapy. Provided putative deviations, procedures selecting for flawless cells are potentially required before applying aged cells in autologous therapy. Allogenic transplantation of stem cells derived from young donors are considered to potentially overcome aging-related limitations (**Figure 1**).

It is further conceivable that the epigenetic status and/or aging as well as pathology-specific changes of cells may be different when isolating MSC from different tissues. It is currently unclear how to predict the optimal MSC source in order to warrant an optimal outcome (Golpanian et al., 2015). Interventions provoking rejuvenation of MSC have therefore been proposed. Extracellular factors, such as oxygen tension or redox status, exert effects on MSC aging (De Barros et al., 2013; Bigot et al., 2015; Sart et al., 2015).

Microenvironmental conditions, epigenetic processes including DNA methylation, telomerase activity, microRNA as well as specific growth factor signaling actively modulate MSC fate (Madonna et al., 2013; Jing et al., 2015; Oh et al., 2015; Okada et al., 2015). For their role in MSC biology, all these aspects provide challenging means to further enhance stem cell efficacy.

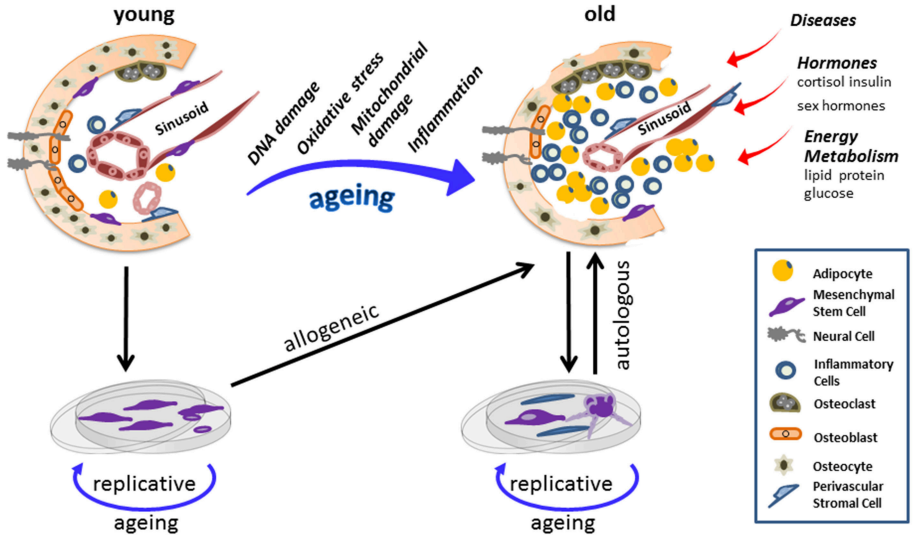

FIGURE 1 | Stem-cell populations are maintained in "niches." Stem cells and the corresponding niche entertain dynamic interactions controlling sustaining tissues regeneration and repair, yet also regulate somatic maintenance. Here a scheme of a bone marrow niche is provided and its potential changes as a consequence of the aging process. Chronological aging diminishes adult stem cells fitness and on the long run induces cellular and anatomical modifications within the niche. In old age mesenchymal stem cell (MSC) population has shrunken, while the number of inflammatory and fat cells increased to a large extent. Alongside, other extracellular factors also influence the aging process of stem cells together with their corresponding niche. Explanation of stem cells and *in vitro* amplifications adds risks of replicative aging to stem cell and progenitor fates. Hence when reintroducing cells in an autologous way, which is more likely undertaken as patients are more often of advanced age, chronologically old/replicative aged cells are applied. Conclusively, aberrations within a stem cell niche, as a consequence of aging, impose both chronological and replicative aging on therapeutic MSC, potentially compromising the success of autologous transplantation in aged donors.

Yet to date methods need to reliably prompt efficacy before being implemented in cell production at authorized manufacturing sites (Oh et al., 2014). Measuring the cellular fitness of MSC has been suggested being a invaluable prognostic value for enhancing the therapeutic success (Wagner et al., 2010). This complex question may however only be tackled by generating a comprehensive database for comparative purposes that stores information on source and MSC characteristics derived from different disease conditions. It is very likely that a pathological state perturbs the tissue milieu (niche) where MSC reside (Mastri et al., 2014). A systemically challenged micro-environment will impinge on the functional integrity of MSC, acknowledging the widely accepted principle of a stem cell niche (Krinner and Roeder, 2014). MSC niche interactions appear to be of mutual benefit since MSC release a wide range of bioactive factors that confer trophic and immune-modulatory effects, such as cytokines and distinct forms of miRNA and tRNA species (Hsiao et al., 2012; Baglio et al., 2015). More and more reports have unraveled the potent features of the MSC secretome, which now is thought to become by itself a regenerative therapeutical tool (Sdrimas and Kourembanas, 2014; Gallina et al., 2015; Succar et al., 2015; Tran and Damaser, 2015).

Considering the knowledge that the patient's disease state pertinently affects MSC functionality, the impact of medication on MSC, be it before or after implantation, is largely neglected and little data are available. MSC are greatly resistant to chemotherapy agents and therefore, therapies in patients undergoing cancer treatment are conceivable (Beane et al., 2014b; Bosco et al., 2015; Yoon et al., 2015) but have yet been not consistently successful (Buttiglieri et al., 2011; Choron et al., 2015). Medications, such as immune suppressants, glucocorticoids, psychopharmaceutics, and contrast agents may interfere with cell viability and proliferation capacity (Georgiou et al., 2012; Jansen Of Lorkeers et al., 2014; Schneider et al., 2015; Tang et al., 2015; Tsuji et al., 2015). These studies provided preliminary though valuable evidences, suggesting the need of more consistent drug interaction studies with special attention given to both short and long-term observations.

CONCLUSION

MSC are considered "work horses" for cell-based therapy. This is because very little ethical concerns have been raised and it is now widely accepted that MSC are largely resistant to malignant transformation. Being ubiquitously present in many tissues, MSC could be successfully isolated from cord blood, fat, skeletal muscles, dental pulp, and several other sources (Ogura et al., 2014). Despite all positive aspects and the remarkable progress in stem cell research, many decisive issues remain to be resolved. Difficult standardization on one hand, the persisting ambiguity regarding MSC-specific markers and the technical challenges with orthotopic transplantations are hampering experimental validations. It is important to unambiguously prove that stromal tissues of various origins contain stromal stem progenitor cells. In contrast to bone marrow-derived MSC, most experimental procedures aiming at identifying MSC in different tissues were solely based on *in vitro* data regarding non-clonal culture and multi-lineage differentiation. Therefore,

the true stem cell character of these MSC has yet not be fully proven (Bhartiya, 2013). For these reasons, their physiology and functions *in vivo* are still scarcely known. Currently, information regarding biological and molecular features, as well as the dominant influences on their surrounding tissue environment, appears insufficient (Murray et al., 2014; New et al., 2015). In fact, sharing the same immune phenotype may be a deceptive indicator for predicting cellular function. Further "known unknowns" are details about migratory properties of MSC. MSC are likely incapable of entering the circulation (Hoogduijn et al., 2014). However, short-distance migration into adjacent tissues is conceivable (Vanden Berg-Foels, 2014), implying that a population of MSC in any given tissue might indeed represent a mixture of local and "migrant" MSC.

In light of these considerations, the question whether special MSC sources are putatively more potent in specific disease treatments requires in depth studies, addressing comparative measures of therapeutic efficacy and safety. Several studies albeit designed and performed in a comparable fashion reported that superficially equal MSC resulted in different outcomes *in vivo* (Meraviglia et al., 2014; Wang et al., 2014; Reinisch et al., 2015). To overcome the present discrepancies, US Food and Drug Administration (FDA) suggested building a database, in which data from MSC subjected to varying culture conditions, derived from diverse tissues, and donors should be compiled.

Once safety of MSC administration can be granted, the next step is to obtain consistent results on sustained curative benefit. Inconsistency or failure in clinical outcomes might not be solely related to MSC preparations, but also attributable to disparate therapeutic protocols. The optimal route of MSC delivery and dosage regime are still debated (Kean et al., 2013; Richardson et al., 2013; Chang et al., 2014; Yavagal et al., 2014). The success of a systemic delivery depends very much on the ability of MSC to home to the site of injury and to access target tissues in case of a damaged or still regenerating vascularization (Cerri et al., 2015). Efficacious outcomes after intravenous delivery have repeatedly been reported (Semedo et al., 2009; Cruz et al., 2015; Rapp et al., 2015). Needless to say that topic administration could be more successful (Antunes et al., 2014; Ishihara et al., 2014; Cerri et al., 2015; Huang et al., 2015).

Specification and definition of efficacy is another pending question to be answered. Multi-lineage differentiation *in vitro* has often been employed to deliberately grant *in vivo* efficacy. As the one has very little to do with the other, in particular because differentiation *in vitro* is performed under tightly controlled culture conditions, better methods have been conceived. *In vivo*, MSC differentiation or complex functions certainly depend on a plethora of parameters, which can be hardly reconstructed *in vitro*. Direct interactions with micro-vascular structures as well as with other cell types, such as endothelial cells appear to play stimulating and inducing roles (Chen et al., 2015; da Silva Meirelles et al., 2015; Tsai et al., 2015). Working along this line, the ability of MSC to suppress T- and B-lymphocytes proliferation and to produce lymphokines was exploited to predict MSC efficacy *in vivo* (Collins et al., 2014; Del Fattore et al., 2015). But once more controversial results have been reported (Sajic et al., 2012).

This pointed at establishing superior methods to verify the functional capacity of MSC prior clinical applications. Thus, tests in living animal were proposed to distinctly assay intrinsic capacities to differentiate into functional tissues or to exert other desired functions. Sequential heterotopic transplantation has proven to fulfill this need. Generation of heterotopic ossicles has demonstrated not only skeletogenic potentials of single clone progenitors but also self-renewal ability. It must be stressed that *in vivo* functional assays also provided means and measures to simultaneously acquire long-term safety data. There is a lack of suitable long-lived models, which would allow the sensitive risk assessment of MSC or its secretome in inducing malignancy (Bruno et al., 2014; Arango-Rodriguez et al., 2015; Schweizer et al., 2015).

A major caveat in most clinical MSC applications is the lack of long-term follow ups. Such studies would reveal those parameters that are essential in determining risk and safety issues in cell therapy. Despite some recent attempts (Brizuela et al., 2014; Caminal et al., 2014; Ciccocioppo et al., 2015; Daltro et al., 2015; Davatchi et al., 2015), we are currently far from a systematic and robust analysis. Only little attention has been given to acquire information on survival after implantation, on the impact of MSC cell therapy on other tissues or on unintended alterations in target tissue as well as in the tissue-borne MSC population itself. Implementation of MSC in therapies raises many practical questions which are beyond scientific consideration. Presently most trials are on a small-scale and mainly performed by academic centers. To acquire reliable and precise information on how to efficiently introduce MSC in clinical therapy, larger trials are needed with the active involvement of industry and the support of interdisciplinary teams.

AUTHOR CONTRIBUTIONS

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REVIEW
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Understanding How the Subcommissural Organ and Other Periventricular Secretory Structures Contribute via the Cerebrospinal Fluid to Neurogenesis

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The dynamic and molecular composition of the cerebrospinal fluid (CSF) and, consequently, the CSF physiology is much more complex and fascinating than the simplistic view held for decades. Signal molecules either transported from blood to CSF or secreted into the CSF by circumventricular organs and CSF-contacting neurons, use the CSF to reach their targets in the brain, including the pre- and postnatal neurogenic niche. The subcommissural organ (SCO), a highly conserved brain gland present throughout the vertebrate phylum, is one of the sources for signals, as well as the choroid plexus, tanycytes and CSF-contacting neurons. The SCO secretes into the fetal and adult CSF SCO-spondin, transthyretin, and basic fibroblast growth factor. These proteins participate in certain aspects of neurogenesis, such as cell cycle of neural stem cells, neuronal differentiation, and axon pathfinding. Through the CSF, the SCO-secretory proteins may reach virtually any target in the embryonic and adult central nervous system. Since the SCO continues to secrete throughout life span, it seems likely that the neurogenetic property of the SCO compounds would be targeted to the niches where neurogenesis continues in adulthood. This review is aimed to bring into discussion early and new evidence concerning the role(s) of the SCO, and the probable mechanisms by which SCO compounds can readily reach the neurogenic niche of the subventricular zone flowing with the CSF to participate in the regulation of the neurogenic niche. As we unfold the multiples trans-fluid talks between discrete brain domains we will have more tools to influence such talks.

Keywords: cerebrospinal fluid, circumventricular organs, CSF-contacting neurons, subcommissural organ, SCO-spondin, transthyretin, integrins, neurogenesis

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INTRODUCTION

The identification of neural stem cells (NSCs) in the adult central nervous system closed down a long-held dogma that neurons are formed exclusively during brain development. The mammalian brain retains the capacity to generate new neurons throughout life in two main locations, the subventricular zone (SVZ) of the lateral ventricles and the hippocampal dentate gyrus (Alvarez-Buylla and Garcia-Verdugo, 2002; Gage, 2002).

Abbreviations: CSF, cerebrospinal fluid; ECM, extracellular matrix; FGF, fibroblast growth factor; NSCs, neural stem cells; RF, Reissner fiber; SCO, subcommissural organ; SVZ, subventricular zone; TTR, transthyretin; VZ, ventricular zone.

The cellular and molecular mechanisms that guide the progression from a dividing NSCs to a functional neuron are far from being understood. A series of components of the neurogenic niche has been identified, including cell–cell interactions, secretory factors, vascular requirements, and specific innervation (Hagg, 2009; Pathania et al., 2010; Faigle and Song, 2013). However, CSF-born signals have largely been overlooked (see below). Key questions remain unsolved. What does control where and how adult neurogenesis occur? Which are the mechanisms and signals underlying neuronal migration, in-fate integration and function? Which are the sources of these signals? How do these signals reach their target?

The design of the CSF-neurogenic niche interphase, i.e., NSC projecting a process to the CSF and bearing a 9+0 cilium, neighboring bi-ciliated and multiciliated cells organized as spatial units around the NSC process (Merkle et al., 2007; Mirzadeh et al., 2008), and the numerous neurotropic, mitogenic, and morphogenic factors, secreted into the CSF, suggest that the CSF should be regarded as a key pathway conveying signals to the pre- and postnatal neurogenic niche. However, this promising research field has largely been neglected. This review aims (1) to bring together early and recent information on the CSF as an integrative pathway; (2) to provide information to understand how the SCO, an ancient brain gland, and other periventricular secretory structures, may contribute to the regulation of embryonic and adult neurogenesis.

THE CEREBROSPINAL FLUID (CSF), A PATHWAY FOR THE DELIVERY OF FACTORS THROUGHOUT THE BRAIN

The CSF results from the secretion by the choroid plexuses and the bulk flow of the interstitial fluid of brain parenchyma to the ventricles and to the subarachnoid space. In humans, approximately 600 ml of CSF is produced each day. The rate of CSF production displays circadian variations, with lowest levels around 06:00 PM and a nightly peak at about 02:00 AM (Nilsson et al., 1992). The CSF moves along the ventricles and subarachnoid space driven by two mechanisms. The bulk of CSF moves from the main site of origin, the choroid plexus of the lateral ventricles, to the sites of reabsorption. Pulsation of large brain arteries contribute to this bulk flow (Iliff et al., 2013). The laminar flow is a supra-ependymal compartment, about 200 μm thick, where the CSF flow is driven by the cilia beating of multiciliated ependyma (Worthington and Cathcart, 1966; Cifuentes et al., 1994; Siyahhan et al., 2014). Molecular, cell biology and neuroimaging research indicates that CSF physiology is more complex than formerly thought. Aspects now being examined include the various sites of CSF formation and re-absorption, CSF proteomic and the changing CSF composition along its pathway (Brinker et al., 2014; Orešković and Klarica, 2014).

Cerebrospinal fluid proteomics is showing a wealth of over 200 proteins (Zappaterra et al., 2007). A long series of peptides and neurotransmitters are also present in the CSF. Some of these compounds move by bulk flow from the interstitial fluid of brain parenchyma, many are secreted by neurons, glia, and ependyma into the CSF, others are transported by specific transport systems from blood to ventricular CSF (choroid plexus) while a few of them originate from cells present in the CSF.

The CSF is a heterogeneous and highly dynamic compartment that changes its molecular composition as it unidirectionally moves through the various ventricular and subarachnoidal compartments. The choroid plexus of the lateral ventricles, the interstitial fluid of the parenchyma surrounding these ventricles and axons endings secreting into these cavities are the source of molecules forming this "first" fluid. At the third ventricle new compounds are added to the CSF by hypothalamic neurons, the pineal gland and the local choroid plexus (Rodríguez, 1976; Nicholson, 1999; Johanson et al., 2008). When entering the Sylvius aqueduct the CSF is enriched by the secretion of the SCO (Vío et al., 2008). Consequently, the CSF of the fourth ventricle is different as compared to that of the lateral ventricles (Zappaterra et al., 2007). This partially explains the different protein composition between the CSF collected from the lateral ventricles and that obtained from a subarachnoid compartment (Vío et al., 2008). Furthermore, at the interphase brain parenchyma/subarachnoid space there is a bidirectional flow of CSF and interstitial fluid along the large paravascular spaces that surround the penetrating arteries and the draining veins. Since water movement along this pathway is mediated by astroglial aquaporin-4 water channels, this paravascular pathway has been termed "glymphatic system" (Iliff et al., 2012, 2013).This pathway facilitates efficient clearance of interstitial solutes and its failure may lead to neurodegeneration (Iliff et al., 2015).

The long series of biologically active proteins, peptides, and neurotransmitters present in the CSF reach this fluid through different mechanisms. (1) Neurotransmitters and their metabolites reach the CSF via the bulk flow of parenchymal fluid. (2) Regulated secretion into the CSF of biologically active compounds by the circumventricular organs (SCO, pineal gland, choroid plexuses, and median eminence), such as SCO-spondin, basic FGF, melatonin, TTR, TTR-T4 complex, TTR-T3 complex, nerve growth factor (NGF), transforming growth factor-β (TGFβ), vascular endothelial growth factor (VEGF), transferrin, and vasopressin (Gross, 1987; Johanson et al., 2008; Rodríguez et al., 2010; Johansson, 2014; **Figure 1**). (3) Selective and circadianly regulated secretion by CSF-contacting neurons of serotonin and neuropeptides such as vasopressin, oxytocin, and somatostatin (Rodríguez, 1976; Vigh-Teichmann and Vigh, 1989; Vígh et al., 2004). (4) Transport of peripheral hormones through the choroid plexus. Most of the transported hormones, such as leptin, prolactin, and thyroxin have specific targets, mostly the hypothalamus (Chodobski and Szmydynger-Chodobska, 2001; Rodríguez et al., 2010; **Figure 1**). Furthermore, recent findings indicate that cells forming the ventricular walls release into the CSF microvesicles containing signaling and intracellular proteins (Marzesco et al., 2005; Street et al., 2012; Chiasserini et al., 2014; Feliciano et al., 2014).

Thus, the early view that the CSF is a medium carrying brain-borne and blood-borne signals to distant targets within the brain (Rodríguez, 1976) has largely been supported by numerous investigations (Wood, 1983; Johnson and Gross, 1993; Johanson

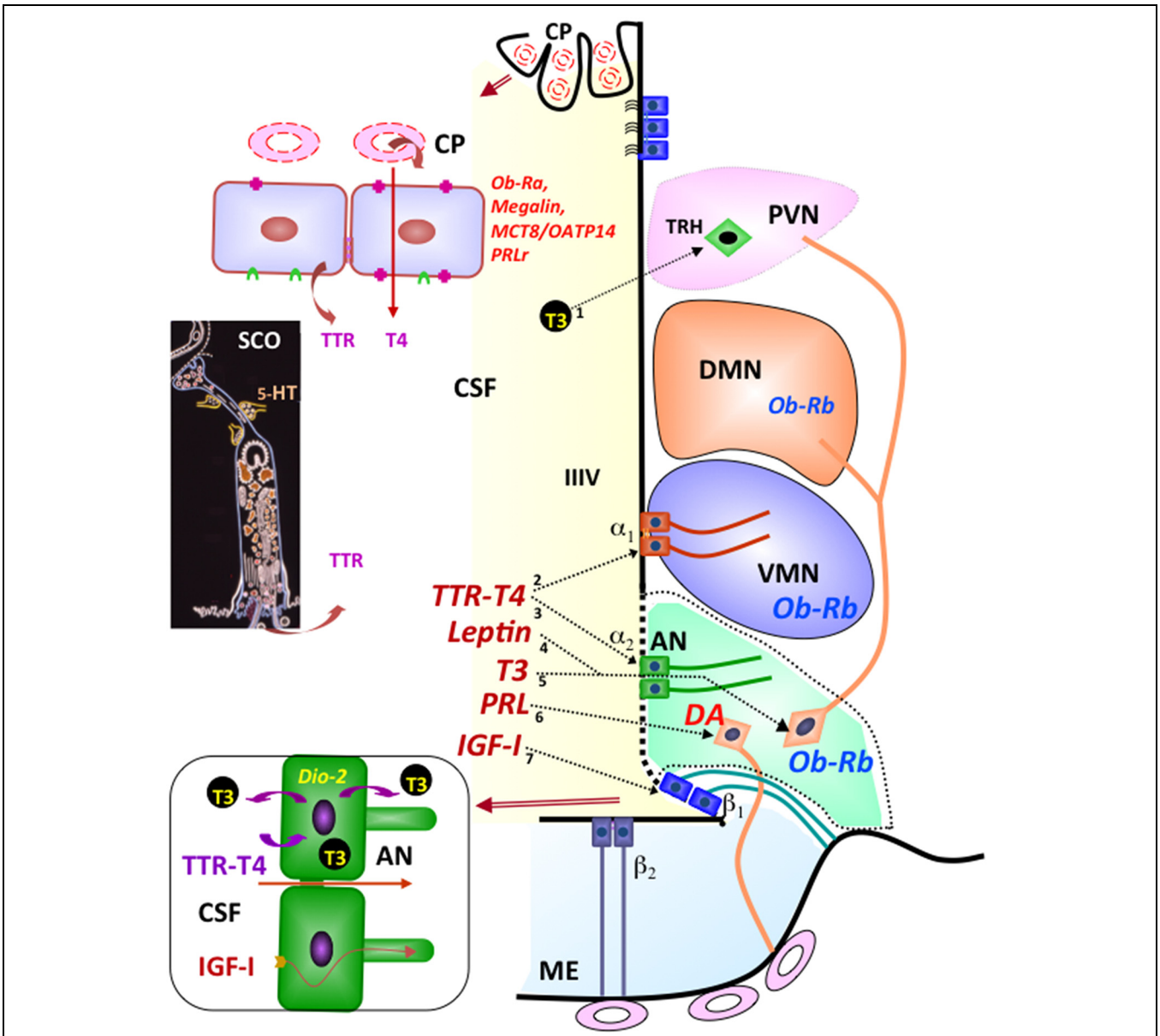

FIGURE 1 | Integrative pathways involving the CSF. By receptor mediated transport at the choroid plexus (CP), leptin (Ob-Ra), insulin growth factor I (megalin), thyroid hormones (MCT8/OATP14), and prolactin (PRLr) are transported from blood to CSF. Transthyretin (TTR) is secreted by choroid plexus and the subcommissural organ (SCO) into the CSF. The secretory activity of the SCO is under serotonin (5-HT) inhibitory control. Most CSF T4 is bound to TTR. TTR-T4 complexes are taken up by tanycytes that express deiodinase 2 (arrows 2, 3). Here (bottom left panel), T4 is converted to T3 and then released into the intercellular space of the arcuate nucleus (arrow 5) or into the CSF to reach the TRH-parvocellular neurons of the paraventricular nucleus (arrow 1). The milieu of the arcuate nucleus (AN; green background) is especially exposed to molecules present in the CSF and closed to the median eminence (ME) and ventromedial nucleus (VMN). Leptin present in the CSF may readily reach the neurons expressing the Ob-Rh receptor of the arcuate (arrow 4), ventromedial and dorsomedial nuclei of the hypothalamus. CSF prolactin (arrow 6) may reach the dopamine-secreting neurons (DA) of the arcuate nucleus that project to the portal capillaries of the median eminence (light-blue background). CSF insulin growth factor I (arrow 7) is internalized by β tanycytes and transported along their processes. Modified after Rodríguez et al. (2010).

et al., 2008; Rodríguez et al., 2010). Worth mentioning here is the much neglected system of CSF-contacting neurons most likely playing receptive functions sensing CSF composition. Most of these neurons are bipolar with the dendritic process reaching the CSF and endowed with a 9+0 single cilium (Vígh et al., 2004; **Figure 4D**).

THE SUBCOMMISSURAL ORGAN

The SCO is an ancient and highly conserved brain gland present throughout evolution of chordates, from amphioxus (Rodríguez and Oksche, 1993; Olsson et al., 1994) to man (Rodríguez et al., 2001; **Figures 2A–E**). The astonishing amphioxus, an

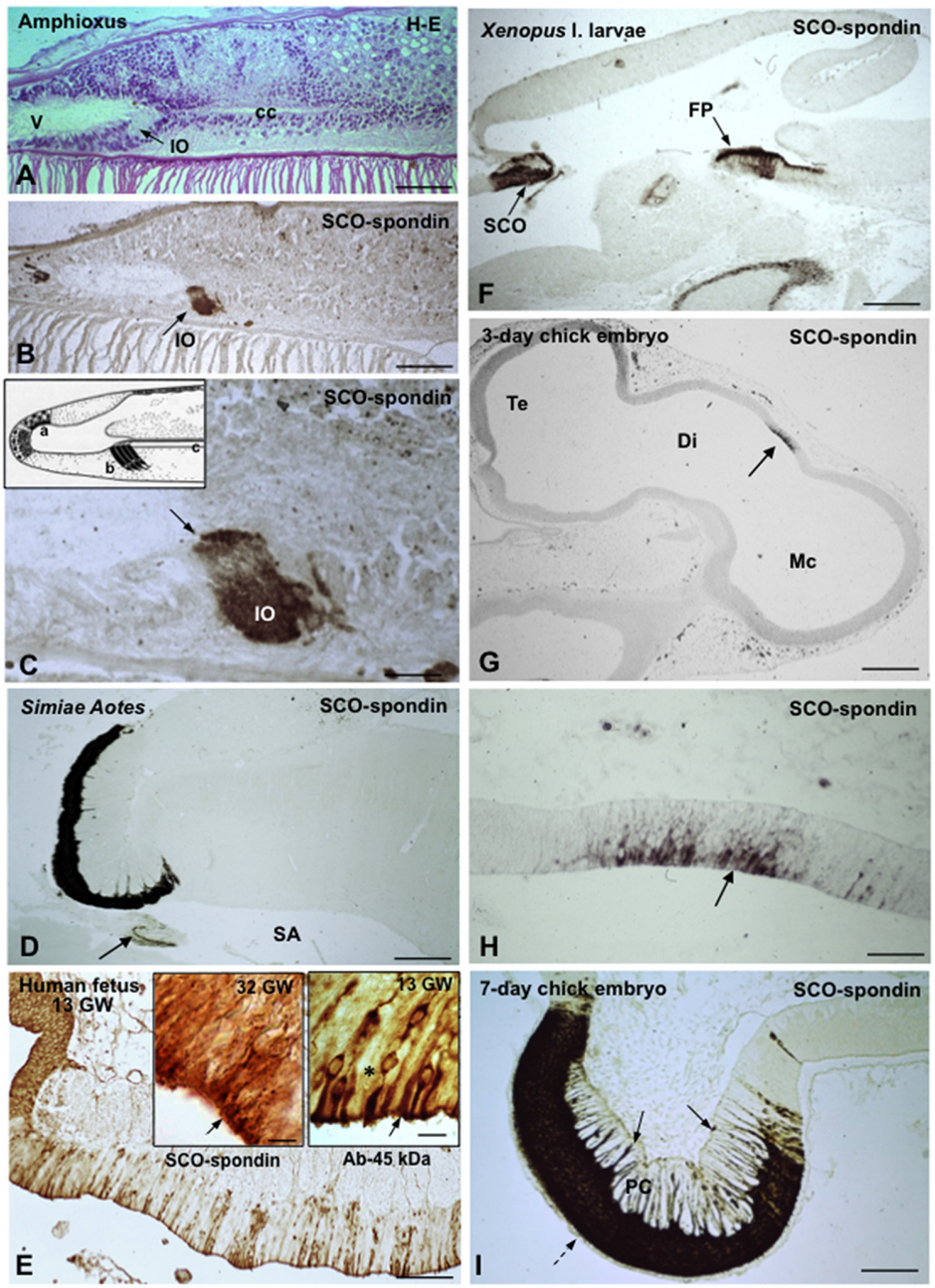

FIGURE 2 | The subcommissural organ is the phylogenetically oldest brain gland and the first to differentiate in ontogeny. (A–E) From amphioxus to primates, 500 million years of evolution. **(A–C)** Sagittal sections through the CNS of the amphioxus (*Branchiostoma lanceolatum*, Acrania), showing the location **(A)** and immunoreactivity **(B,C)** of the cells forming the Infundibular organ (IO). V, ventricle; cc, central canal; from Olsson et al. (1994). **(C)** Line drawing of the CNS of the amphioxus showing a secretory ependyma in the recessus neuroporicus (a), the infundibular organ (b) and the central canal with Reissner fiber (c); from Olsson and Wingstrand (1954). **(D)** Subcommissural organ and Reissner fiber (arrow) of the primate Aotes. SA, sylvius aqueduct; from Rodríguez et al. (1993). **(E)** Sagittal section through the epithalamus of a 13-weeks-old human fetus immunostained with an antiserum against a 45 kDa compound (most likely corresponding to TTR) obtained from the CSF of a hydrocephalic fetus. A population of ependymocytes are strongly immunoreactive; from Rodríguez et al. (1993). **Right inset** detailed magnification of previous figure showing immunoreactive (arrow) and immunonegative (asterisk) ependymal cells; **left inset** SCO from a 32 GW fetus immunostained for SCO-spondin; all cells are immunoreactive (arrow). **(F)** Sagittal section through the CNS of a *Xenopus* l larvae. The cells of the subcommissural organ (SCO) and the floor plate (FP) strongly express SCO-spondin. **(G)** Sagittal section through the CNS of a 3-days-old chick embryo. A small group of neuroependymal cells located a the roof of the diencephalic vesicle (Di) expresses SCO-spondin (arrow). Te, telencephalon; Mc, mesencephalon. **(H)** Detailed view of previous figure showing that SCO-spondin is mainly located in the apical region of the neuroependymal cells (arrow). **(i)** At the 7th day of incubation, the chick SCO is fully differentiated with SCO-spondin located in the cell body of ependymocytes (broken arrow) and along their basal processes ending at the pial membrane (full arrows). PC, posterior commissure; from Schoebitz et al. (1986). Scale bars: **(A,B)** 80 μm; **(C)** 16 μm; **(D)** 400 μm; **(E)** 100 μm; right Inset 9 μm; left inset 8 μm; **(F)** 300 μm; **(G)** 280 μm; **(H)** 56 μm; **(I)** 85 μm.

evolutionary leap made at the bottom of the ocean over 500 million years ago, already has a small group of cells secreting a very thin Reissner fiber (RF) (Olsson and Wingstrand, 1954; **Figure 2C**, inset) that immunoreacts with antibodies against mammalian SCO-spondin (Olsson et al., 1994). The ancient SCO-spondin-secreting cells symbolize a family resemblance between amphioxus and primates (compare **Figures 2B,D**). SCO-spondin could be considered a member of an exclusive group of proteins accompanying the brain through its long lasting evolution what, in turn, highlight the functional significance of this molecule.

In ontogeny, the SCO is one of the first brain structure to differentiate (Schoebitz et al., 1986; **Figures 2F–I**). In the human, the SCO can be morphologically distinguished in 7-weeks-old embryos. By the 13th gestational week (**Figure 2E**), the SCO is a fully differentiated gland that remains secretory active throughout the fetal life, releasing CSF-soluble proteins (Rodríguez et al., 2001). During childhood the secretory parenchyma of the SCO is confined to islets of secretory ependymal cells. In non-human species, the SCO is a highly differentiated gland during most of the fetal period and throughout life span (Rodríguez et al., 1984a; Schoebitz et al., 1986, 1993; **Figure 2D**).

The SCO is located in the dorsocaudal region of the third ventricle, at the entrance of the Sylvian aqueduct (**Figures 3A,B**). The secretory cells of the SCO are arranged into two different layers, the ependyma and the hypendyma.

The ependymal cells of the SCO are bipolar, with and apical pole contacting the ventricular CSF and a basal process projecting to local capillaries and to the subarachnoid space (Leonhardt, 1980; Rodríguez et al., 1992, 2001; **Figure 3D**). The cell body presents a clear zonation, which has facilitated the investigation of the secretory process. Different phases of this process occur in discrete but separate areas of the cell, namely, (1) synthesis in the perinuclear and intermediate regions, (2) storage of precursor forms in big RER cisternae located in the subnuclear region, (3) processing and packaging in the intermediate region, (4) transport in the subapical region, (5) storage of processed forms and release in the apical cell pole (Rodríguez et al., 1992, 2001; **Figures 3C,D**). Further, the SCO offers a unique feature: the secretory material upon release condenses, first as a film on the surface of the organ and then, after further packaging, into RF (**Figures 3D–F**). Most of the ultrastructural characteristics of the hypendymal cells are similar to those described for the ependymal cells.

In non-mammalian species all ependymal cells of the SCO display long and slender processes that traverse the posterior commissure and end on the external basement membrane of the brain (**Figure 2I**). Their terminals are loaded with secretory granules. The most likely fate of this secretion is the local leptomeningeal cistern (there is no continuous subarachnoid space in non-mammalian species). In mammals, the basal processes of the SCO cells containing secretory granules either project to the subarachnoid space or to the subependymal capillaries. Here, the processes end on a network of extensions of the perivascular basement membrane formed by long-spacing collagen, a unique arrangement and a landmark of the SCO (Rodríguez et al., 1992, 2001). The basal processes of ependymal and hypendymal cells receive abundant synaptic contacts of various nature (see innervation below; **Figure 3D**).

The whole arrangement of the SCO cells indicates that (i) they secrete compounds to the ventricular CSF, the subarachnoidal CSF and probably to blood; (ii) this secretory activity is under neural control. The nature of the compounds secreted into ventricular CSF is only partially known (i.e., SCO-spondin, TTR and probably basic FGF), whilst that of the compounds contained in the secretory granules stored at the perivascular and subarachnoidal ependymal terminals is unknown.

In most circumventricular organs the blood-brain-barrier has been displaced from the vascular side to the ependymal side so that they are open to blood and tightly closed to both the CSF and the neighboring neural parenchyma (see Rodríguez et al., 2010). Due to the design of its barriers, the SCO is closed to blood and to the CSF, becoming a sort of an *island within the brain* (Rodríguez et al., 1992, 1998). The functional meaning of this unique arrangement is unknown.

THE SECRETORY PRODUCTS OF THE SUBCOMMISSURAL ORGAN

The SCO secretes into the ventricular CSF two classes of proteins, the ones that remain soluble in the CSF and that, consequently, go with the flow and those that aggregate to form an insoluble, ever-growing structure, the RF (**Figures 3A,B**).

RF-Glycoproteins

The ependymal cells secrete *N*-linked glycoproteins of high molecular mass that, upon release undergo a progressive packaging until forming a fully packaged RF in the postnatal life (Sterba, 1969; Nualart et al., 1991). By addition of newly released glycoproteins to its proximal end, RF grows caudally and extends along the aqueduct, fourth ventricle, and the whole length of the central canal of the spinal cord (Sterba, 1969; Leonhardt, 1980; Caprile et al., 2003; **Figures 3B,D,F**). RF material continuously arrives at the dilated caudal end of the central canal, known as the terminal ventricle or ampulla, where RF-glycoproteins undergo chemical modifications (loss of sialic acid residues), disaggregate and then escape through openings in the dorsal wall of the ampulla to finally reach local vessels (Olsson, 1958; Peruzzo et al., 1987; Rodríguez et al., 1987).

SCO-Spondin

Molecular procedures have led to the identification of SCO-spondin as a multidomain, large-molecular mass glycoprotein (540 kDa) secreted by the SCO into the ventricular CSF, where it contributes to form the RF (Nualart et al., 1991; Gobron et al., 1996; Meiniel, 2001; see further below; **Figures 3A,B**). At variance with SCO-spondin forming RF, there are compounds of 200, 63, 50, and 25 kDa molecular mass that are consistently found in the CSF of rodents (Vío et al., 2008) and humans (**Figure 3A**, inset). These compounds react with specific antibodies against SCO-spondin and most likely result from a further processing

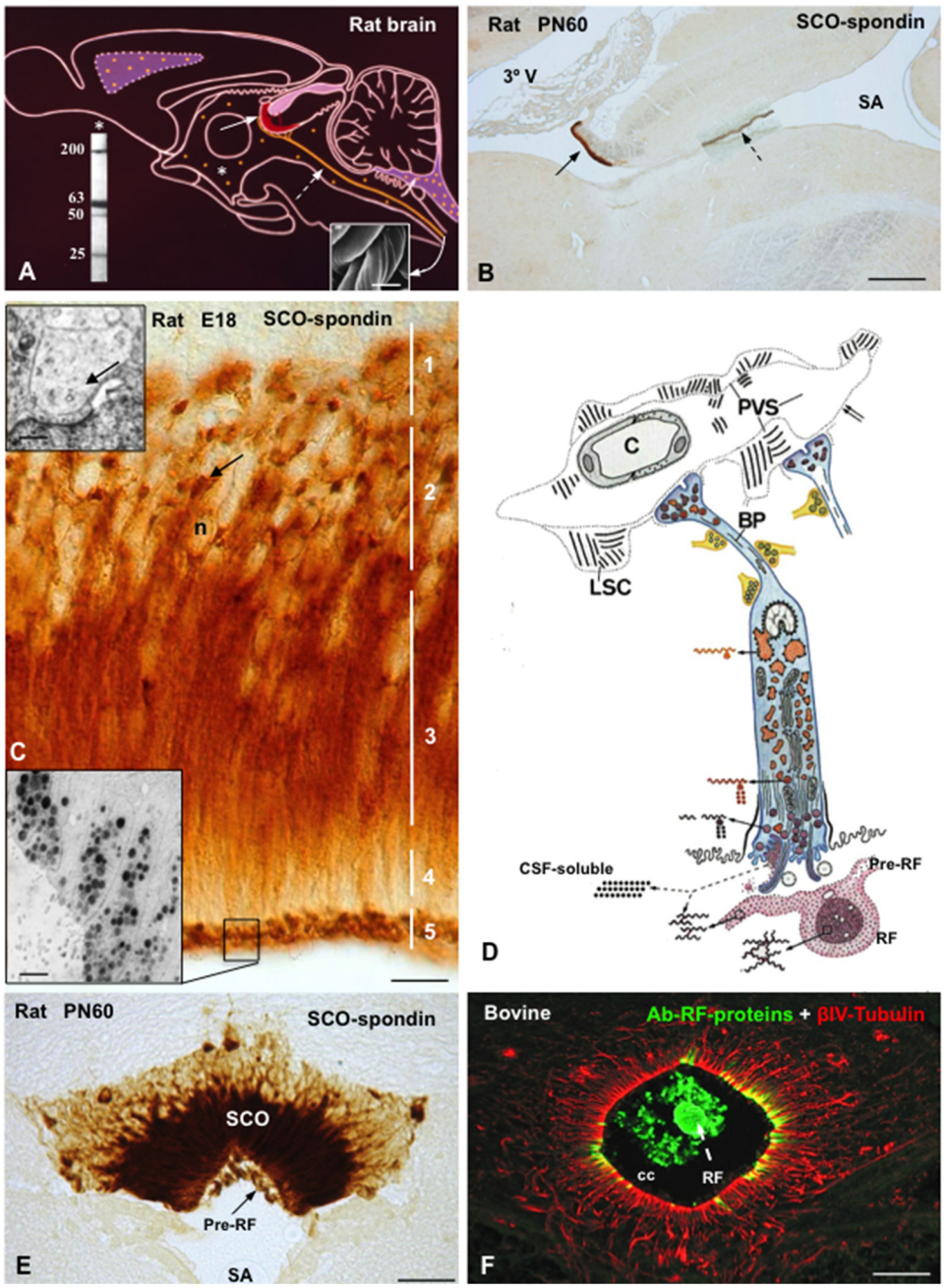

FIGURE 3 | The subcommissural organ-Reissner fiber complex. (A) Drawing depicting the rat subcommissural organ (red, full arrow)- Reissner fiber (orange, broken arrow) complex and the CSF-soluble secretion (orange dots, asterisk). (Right inset) Scanning electron microscopy of bovine RF collected from the central canal. Left inset. Western blot of CSF of PN30 rats, immunoreacted with antibodies against SCO-spondin. CSF-soluble compounds of 200, 63, 50, and 25 kDa of molecular weight are shown. **(B)** Sagittal section of a rat brain immunostained with anti-SCO-spondin at postnatal day 60. The SCO (full arrow)-RF (broken arrow) complex is selectively immunoreactive. **(C)** High magnification of the SCO of a rat embryo (E18) immunostained with anti-SCO-spondin. Zonation of a SCO-cell (1–5) is shown. Arrow points to paranuclear immunoreactive masses corresponding to RER. Upper inset. Electron microscopy of dilated RER cisternae (arrow). Lower inset. Electron microscopy immunocytochemistry using anti-SCO spondin showing secretory granules stored at the apical cell pole. **(D)** Drawing depicting the ultrastructure and the secretory process of a SCO-ependymal cell. They are bipolar cells, with and apical pole contacting the ventricular CSF and a basal process projecting to local capillaries and to the subarachnoid space. Glycoproteins secreted by the SCO cells either remain soluble into CSF or polymerize forming the RF. The secretory material upon release condenses, first as a film on the surface of the organ (pre-RF) and, after further packaging, into RF. The basal processes of ependymal cells (BP) receive abundant serotonergic, gabaergic, and catecholaminergic neural inputs and end on a network of basal lamina containing long spacing collagen (LSC). PVS, perivascular space. **(E)** Frontal section of a rat brain at PN60 immunostained with anti-SCO-spondin. SCO and pre-RF are strongly reactive. **(F)** Frontal section of the bovine spinal cord processed for double immunofluorescence using anti-RF proteins (green) and βIV-tubulin (red). The central canal (cc) contains Reissner fiber (RF, green) and is lined by tanycytes-like ependymal cells (red). Scale bars: **(B)** 200 μm; **(C)** 10 μm; **(E)** 40 nm; **(F)** 20 μm. From Rodríguez et al. (1993); Vío et al. (2008), Ortloff et al. (2013).

of SCO-spondin. We regard these proteins as CSF-soluble SCO-spondin-derived compounds. In adulthood, the CSF contains both RF-SCO-spondin and the soluble SCO-spondin related compounds (Vío et al., 2008). During the embryonic period, the very active SCO of all species studied (**Figure 2I**), including the human (**Figure 2E**, left inset), secretes CSF-soluble SCO-related proteins while RF is missing (Rodríguez et al., 1998, 2001; Hoyo-Becerra et al., 2006; Vío et al., 2008).

At early developmental stages SCO-spondin is also expressed by the floor plate cells that release it into the fetal CSF and also transport it along their basal processes (paracrine effect?; Yulis et al., 1998; Richter et al., 2001; **Figure 2F**). The floor plate, a key structure in brain development, participates in the neural patterning and axon guidance of the ventral neural tube.

Transthyretin

Transthyretin, a protein involved in the transport of thyroid hormone and retinol in the CSF (Chanoine and Braverman, 1992; Bernal, 2002), is expressed by the ependymal cells of the SCO (Montecinos et al., 2005). The mRNA encoding TTR and the 14 kDa protein are expressed in the SCO under *in vivo* and *in vitro* conditions. Organ cultured SCO secretes TTR into the culture medium, indicating that the SCO synthesizes TTR and secretes it into the CSF (Montecinos et al., 2005). The SCO possesses two populations of secretory cells, one secreting both RF-glycoproteins and TTR and the other secreting only the former (**Figures 2E** and **8H**). TTR was detected in the SCO of bovine embryos and human embryos (**Figure 2E**) suggesting that this ependymal gland is a source of TTR during brain development SCO (Montecinos et al., 2005).

Other Proteins

Antibodies raised against "CSF-specific" glycoproteins (glycoproteins present in the CSF but missing from the plasma) obtained from the CSF of hydrocephalic children react with the human and rat SCO (Rodríguez et al., 1993, 2001; Montecinos, 1995). Immunoreactive-basic fibroblast growth factor (bFGF) has been also detected in the SCO (Cuevas et al., 1996).

The detection in the CSF of the lateral ventricle and cisterna magna of CSF-soluble compounds secreted by the SCO (Rodríguez et al., 1993; Vío et al., 2008) indicates that such a material circulates in the ventricular and subarachnoidal CSF (**Figure 3A**). Because both CSF compartments are in open communication with the brain tissue, the SCO-soluble secretion could reach any region of the central nervous system, with the exception of the other circumventricular organs that have a tight barrier with the CSF.

The secretory activity of the SCO is under neural control. This include serotonergic (Bouchaud, 1979; Jiménez et al., 2001), gabaergic and catecholaminergic (Balaban et al., 1994; Tomé et al., 2004) inputs (**Figure 3D**). SCO-cells also express receptors for angiotensin II (Ghiani et al., 1988), endothelin 1 and bradykinin (Schöniger et al., 2009). The serotonergic input exerts and inhibitory control on the expression and release of SCO-spondin (Richter et al., 2004).

THE CEREBROSPINAL FLUID, THE SUBCOMMISSURAL ORGAN, AND THE NEUROGENIC NICHE

All cells forming the central nervous system are generated from a common source, neuroepithelial/NSCs located in the ventricular zone (VZ) of the developing brain. After birth, and during life span, neurogenesis continues at specific brain areas, known as neurogenic niches. Adult neurogenesis is mostly confined to two brain regions, the SVZ of the lateral ventricles (**Figure 4A**) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Alvarez-Buylla and Garcia-Verdugo, 2002; Gage, 2002). Several publications have also reported the generation of new neurons in other regions of the adult brain, including the neocortex, the amygdala, the hypothalamus, the circumventricular organs, the striatum and the substantia nigra (Dellmann and Rodríguez, 1970; Bennett et al., 2009; Migaud et al., 2010; Furube et al., 2015).

The molecular mechanisms that control neurogenesis are being extensively studied (reviewed by Urban and Guillemot, 2014). It is becoming evident that NSCs of the embryonic and adult brain are not as multipotential as previously thought. Instead, subpopulations of NSCs appear to be committed to generate specific types of neural cells (Alvarez-Buylla et al., 2008; Taverna et al., 2014). The mechanisms underlying the NSCs heterogeneity are among the most exciting questions in the field (DeCarolis et al., 2013; Encinas et al., 2013; Giachino et al., 2014). Neurogenesis involves several steps such as proliferation, commitment of the new cells to a neuronal phenotype, their migration and maturation and, finally, the establishment of appropriate synaptic contacts (Abrous et al., 2005; Braun and Jessberger, 2014). These steps are regulated by intrinsic and extrinsic factors. Intrinsic factors include cell-to-cell interactions and niche-derived morphogens released by stem cells, ependyma cells, and endothelial cells (**Figure 4A**); extrinsic factors include signals generated in the vicinity of the niche as well as blood-borne and CSF-borne compounds (Sawamoto et al., 2006; Riquelme et al., 2008; Hagg, 2009; Pathania et al., 2010; Faigle and Song, 2013; **Figures 4A–E**).

The NSCs of the embryonic VZ are characterized by projecting a 9+0 single cilium to the fetal CSF (Sotelo and Trujillo-Cenóz, 1958; Tramontin et al., 2003). There is evidence that molecules present in the fetal CSF are cues for the NSCs (Parada et al., 2006; Zappaterra et al., 2007) and that receptors for insulin and insulin-like growth factors 1 and 2, FGF, sonic hedgehog and BMP, localize at the apical plasma membrane (Lehtinen and Walsh, 2011). Similar to the embryonic NSCs, the NSCs of the adult SVZ project a process that reaches the ventricular CSF and bears a single 9+0 cilium (Doetsch et al., 1999). Although virtually nothing is known about the molecular characteristic of this cilium, it seems most likely that it is receptive to signals present in the fetal and adult CSF (**Figure 4A**). Interestingly, primary cilia ablation leads to disruption of hedgehog signaling which plays key roles in brain development and in adult neurogenesis (Tong et al., 2014).

Cerebrospinal fluid-long-distance cues may act directly on NSC and progenitor cells to regulate neurogenesis (Johanson

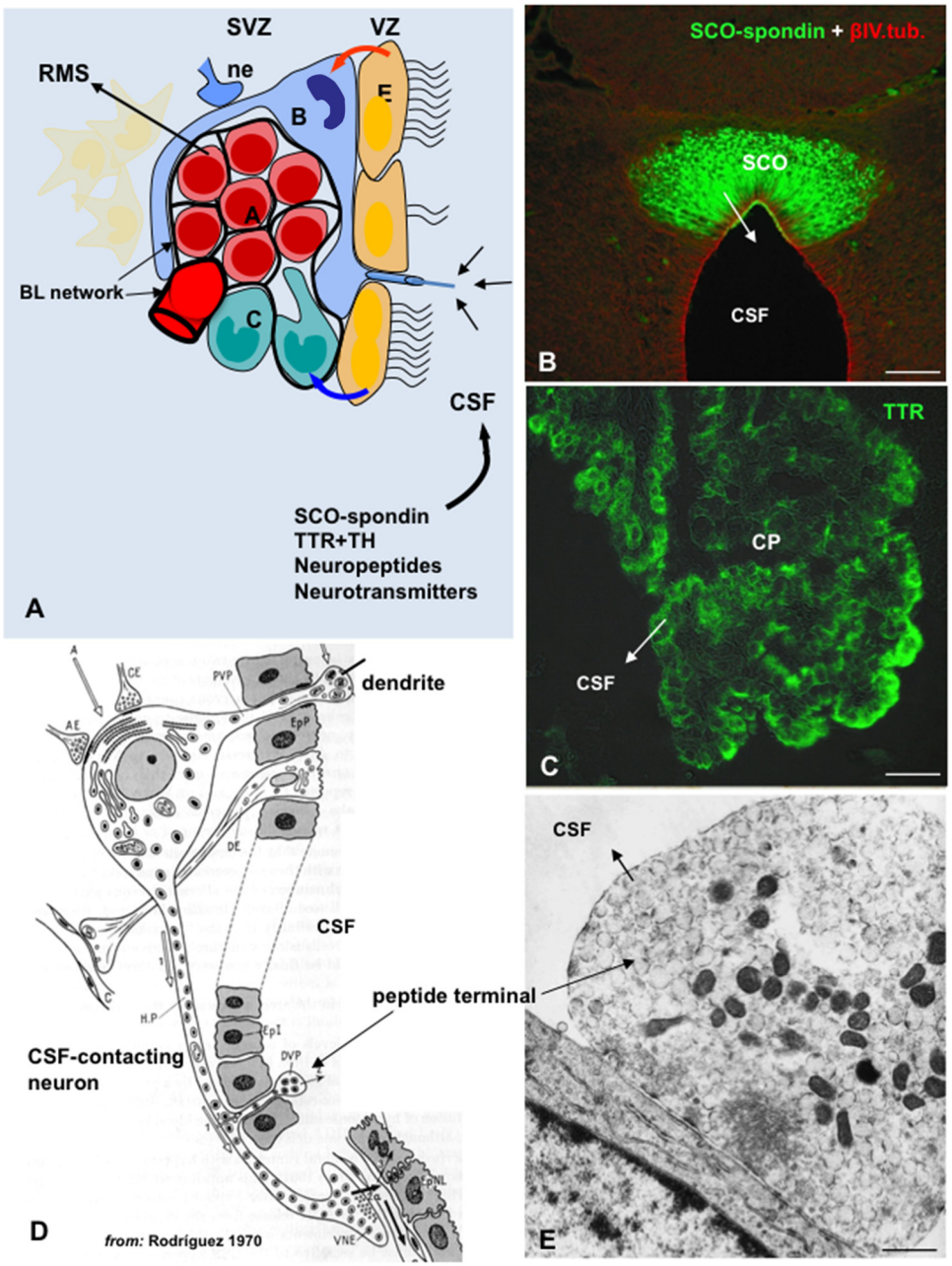

FIGURE 4 | The cerebrospinal fluid is a pathway for the delivery of neurotropic factors to the adult SVZ niche. (A) Cell organization of SVZ niche in the adult brain. SVZ astrocytes (**B**, blue) are stem cells which generate migrating neuroblasts (**A**, red) destined for the olfactory bulb via rapidly dividing transit-amplifying cells (**C**, green). A specialized basal lamina (BL, black) extends from perivascular cells and contacts all cell types, including multiciliated ependyma cells (**E**, orange). Ependymal cells, neural terminals (ne), the extracellular matrix (ECM)-basal lamina (BL) network, and the cerebrospinal fluid (CSF) are key components of the niche and regulator of the adult neurogenesis. Stem cells display a single 9+0 cilium to sensor CSF signals. Compounds secreted into the CSF by circumventricular organs such as the subcommissural organ (SCO) and choroid plexus (CP), or by CSF-contacting neurons can readily reach the SVZ (modified after Riquelme et al., 2008). **(B)** Frontal section of the rat SCO immunostained with antibodies against SCO-spondin and βIV-tubulin (from Ortloff et al., 2013). **(C)** Choroid plexus immunostained for TTR. **(D)** Drawing depicting a hypothalamic peptidergic CSF-contacting neuron with a dendrite projecting to the ventricle bearing a 9+0 cilium, and axon projecting to the capillaries of the pituitary gland and bearing and axonal branch reaching the ventricle (from Rodríguez, 1976). **(E)** Electron microscopy of a peptide terminal within the ventricle, with neurosecretory granules undergoing exocytosis. Scale bars: **(B)** 120 μm; **(C)** 35 μm; **(E)** 700 nm.

et al., 2008; Johansson, 2014). Many of the CSF compounds secreted by the CSF-contacting neurons and circumventricular organs, such as the SCO and the choroid plexuses, are good candidates to signal the receptive "CSF-contacting NSCs" of the SVZ niche (**Figures 4B–E**). The design of the CSF-neurogenic niche interphase and the numerous neurotropic

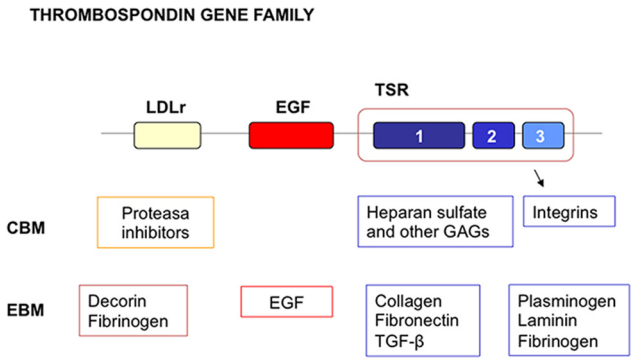

FIGURE 5 | Multidomain organization of thrombosponin type 1 molecules. LDL receptor domains are indicated by the yellow box. EGF like domains are indicated by the red box. Thrombospondin types 1, 2, and 3 repeats (TSRs) are indicated by the blue boxes. A number of cellular and extracellular binding molecules for the domains have been identified. Many of these are components of ECM. CBM, cellular binding molecules; EBM, extracellular binding molecules.

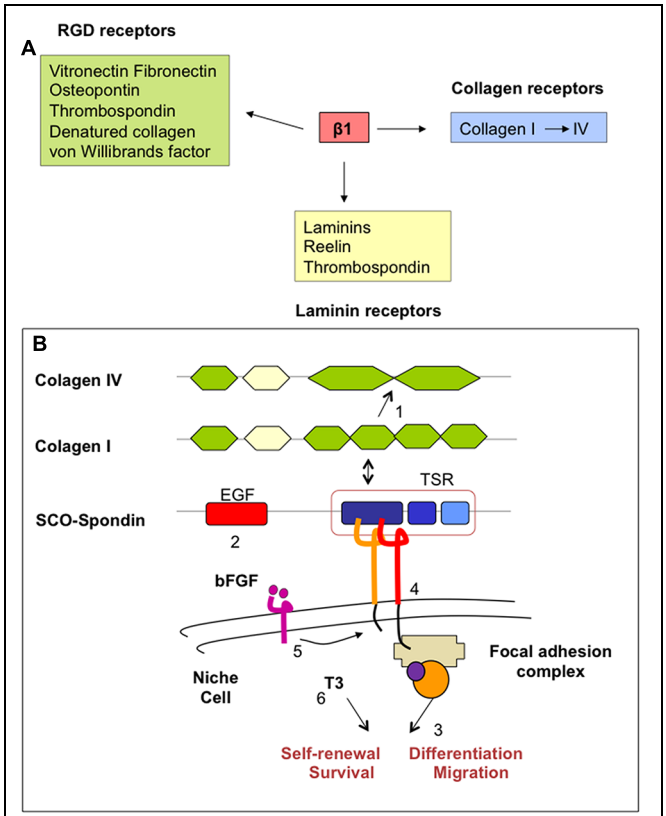

FIGURE 6 | (A) Ligands for integrin-β1 heterodimers. Many of these ligands are components of ECM. **(B)** Simplified schematic drawing of how SCO-spondin might promote neurogenesis in the adult SZV niche. SCO-spondin (1) may change the composition of ECM (i.e., transforming the type of collagen) and (2) the availability of growth factors in the niche, modifying (3) the immediate microenvironment and behavior of niche cells. Some of these functions could be mediated (4) by interaction of SCO-spondin with integrin-β1 signaling and (5) cross talking with other essential pathways, like those regulated by bFGF and TTR/thyroid hormones (6).

factors secreted into the CSF, point to the CSF as a key milieu for the SVZ niche. A further thought concerns the properties of the CSF–SVZ barrier. Neither the cell junction complexes between the different component of the ependymal component of the niche (NSC processes, bi- and multi-ciliated ependymal cells) nor the barrier properties of this cell layer have been properly investigated. This information is required for a better understanding of the relationships between the processes taking place in the SVZ and, via the CSF, in other brain regions.

EFFECTS OF SCO-COMPOUNDS ON FETAL NEUROGENESIS

The fetal CSF may be regarded as the main component of the milieu of stem cells and progenitor cells of the germinal zone providing signals participating in embryonic brain growth and differentiation (Miyan et al., 2003; Gato and Desmond, 2009; Gato et al., 2014). Quality and quantity of proteins of fetal CSF vary throughout development (Mashayekhi et al., 2002; Zappaterra et al., 2007; Vío et al., 2008), and differ from those of adult CSF (Vío et al., 2008). In all species, including the human, the SCO secretes CSF-soluble proteins during most of the fetal period. SCO-spondin, SCO-spondin-derived polypeptides, TTR and other detected but not-yet identified secretory compounds are released by the ependymal cells of the SCO into the ventricular CSF, while the secretory hypendymal cells secrete into the subarachnoid space a material reacting with antibodies against RF-glycoproteins and likely corresponding to SCO-spondin-derived compounds (Rodríguez et al., 1984a,b, 1993; Schoebitz et al., 1993; Hoyo-Becerra et al., 2006; Vío et al., 2008). Eight bands immunoreacting with antibodies against RF-glycoproteins are consistently found in CSF samples from rats at E18, E20, and PN1. Only four of these compounds are detected in the CSF of PN30 rats, indicating that secretion and/or processing of SCO secretory proteins in the fetal period is different from that of adult life (Vío et al., 2008).

Subcommissural organ-spondin, promotes neuronal growth and differentiation during the embryonic development (Monnerie et al., 1995; Gobron et al., 2000; Meiniel, 2001; Stanic et al., 2010; Grondona et al., 2012; Vera et al., 2013). In chick embryos, SCO-spondin is released into the embryonic CSF at early stages of development (Schoebitz et al., 1993; Hoyo-Becerra et al., 2006). Inhibition of SCO-spondin by injecting antibodies into the embryonic CSF or using shRNA to knockdown this protein drastically decreases the neurodifferentiation process (Vera et al., 2013). This effect appears to be mediated by interaction of SCO-spondin with low density lipoproteins from embryonic CSF (Vera et al., 2015). During the fetal period, the basal route of secretion of the SCO via the processes of the hypendymal cells is more developed than in the postnatal period (Schoebitz et al., 1986; **Figure 2I**). There is evidence that SCO-spondin is

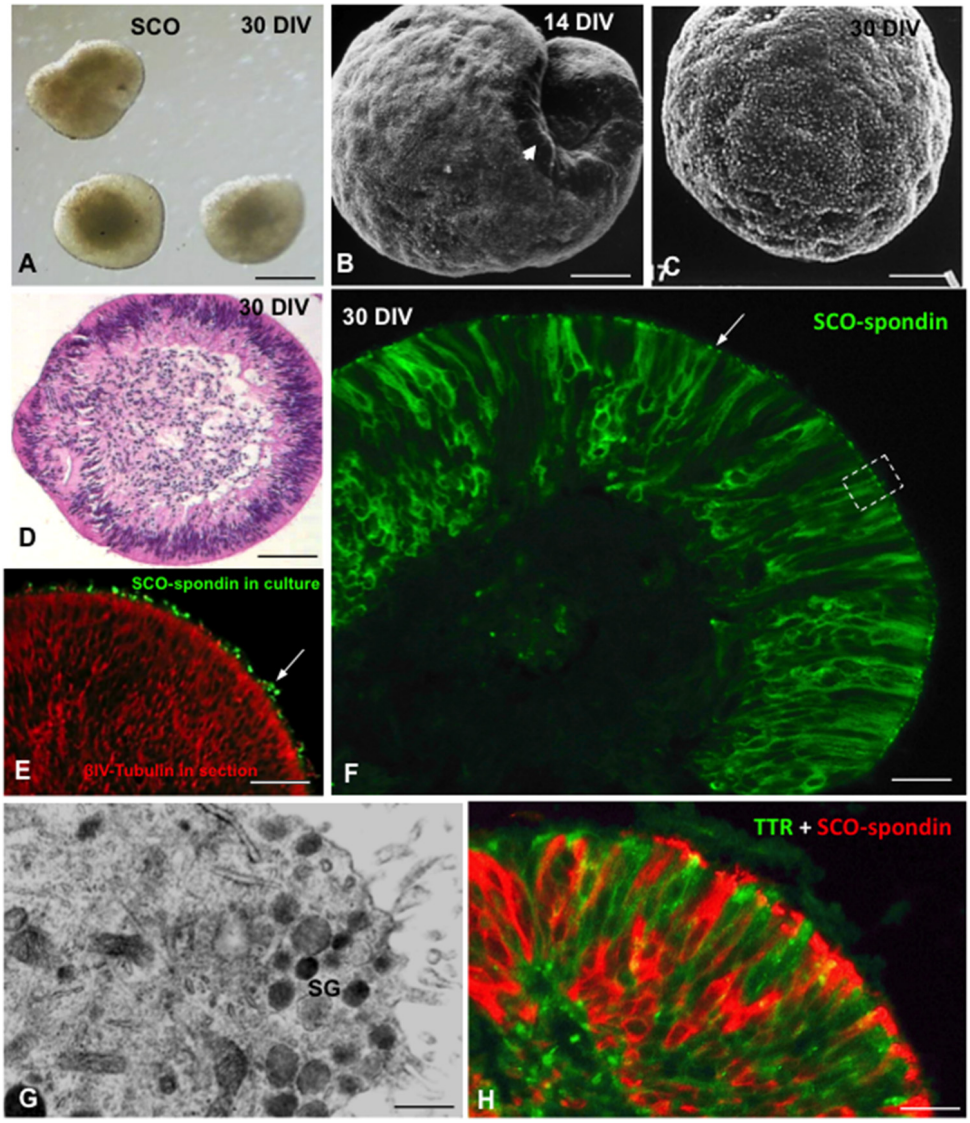

FIGURE 7 | Organ culture of bovine subcommissural organ. After 30 days in culture, SCO explants organize forming spheres of secretory ependymocytes. **(A)** Phase contrast microscopy. **(B,C)** Scanning electron microscopy after 14 **(B)** and 30 DIV **(C)**. **(D)** Section of a SCO-explant stained with haematoxylin-eosin. **(E)** Secretory evidence of secretion. Explants were cultured in the presence of antibodies against SCO-spondin. After histological procedure, sections were incubated with anti-IgG conjugated with alexa 488. Immunofluorescence reveals the presence of SCO-spondin aggregates associated to cilia (green, arrow). **(F)** Section of a SCO-explant immunostained for SCO-spondin showing the intracellular and extracellular (arrow) location of the protein. **(G)** Ultrathin section of an area similar to that framed in previous figure, showing the ultrastructure of the apical cell pole loaded with secretory granules (sg). **(H)** Section of a SCO-explant. Double immunofluorescence for SCO-spondin (red) and TTR (green). Scale bars: **(A)** 60 μm; **(B–E)** 25 μm; **(F)** 10 μm; **(G)** 500 nm; **(H)** 10 μm. From Schöebitz et al. (2001), Montecinos et al. (2005).

released from these processes becoming part of the ECM (Caprile et al., 2009) contributing to the organization of the axons forming the posterior commissure (Stanic et al., 2010; Grondona et al., 2012). This effect appears to be mediated by the interaction of SCO-spondin with β1-integrin (Caprile et al., 2009).

After early studies had shown that insufficient thyroid hormone supply to the brain leads to neurodevelopmental defects and mental retardation (revised by Morreale de Escobar, 2001), the effects of thyroid hormones on brain development have been thoroughly investigated. Transthyretin (TTR), secreted by the choroid plexus (Dickson et al., 1986; Buxbaum and Reixach, 2009; Johansson, 2014) and the SCO (Montecinos et al., 2005) in ontogeny, is a CSF protein delivering thyroid hormones and retinol to areas involved in pre- and postnatal neurogenesis (Chanoine and Braverman, 1992; Kassem et al., 2006; Richardson et al., 2007; Alshehri et al., 2015). It is worth noting that TTR is not essential for thyroid hormones distribution to most tissues in adult mice, one notable exception being the SVZ of the brain (Monk et al., 2013). Here, thyroid hormones regulate the cell

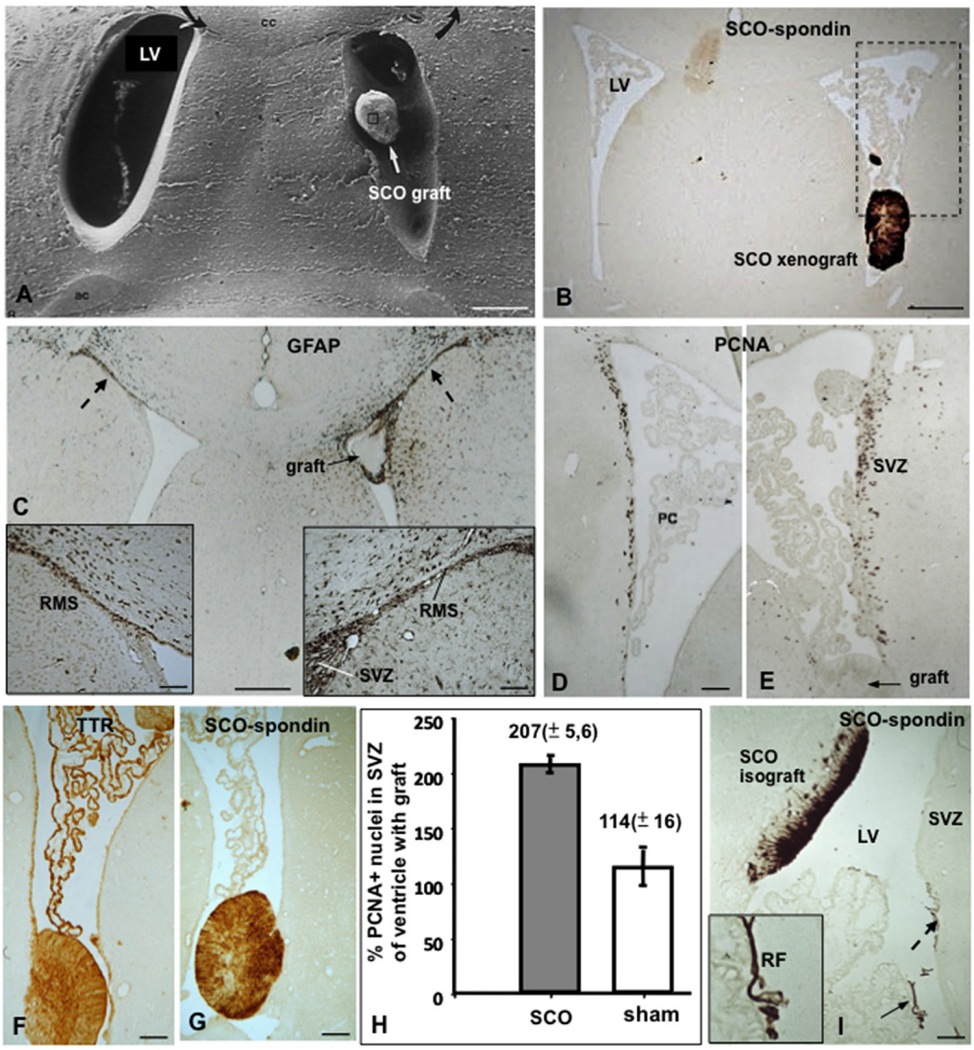

FIGURE 8 | Xeno- and isografting of SCO-explants into the lateral ventricle of adult rats. (A–H) Bovine SCO-explants 30 DIV were grafted into the lateral ventricle of adult rats. **(A)** Scanning electron microscopy showing a SCO-explant in the ventricle. **(B)** Frontal section of the brain of a grafted animal immunostained with AFRU. The grafted SCO is strongly reactive. The area framed is shown in figures D and E. LV, lateral ventricle**. (C)** Frontal section of the brain of a grafted animal immunostained for GFAP. Astrocytes forming the rostral migratory stream (RMS) are shown. In the grafted ventricle the RMS is hypertrophied (right inset) as compared to that of the contralateral ventricle (left inside). **(D,E)** Areas similar to that framed in figure **(B)**, immunostained for PCNA. In the grafted ventricle **(E)** proliferation is significantly higher than in the contralateral ventricle **(D)**. **(F,G)** The grafted SCO expresses TTR and SCO-spondin. TTR is also expressed by the choroid plexus. **(H)** Quantitative analysis of PCNA+ nuclei after SCO grafting in a lateral ventricle of an adult normal rat. The results are expressed as percentage of the number of labeled nuclei in the SVZ of the ventricle carrying the grafts with respect to that of the contralateral ventricle, taken as 100%. Sham operated rats underwent surgery as for transplantation, but received no graft. There is a twofold increase of PCNA+ nuclei in the grafted ventricle. **(I)** Rat SCO explant grafted into the lateral ventricle of an adult rat. The graft becomes integrated into the wall of the lateral ventricle (LV) with the ependymal cells secreting SCO-spondin into the ventricle aggregated on the ependyma of the subventricular zone (broken arrow; SVZ) and forming a Reissner fiber (RF; full arrow; inset). Scale bars: **(A–C)** 120 μm; **(D,E)** 60 μm; **(F,G)** 40 μm; **(I)** 60 μm. From Rodríguez et al. (1999); González (2007).

cycle of NSC and neural progenitor cells by influencing both proliferation and apoptosis (Lemkine et al., 2005; Richardson et al., 2007). Further, T3 exerts a role in NSC commitment toward neuroblasts (Kapoor et al., 2012; López-Juárez et al., 2012). T4 and T3 might also influence oligodendroglial differentiation (Almazan et al., 1985; Franco et al., 2008; Fernández et al., 2009).

The proteomic screening of CSF has revealed differences in the CSF proteins of non-affected and hydrocephalic rats, in particular with respect to SCO-secretory proteins and TTR (Ortloff et al., 2013). TTR concentration is higher; it is speculated that it would be involved in neuroprotection. In addition, immature forms of SCO-spondin and SCO-spondin related compounds have been detected into the hydrocephalic CSF (Ortloff et al., 2013). Such an abnormal CSF plays a role in the deficient cortical development of this mutant (Mashayekhi et al., 2002). Recent findings in HTx rats and hydrocephalic human fetuses strongly indicate that hydrocephalus and abnormal

neurogenesis are two inseparable phenomena (Guerra et al., 2015).

EFFECTS OF SCO-COMPOUNDS ON ADULT NEUROGENESIS

In all species but human (see above), the SCO remains highly differentiated and secretory active through life span (Rodríguez et al., 1984a). During this long period, the SCO continues to secrete SCO-spondin, SCO-spondin-derived compounds and TTR. The latter two are CSF-soluble and go with the CSF flow. What is the fate and target of these compounds in the adult brain? Would their early neurogenetic properties also be expressed in adulthood? The evidence collected during recent years points to a positive answer (see below).

Basic Fibroblast Growth Factor

Multiple studies demonstrate the important role of bFGF in regulating neurogenesis and mediating brain repair processes. bFGF has been shown to be a potent mitogenic factor for NSC and progenitor cells both *in vitro* and *in vivo* (Gage et al., 1998; Wagner et al., 1999; Cheng et al., 2001). Evidence indicates that bFGF exerts proliferative effects on quiescent NSC (Zheng et al., 2004; Wang et al., 2011).

Transthyretin

Transthyretin synthetized by the choroid plexus and the SCO is secreted into the CSF. A marked difference between these two sources of TTR is that the SCO cells, at variance with the choroidal cells, are not open to the blood stream and their secretory activity is under the control of a complex neural input (**Figure 1**). Within the choroidal cells, TTR binds thyroxin (T4) that has entered these cells either by passive diffusion or by specific transporters (Alshehri et al., 2015). Via the CSF, the TTR-T4 complexes are carried to specific brain areas (**Figure 1**).

T4 is the predominant iodothyronine in plasma. However, T3 is the major receptor-active form of thyroid hormones. Consequently, T4 has to be converted by the effect of diodinase 2 into T3. The conversion of thyroxin present in the CSF into T3 takes place, exclusively, in the tanycytes located in the hypothalamus (Lechan and Fekete, 2005, 2007; Rodríguez et al., 2005, 2010). Tanycytes are virtually the only cell type exposed to the CSF that expresses diodinase 2 (Guadano-Ferraz et al., 1997; Diano et al., 2003; Lechan and Fekete, 2005, 2007). Tanycytes take up T4-TTR and/or T4 from the CSF and pour T3 back to the CSF where it forms T3-TTR. The T3-TTR complex has receptors at specific brain regions (Rodríguez et al., 2010; **Figure 1**).

These findings point to a functional relationship, via the CSF, between three different types of ependymal cells, namely, the ependymocytes of the SCO, the choroidal cells of the choroid plexus and tanycytes. The outcome of such an association is to provide signals to the neurogenic niche (**Figure 1**).

SCO-Spondin and SCO-Spondin-Derived Compounds

The complex multidomain organization of SCO-spondin allow to speculate about probable mechanism(s) by which SCO-spondin and SCO-spondin-derived compounds would promote neurogenesis in the adult SVZ niche. This protein displays a unique arrangement of several conserved domains, including 26 thrombospondin type 1 repeats (TSRs), 9 low density lipoprotein receptor (LDLr) type A domains, 2 epidermal growth factor (EGF) like domains, and NH2 and COOH von Willebrand cysteine-rich domains (vWD; Meiniel et al., 2008). All these consensus sequences represent potential sites of protein–protein interaction. Potential binding sites to proteoglycans and growth factors have also been identified (Gobron et al., 2000; Meiniel, 2001; **Figure 5**). Due to the large number of TSR, SCO-spondin is regarded as an extra cellular matrix-like protein belonging to the TSR superfamily. It is involved in multiple functions including cell attachment, motility, proliferation, cell–cell contact, cell aggregation and angiogenesis, all of which are thought to contribute to vascular homeostasis and brain functions (Adams, 2001; Tucker, 2004). This is consistent with the role of SCO-spondin to promote cell differentiation and neurite outgrowth of various neuronal cell populations in cell culture (Monnerie et al., 1995; Meiniel et al., 2003), and the proposed role of SCO-spondin in the formation of posterior commissure during the embryonic development (Stanic et al., 2010; Grondona et al., 2012). Interestingly, through TSR motifs SCO-spondin could bind β1-integrin (**Figure 6A**). This interaction may be essential for the neurite outgrowth induced by SCO-spondin *in vitro* (Bamdad et al., 2004) and for the posterior commissure development *in vivo* (Caprile et al., 2009; Grondona et al., 2012).

In the adult SVZ niche, β1-integrin is highly expressed by NSC, progenitor cells, neuroblasts, and endothelial cells (Shen et al., 2008). Here, integrins provide NSC the capacity to regulate their responsiveness to growth factors (Fuchs et al., 2004; Campos, 2005). Furthermore, β1-integrin is required for maintaining the integrity of the glial tubes in the rostral migratory stream (Jacques et al., 1998; Belvindrah et al., 2007). SCO-spondin and SCO-spondin-derived compounds present in the CSF may reach the SVZ niche through the ependyma devoid of tight junctions. Due to its multidomain organization, SCO-spondin and its derivatives behave as a ligand for β1-integrin, collagen, and laminins of the ECM of the adult neurogenic niche. According to the evidence discussed above, these interactions could lead to changes in the microenvironment (basal lamina, ECM, growth factors, availability) and behavior of niche cells (NSC, neural progenitors, endothelial and ependymal cells; **Figure 6B**). Interestingly, bFGF, also secreted by SCO-cells, increases the expression of β1-integrin (Enenstein et al., 1992). Further, the effect of thyroid hormones on integrin signaling appears to be crucial for a normal neurogenesis (Stenzel et al., 2014). Cross-talking of SCO-spondin with other signaling pathways, such as those regulated by bFGF, thyroid hormones and low density lipoproteins could be envisaged.

EXPERIMENTAL APPROACHES: GRAFTING OF SUBCOMMISSURAL ORGAN TO PROMOTE NEUROGENESIS IN THE ADULT SVZ NICHE

Under proper culture conditions, SCO explants can be organ cultured for several months. After 3–4 weeks in culture, the explants form spheres lined by fully differentiated ependymal secretory cells (**Figure 7**). Explants synthetize (**Figure 7H**) and secrete SCO-spondin and TTR into the culture medium (Schöebitz et al., 2001; Montecinos et al., 2005).

Subcommissural organ explants grafted under the kidney capsule keep their secretory properties similar to the *in situ* SCO (Rodríguez et al., 1989). A network of processes of the perivascular basal lamina, resembling that found in circumventricular organs (Rodríguez, 1969; Rodríguez et al., 1979; Dellmann et al., 1987) and in the niche of the SVZ (Mercier et al., 2002; Kerever et al., 2007) connects the secretory cells to newly formed capillaries re-vascularizing the grafted SCO. Long-spacing collagen appears in expanded areas of such laminar networks and also in the perivascular space supporting that: (i) formation of long-spacing forms of collagen is triggered by factors provided by the SCO-secretory cells, and (ii) secretory material of the grafted ependymal and hypendymal cells reaches the extended network of the basal lamina processes (Rodríguez et al., 1989).

Rat SCO explants grafted into a lateral ventricle of normal adult rats become re-vascularized and secrete RF-glycoproteins into the CSF forming a RF, now located in the lateral ventricle (**Figures 8A,B,F**). The basal lamina of the newly formed capillaries, but not the capillaries of the neighboring brain parenchyma, contains long spacing collagen, indicating that the expression of this special type of collagen is triggered by signals of the grafted SCO cells (Rodríguez et al., 1999). Xenografts of bovine SCO explants into a lateral ventricle of normal and hydrocephalic rats survive for weeks, secrete SCO-spondin and TTR to the host CSF and promote neurogenesis in the ipsilateral SVZ niche (Rodríguez et al., 1999; González, 2007; Jara et al., 2014; **Figures 8A–E,G–I**).

CONCLUSION AND FUTURE DIRECTIONS

A good body of evidence is revealing that the dynamic and molecular composition of the CSF and, consequently, the CSF physiology is much more complex and fascinating than the simplistic view held for decades. Signal molecules either specifically transported from blood to CSF or secreted into the CSF by a series of periventricular structures, use the CSF to reach their targets in the brain. This allows a cross talk between brain regions located beyond the blood-brain-barrier, thus keeping the brain milieu private. One of these brain target is the neurogenic niche, and the SCO, choroid plexus, and tanycytes are some of the sources of signals that reach this target via the CSF. Thus, the CSF path has made it possible for these four brain structures to become good functional partners.

As we unfold the multiples trans-fluid talks between discrete brain domains we will have more tools to influence, in one way or another, such talks. The CSF may become an appropriate medium to deliver foreign molecules or to host cell grafts.

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Expression of Lymphatic Markers in the Adult Rat Spinal Cord

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Under physiological conditions, lymphatic vessels are thought to be absent from the central nervous system (CNS), although they are widely distributed within the rest of the body. Recent work in the eye, i.e., another organ regarded as alymphatic, revealed numerous cells expressing lymphatic markers. As the latter can be involved in the response to pathological conditions, we addressed the presence of cells expressing lymphatic markers within the spinal cord by immunohistochemistry. Spinal cord of young adult Fisher rats was scrutinized for the co-expression of the lymphatic markers PROX1 and LYVE-1 with the cell type markers Iba1, CD68, PGP9.5, OLIG2. Rat skin served as positive control for the lymphatic markers. PROX1-immunoreactivity was detected in many nuclei throughout the spinal cord white and gray matter. These nuclei showed no association with LYVE-1. Expression of LYVE-1 could only be detected in cells at the spinal cord surface and in cells closely associated with blood vessels. These cells were found to co-express Iba1, a macrophage and microglia marker. Further, double labeling experiments using CD68, another marker found in microglia and macrophages, also displayed co-localization in the Iba1+ cells located at the spinal cord surface and those apposed to blood vessels. On the other hand, PROX1-expressing cells found in the parenchyma were lacking Iba1 or PGP9.5, but a significant fraction of those cells showed co-expression of the oligodendrocyte lineage marker OLIG2. Intriguingly, following spinal cord injury, LYVE-1-expressing cells assembled and reorganized into putative pre-vessel structures. As expected, the rat skin used as positive controls revealed classical lymphatic vessels, displaying PROX1+ nuclei surrounded by LYVE-1-immunoreactivity. Classical lymphatics were not detected in adult rat spinal cord. Nevertheless, numerous cells expressing either LYVE-1 or PROX1 were identified. Based on their localization and overlapping expression with Iba1, the LYVE-1+ cell population likely represents a macrophage subpopulation, while a significant fraction of PROX1+ cells belong to the oligodendrocytic lineage based on their distribution and the expression of OLIG2. The response of these LYVE-1+ and PROX1+ cell subpopulations to pathological conditions, especially in spinal cord inflammatory conditions, needs to be further elucidated.

Keywords: spinal cord, macrophage, PROX1, LYVE-1, Iba1, OLIG2, lymphatics, spinal cord injury

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INTRODUCTION

Two crucial roles are carried out by the lymphatic system: (1) fluid homeostasis via the drainage of extracellular fluid; and (2) immune defense through the transport of antigens and immune cells to the lymph nodes (Alitalo, 2011; Card et al., 2014). To fulfill these functions, the lymphatic system is widespread throughout the human body (Breslin, 2014). Nevertheless, a few tissues, such as the (inner) eye (Streilein, 2003) and the central nervous system (CNS; Ransohoff and Engelhardt, 2012), lack a "classical" lymphatic system and are therefore considered to be alymphatic. The epithet "classical" refers to the presence of vessels with a lymphatic phenotype, i.e., with endothelial cells expressing the surface receptors VEGFR3 or LYVE-1 and membrane bound components, such as podoplanin, or transcription- factors, such as PROX1 or FOXC2 (Banerji et al., 1999; Jackson et al., 2001; Sleeman et al., 2001; Jackson, 2007).

Over the last decades, research of the lymphatic system underwent a tremendous boost based on the introduction of these aforementioned markers. While identification of lymphatics within a tissue remains challenging due to the small caliber of the vessels and their structural similarity with small-caliber blood vessels, the markers introduced now allow reliable identification (Sleeman et al., 2001). The crux with lymphatic markers however is that no exclusive marker has been identified so far (Sleeman et al., 2001) since most lymphatic markers are also expressed on cells other than lymphatic endothelium (Matsui et al., 1999; Schroedl et al., 2008). Therefore, for the unequivocal identification of lymphatics, there is a consensus that a combination of several markers is necessary, as recently stated for the inner eye (Schroedl et al., 2014) or under pathological conditions (Van der Auwera et al., 2006). Hence, we chose for this study the combination of the transcription factor PROX1, a homeo-box protein that retains its activity in nuclei of lymphatic endothelium in adulthood (Wigle and Oliver, 1999; Wilting et al., 2002) and LYVE-1, a membrane-bound glycoprotein and one of the best characterized markers of lymphatic endothelium (Jackson, 2004; Baluk and McDonald, 2008).

Being part of the CNS, which is considered to be alymphatic, the existence of structures or cells expressing lymphatic markers has not been thoroughly investigated within the spinal cord. The characterization of these putative lymphatic cells is particularly relevant for repair mechanisms considering that lymphatic cells can organize into a lymphatic system during pathological processes (Paavonen et al., 2000; Kerjaschki et al., 2004; Maruyama et al., 2005; Zumsteg et al., 2009; Tammela and Alitalo, 2010; Kerjaschki, 2014; Tempfer et al., 2015).

In this study, we scrutinize with histological methods the presence of potential lymphatic system or cells expressing lymphatic markers in the healthy, as well as the injured, adult rat spinal cord.

MATERIALS AND METHODS

Specimens

All experiments were performed in conformity with the Directive (2010/63/EU) of the European Parliament and of the Council and were approved by the national animal health commission (Land Salzburg, Referat Gesundheitsrecht und Gesundheitsplanung Referat 9/01). Female Fisher 344 rats of approximately 3 months of age were used for this study. For histological analysis, rats received an overdose of anesthetics (ketamine/xylazine/acepromazine; i.p.) prior to transcardial perfusion with NaCl 0.9% followed by phosphate buffered saline (PBS) containing 4% formaldehyde. The spinal cord and skin samples from the thigh were dissected, further fixed by immersion (1 h, room temperature, RT) and rinsed in PBS. Spinal cord and skin samples were transferred into PBS containing 15% sucrose (12 h at 4°C), embedded in tissue embedding medium (Slee Technik, Mainz, Germany) and frozen using liquid nitrogen-cooled methylbutane and stored at −20°C until further processing.

Spinal Cord Contusion Injury (SCI)

Rats were anesthetized using 1.6% Isoflurane-oxygen mix. For analgesia, 0.03 mg/kg bodyweight (bw) Buprenorphine (Bupaq®, 0.3 mg/mL, Richterpharma, Wels, Austria) was injected sub-cutaneous (SC) 30 min prior to surgery. To prevent hypothermia, body temperature was maintained by a rectal sensor-coupled to a heating pad. Heart frequence and oxygen saturation was monitored throughout the whole surgical procedures (SomnoSuite®, KENT). During surgery, the dorsal aspect of the vertebra at thoracic level 8 was removed to expose the dura mater and the spinal cord. Contusion was performed using an Infinite Horizon impactor (IH-Impactor®, Precision Systems and Instrumentation, LLC with a force of 200 kdyn with immediate withdrawal). After that the absence of bleeding has been confirmed, paravertebral muscles were sutured and the skin was closed using a skin stapler. To prevent pain and infections after surgery, 1–2 mg/kg bw Meloxicam (Metacam® 5 mg/ml) and 10 mg/kg bw Enrofloxacin (Baytril® 25 mg/ml) was injected SC daily for 5 days. Additionally, 0.01 mg/kg bw Buprenorphine injected SC twice a day for 2 days post surgery. As bladder function was impaired after SCI, bladder was manually voided 2–3 times per day. Perfusion for histological analysis was performed 14 days post-lesion.

Immunohistochemistry

Spinal cord and skin samples were sectioned with a cryostat (HM 550, Microm, Walldorf, Germany) in serial sections of 16 μm, collected on adhesion slides (Superfrost Plus; Thermo Scientific, Wien Austria) and air-dried for 1 h at RT. Sections were rinsed 5 min in Tris-buffered saline (TBS; Roth, Karlsruhe, Germany) and incubated for 1 h at RT in TBS containing 5% donkey serum (Sigma-Aldrich, Wien, Austria), 1% bovine serum albumin (BSA; Sigma-Aldrich), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). Sections were then rinsed for 5 min in TBS and further incubated for single and double immunohistochemistry

with the primary antibodies (**Table 1**) diluted in TBS, containing 1% BSA and 0.5% Triton X-100, for 12 h at RT. Sections were rinsed four times for 5 min in TBS and the primary antibodies visualized by corresponding Alexa488-, or Alexa555-conjugated antibodies (Invitrogen, Karlsruhe, Germany) diluted 1:1000 in TBS, containing 1% BSA and 0.5% Triton X-100 (1 h at RT). Subsequently, the sections were rinsed 5 min in TBS followed by a 10 min nuclear labeling with 4′,6-Diamidino-2 phenylindole dihydrochloride (DAPI, 0.25 μg/mL; VWR, Vienna, Austria). Finally, sections were rinsed three times for 5 min in PBS and embedded in TBS-glycerol (1:1 at pH 8.6).

Rat skin sections were used to validate the LYVE-1 and PROX1 primary antibodies. Additionally, to avoid possible cross-reactivity in experiments involving two or more primary antibodies, successive incubations have been performed (i.e., primary and secondary antibodies for epitope one, followed by incubation with primary and secondary antibodies for epitope two). Negative controls were performed by omission of the primary antibodies during incubation and resulted in absence of immunoreactivity.

Documentation

Micrographs of immunohistostainings were acquired using a confocal laser scanning unit (Axio ObserverZ1 attached to LSM710, Zeiss, Göttingen, Germany; ×20 dry or ×40 and ×60 oil immersion objektive lenses, with numeric apertures 0.8, 1.30, and 1.4, respectively; Zeiss). All images presented here consist of confocal images in single optical section mode. Negative controls were recorded with identical laser settings as used for documentation of corresponding primary antibodies.

RESULTS

In the rat adult spinal cord, PROX1-immunoreactivity was detected in numerous nuclei evenly distributed throughout the gray and white matter. Nuclei of the central canal lacked PROX1-immunoreactivity (**Figure 1A**). Absence of immunoreactivity was observed in the negative controls (**Figure 1B**), whereas in rat skin, used as positive control, PROX1-positive nuclei were bordering luminal structures (**Figure 1C**). Detection of the lymphatic endothelial marker LYVE-1 revealed numerous LYVE-1-expressing cells on the surface of the spinal cord, surrounding the ventral spinal artery, and following the sulcal arteries into the anterior median sulcus (**Figure 1D**). Double labeling experiments demonstrated that cells expressing LYVE-1 did not possess PROX1-positive nuclei (**Figure 1D**). Corresponding negative controls were lacking immunoreactivity (**Figure 1E**), whereas controls in rat skin showed cells with PROX-1 positive nuclei surrounded by LYVE-1 immunoreactivity (**Figure 1F**). Identical results were obtained on the dorsal side of the spinal cord, i.e., LYVE-1-immunoreactive cells were detected on the spinal cord surface and also in association with vessels of the dorsal spinal arteries entering the parenchyma (**Figures 1G,H**). Occasionally, PROX1-immunoreactive nuclei were in close vicinity with LYVE-1+ structures, which likely reflected expression of single markers in adjacent cells (**Figure 1H**). Nevertheless, the overwhelming majority of LYVE-1-positive cells surrounding vessels lacked PROX1 immunoreactivity (**Figure 1I**).

Double immunohistochemistry with LYVE-1 and Iba1 revealed a co-localization of both markers in cells located at the spinal cord surface (**Figure 2A**). Within the spinal cord, LYVE-1+/Iba1+ cells surrounding blood vessels were detected, whereas cells within the spinal cord parenchyma solely displayed immunoreactivity for Iba1 (**Figures 2D,E**). Cells displaying immunoreactivity exclusively for LYVE-1 were not observed. Double immunohistochemistry for CD68 and Iba1 revealed an identical pattern, i.e., CD68+/Iba1+ cells were detected on the spinal cord surface (**Figure 2F**), following the spinal cord arteries (**Figures 2F,G**). Cells immunoreactive exclusively for CD68 were not observed. Corresponding negative controls ascertained the absence of immunoreactivity (**Figure 2B**). In contrast, in the rat skin positive controls, two distinct cell populations were observed expressing either LYVE-1 or Iba1 without apparent co-localization (**Figure 2C**).

To further characterize the PROX1+ cells within the spinal cord, PROX1-immunohistochemistry was combined with either Iba1, the pan-neuronal marker PGP9.5, or the oligodendrocyte lineage marker OLIG2. These double labeling experiments revealed that PROX1+ nuclei were not associated with Iba1 (**Figure 3A**), while corresponding controls showed absence of immunoreactivity (**Figure 3B**). Similarly, PGP9.5-immunoreactivity was not associated with PROX1+ nuclei in both small- and large-sized neurons (**Figure 3C**). Corresponding negative controls were not immunoreactive (**Figure 3D**). In contrast, the combination of PROX1 and OLIG2 revealed extensive co-localization of both markers in nuclei throughout the spinal cord

TABLE 1 | Primary antibodies used in this study.

Markers	Symbols	Hosts	Company	Dilution
Prospero homeobox protein 1	PROX1	Mouse	Acris; Herford, Germany	1:500
Lymphatic vessel endothelial hyaluronan receptor 1	LYVE-1	Rabbit	Acris; Herford, Germany	1:50
Cluster of differentiation 68	CD68	Rabbit	Abcam; Cambridge, UK	1:500
Ionized calcium-binding adapter molecule 1	Iba1	Goat	Wako Chemicals; Neuss, Germany	1:500
Protein-gene product 9.5	PGP9.5	Guinea pig	Merck Millipore; Vienna, Austria	1:500
Oligodendrocyte transcription factor 2	OLIG2	Rabbit	Merck Millipore; Vienna, Austria	1:300
Major histocompatibility complex class II	MHCII	Mouse	Abcam; Cambridge, UK	1:250

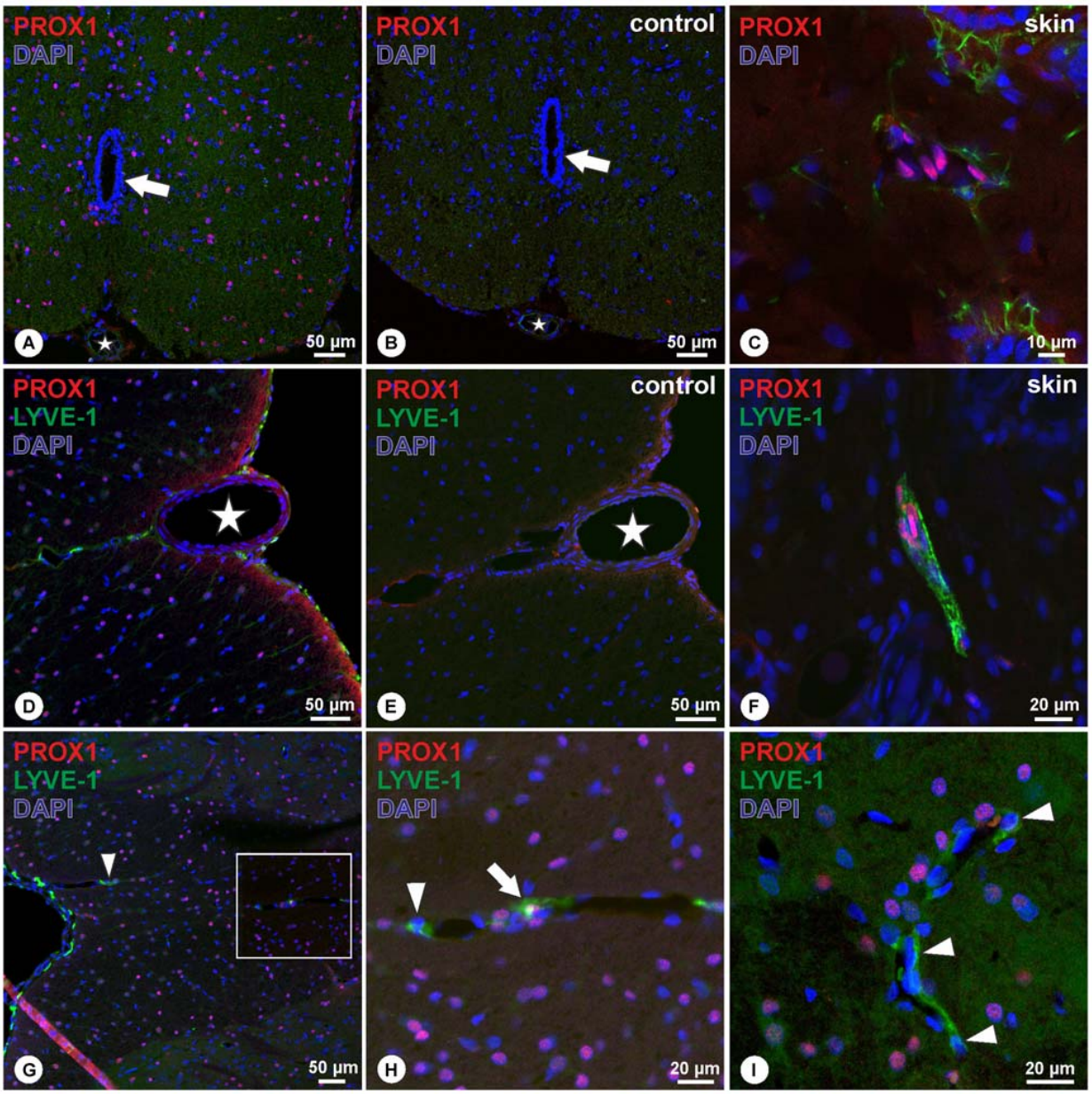

FIGURE 1 | (A–C) In cross sections of the spinal cord, PROX1-immunoreactivity (red) was detected in numerous nuclei (DAPI, blue) throughout the gray and white matter without preference, as indicated by purple mixed color **(A)**, whereas negative controls revealed absence of immunoreactivity **(B)**. Asterisk **(A,B)** indicates ventral spinal artery, arrow **(A,B)** indicates the central canal. Controls in cross sections of rat skin **(C)** revealed nuclear Prox1 immunoreactivity as in **(A)**. **(D–F)** Double-immunohistochemistry of PROX1 (red) and LYVE-1 (green) reveals LYVE-1+ cells lacking PROX1-immunoreactivity on the surface of the spinal cord and following the anterior sulcal arteries **(D)**. Immunoreactivity was absent in negative controls **(E)**. Asterisk in **(D,E)** indicates ventral spinal artery. Skin controls revealed PROX1+ nuclei surrounded by LYVE-1-immunoreactivity **(F)**. Blue: DAPI. **(G–I)** Double-immunohistochemistry of PROX1 (red) and LYVE-1 (green) reveals LYVE-1+ cells on the surface of the posterior spinal cord lacking PROX1-immunoreactivity **(G)**, following the posterior spinal arteries (arrowhead). While occasionally cells were detected with apparent association of PROX1 and LYVE-1 (arrow in **H**), the majority of cells displayed immunoreactivity for LYVE-1 only (arrowheads in **H,I**). **(H)** represents magnification of the boxed area in **(G)**. Blue: DAPI.

(**Figure 3E**). Characterization in five randomly chosen micrographs of approximately 200 nuclei expressing either marker revealed that 46% were PROX1+/OLIG2+, whereas 42% showed immunoreactivity for OLIG2 only and 12% displayed immunoreactivity for PROX1 only. PROX1/OLIG2 immunoreactivity was absent in corresponding negative controls (**Figure 3F**).

Preliminary investigation in lesioned spinal cord revealed cell debris and cells accumulation within the lesion site. Many cells displayed immunoreactivity for CD68 (**Figure 4A**), while

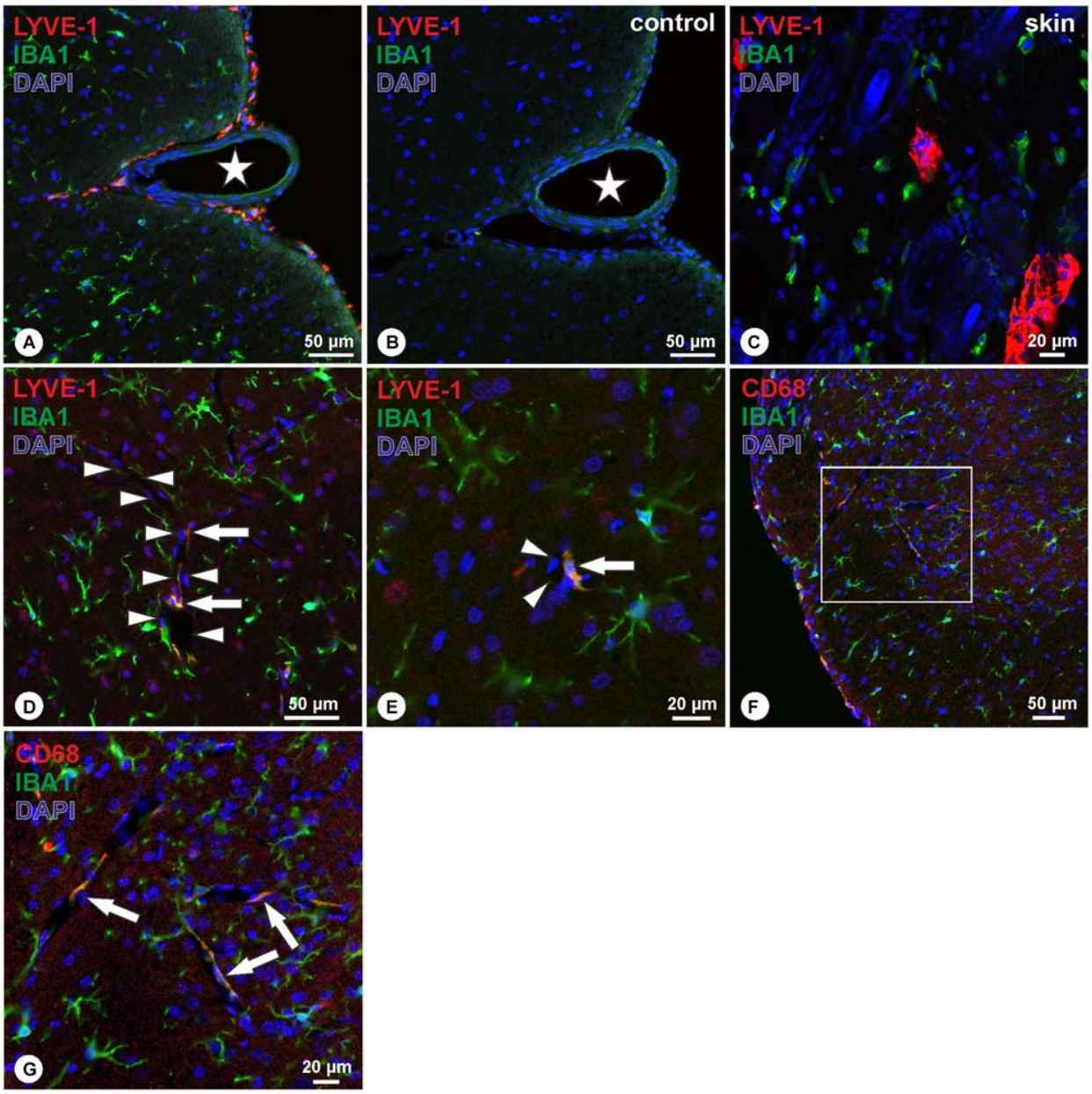

FIGURE 2 | (A–C) Double-immunohistochemistry of LYVE-1 (red) and Iba1 (green) reveals LYVE-1+/Iba1+ cells on the surface of the spinal cord, following the anterior sulcal arteries **(A)**, whereas negative controls revealed absence of immunoreactivity **(B)**. Asterisk in **(A,B)** indicates ventral spinal artery. Skin controls revealed DAPI+ nuclei (blue) surrounded by LYVE-1-immunoreactivity **(C)**, and dispersed cells showing immunoreactivity for Iba1 only. **(D,E)** Within the spinal cord parenchyma, double immunohistochemistry for LYVE-1 (red) and Iba1 (green) reveals cells co-localizing for both markers (arrows in **D,E**) bordering spinal cord blood vessels (arrowheads in **D,E**), whereas the majority of cells displayed immunoreactivity for Iba1 only. Blue: DAPI. **(F,G)** Double-immunohistochemistry of CD68 (red) and Iba1 (green) reveals CD68+/Iba1+ cells on the surface of the spinal cord **(F)**, and on cells bordering spinal cord blood vessels (arrows in **G**). **(G)** Higher magnification of boxed area in **(F)**. Blue: DAPI.

a subpopulation of CD68+ cells was also immunoreactive for MCHII (**Figure 4A**). Iba1-immunoreactive cells were interspersed between CD68+/MHCII+ cells (**Figure 4A**). The amount of CD68-immunoreactive cells decreases towards the periphery of the lesion site, and similarly Iba1+ cells were less frequently detected. Within the lesion site, LYVE-1+ cells assembled in structures that were not observed in the unlesioned tissue (**Figures 4B,C**). These LYVE-1+ structures appeared organized (**Figure 4C**), where associated with elongated nuclei, and displayed vessel-like formation (**Figures 4C,D**). Double immunohistochemistry with LYVE-1 and PROX1 revealed that the majority of LYVE-1 immunoreactive structures were not associated with PROX1-poitive nuclei (**Figure 4E**). However, in few

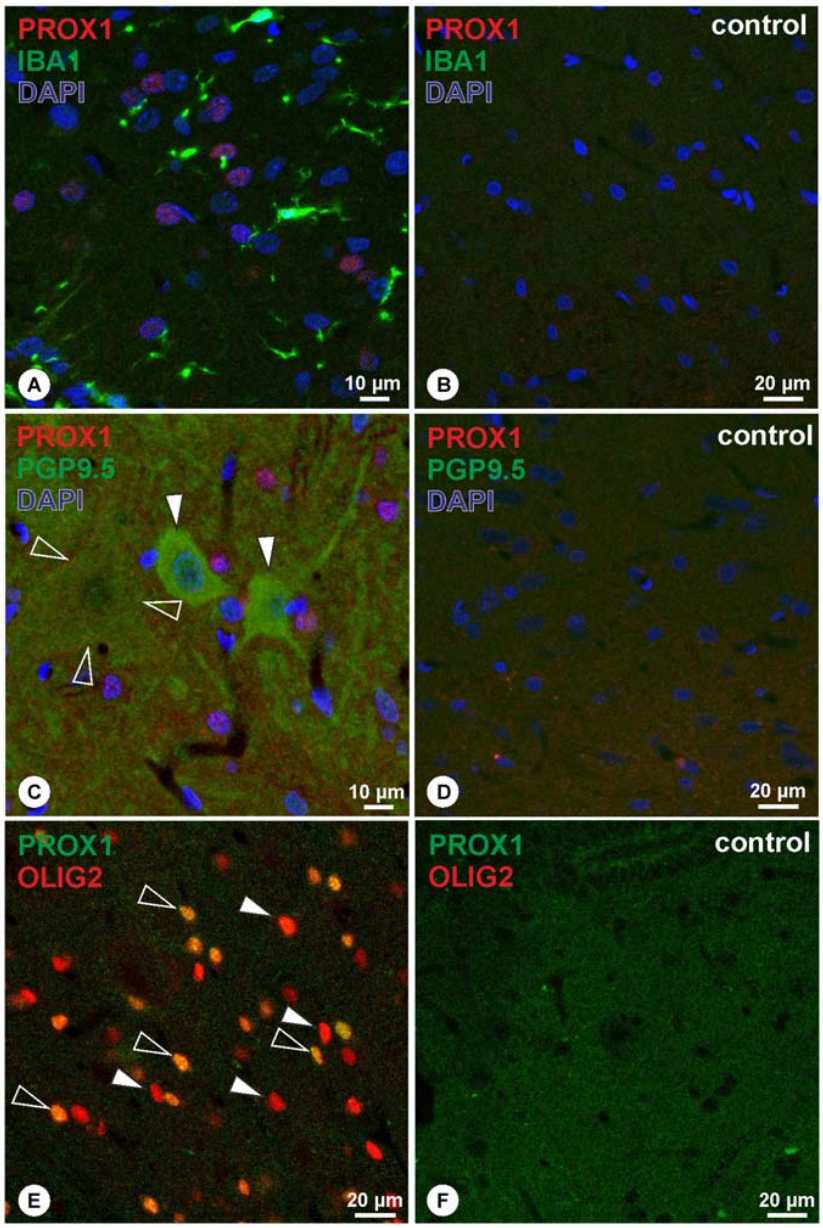

FIGURE 3 | (A,B) Double-immunohistochemistry of PROX1 (red) and Iba1 (green) reveals that PROX1 positive nuclei (purple mixed color) are not associated with Iba1-positive cells **(A)**, whereas negative controls lack immunoreactivity **(B)**. Blue: DAPI. **(C,D)** Double-immunohistochemistry of PROX1 (red) and the pan-neuronal marker PGP9.5 (green) reveals that PROX1-positive nuclei (purple mixed color) are not associated with neurons of the spinal cord (**C**; arrowheads point to small neurons, open arrowheads outline a faint immunoreactive large neuron), whereas negative controls lack immunoreactivity **(D)**. Blue: DAPI. **(E,F)** Double-immunohistochemistry of PROX1 (green) and OLIG2 (red) reveals co-localized nuclei (**E**; yellow mixed color, open arrowheads), whereas some nuclei display OLIG2-immunoreactivity only (**E**; arrowheads). Immunoreactivity was absent in corresponding negative controls **(F)**.

instances, PROX1-positive nuclei were closely related to LYVE-1 immunoreactive structures (**Figure 4F**).

DISCUSSION

The dominant opinion in current textbooks considers the CNS as an alymphatic environment (Iliff and Nedergaard, 2013). However, with the availability of lymphatic markers, this dogma has been recently revisited and challenged (Aspelund et al., 2015; Louveau et al., 2015; Wood, 2015). Therefore, we investigated adult rat spinal cord for the expression of components specific for lymphatic endothelium, namely the membrane bound glycoprotein LYVE-1 in combination with the transcription factor PROX1. LYVE-1 as a marker

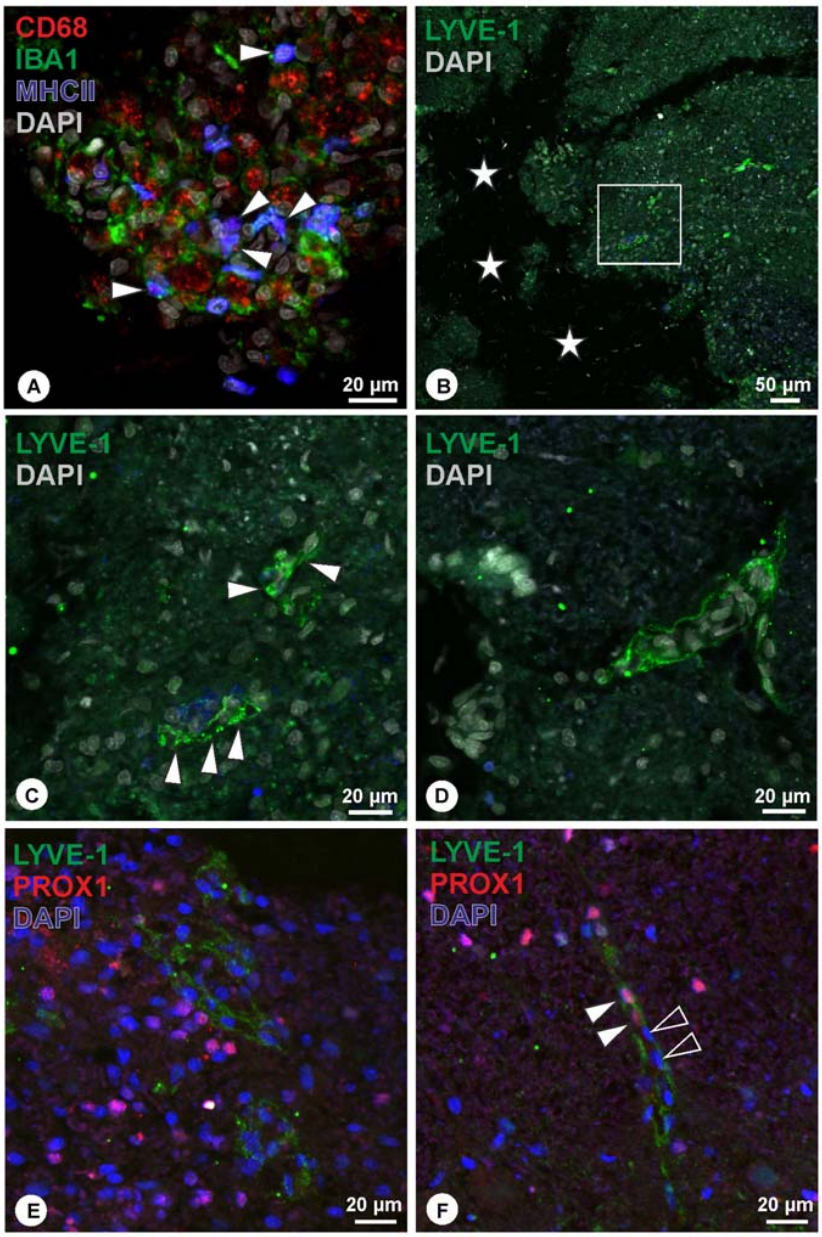

FIGURE 4 | (A) Within the lesion site, many cells were detected displaying immunoreactivity for CD68 (red), a subpopulation of which co-localized for MHCII (blue, arrowheads), while Iba1-immunoreactive cells (green) were intermingling. **(B,C)** Close to the lesion center (asterisks), organized LYVE-1 immunoreactive structures (green) were detected (**C**, arrowheads; magnification of boxed area in **B**), displaying vessel-like appearance. DAPI: gray. **(D)** Another example of vessel-like structure expressing LYVE-1. DAPI: gray. **(E,F)** Double immunohistochemistry of LYVE-1 (green) and PROX1 (red) revealed that the majority of LYVE-1 immunoreactive structures were not associated with PROX1-positive nuclei **(E)**. In few instances, PROX1 positive nuclei were detected closely associated with LYVE-1 immunoreactive structures (**F**, arrowheads), while other nuclei in proximity were lacking PROX1 (**F**, open arrowheads).

for lymphatic endothelium was first identified in 1999 by Banerji et al. (1999). However, its expression is not exclusive for lymphatics, since it is also detected among others on liver sinusoids (Mouta Carreira et al., 2001), pulmonary (Favre et al., 2003) and renal glomerular capillaries (Lee et al., 2011) and its expression pattern may also change along the lymphatic vascular tree (Baluk and McDonald, 2008).

The function of LYVE-1 in cells of these various systems is still not fully understood (Jackson, 2009). On the other side, PROX1 is a homeobox transcription factor and a key player in the development of many organ systems, such as the enterohepatic system or heart (Oliver et al., 1993; Sosa-Pineda et al., 2000). In the CNS, PROX1 is known as a critical regulator of neurogenesis and neuronal differentiation (reviewed in Stergiopoulos et al., 2014). In nuclei of lymphatic endothelial cells, PROX1 expression

persists into adulthood in physiological as well as in pathological conditions (Wilting et al., 2002). Here, PROX1 is considered to be a master gene (Hong and Detmar, 2003) in lymphatic endothelial progenitor cells controlling the expression of other lymphatic markers (for review, see Yang and Oliver, 2014).

Although we could readily detect these two markers closely associated in lymphatic endothelial cells of the skin, our lymphatic control tissue, our study demonstrated the absence of PROX1+/LYVE-1+ cells in the spinal cord. On the other hand, we detected numerous cells expressing either of these markers, however, under physiological conditions, these were not associated with lymphatic vessel-like structures. The LYVE-1+ cells were found at the surface of the spinal cord or closely located to spinal cord blood vessels. These LYVE+ cells co-localized with the macrophage and microglia markers Iba1, and most likely also CD68, as observed by the almost overlap of the latter with Iba1-immunoreactivity. This marker combination, together with the localization in vicinity to blood vessels highly suggests that this cell population represents macrophages.

Indeed, brain macrophages are mainly situated close to cerebral blood vessels under physiological conditions (Bogie et al., 2014) and LYVE-1 expression in macrophages is well established in other systems (Schledzewski et al., 2006; Cho et al., 2007; Schroedl et al., 2008). While macrophages play an important role during pathological processes (Shechter and Schwartz, 2013), the role of the LYVE-1+ macrophage sub-population in the spinal cord in physiological and pathological conditions remains to be elucidated. Following spinal cord contusion injury, we could detect a re-organization and redistribution of cells expressing LYVE-1 within the lesioned parenchyma. Some of these cells reorganized in structures resembling putative pre-vessels (**Figure 4**). Therefore, following lesion, there is a solid evidence to reconsider the possibility of lymphatics within the spinal cord, and most likely also within the brain.

PROX1+ cells were found widespread throughout the spinal cord parenchyma. These PROX1-positive nuclei were neither detected in association with the microglia/macrophage marker Iba1, nor with the pan-neuronal marker PGP9.5, thus ruling out that they represented microglia or neurons, respectively. Instead, PROX1+ nuclei were co-localized with the oligodendrocyte lineage marker OLIG2. The co-existence of these two markers within a cell population is particularly puzzling since Olig2 and PROX1 have been reported to reciprocally suppress the expression of each other during development and in forced-expression experiments (Kaltezioti et al., 2014; Stergiopoulos et al., 2014). Nevertheless, in a study addressing the gene expression pattern found in the various cell populations of the CNS, Cahoy et al. (2008) found that Olig2 and PROX1 are enriched in the oligodendroglial lineage. Moreover, the expression of PROX1 was five times higher in myelin-producing oligodendrocytes as compared to oligodendrocyte precursor cells (OPCs). Hence, the PROX1+/Olig2+ cell population detected in the spinal cord probably consists of mature oligodendrocytes. Whether the small percentage of cells expressing solely PROX1 constitutes a distinct population, or a population with low Olig2 expression, remains to be deciphered.

With this study, we confirmed the assumption that classical lymphatic vessels are absent from the healthy adult rat spinal cord. Nevertheless, recent data generated under pathological conditions suggested the development of lymphatic structures in otherwise alymphatic environments, as seen for example in cornea (Cursiefen et al., 2002) or tendon lesions (Tempfer et al., 2015). On the other hand, the existence of structures capable of fulfilling lymphatic vessel-like functions, as recently described for e.g., the Schlemm's Canal of the eye (Aspelund et al., 2014), remains to be demonstrated in the spinal cord. The response of the LYVE-1 positive macrophages observed following spinal cord lesion needs to be further investigated as they may be the prerequisite for the formation of lymphatic-like structures (Maruyama et al., 2005; Alitalo, 2011; Ran and Montgomery, 2012; Kerjaschki, 2014).

AUTHOR CONTRIBUTIONS

FS, AK-E, SC-D, HAR conceived and designed the study and AK-E, FS, CK, LB, PZ performed the experiments or contributed to data acquisition. FS, AK-E, CK, LB, SC-D analyzed the data and AT, PZ, CK, HT, BB, CR contributed to data interpretation. AK-E, FS, SC-D wrote the manuscript and AK-E, FS, SC-D, HAR, HT, AT, PZ, LB, CK, AT, BB, CR critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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OPINION
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Beyond Clotting: A Role of Platelets in CNS Repair?

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"NON-CANONIC" ROLES OF PLATELETS: INFLAMMATION, ANGIOGENESIS AND TISSUE REGENERATION

Platelets are small, oval, circulating, anucleate cells that upon endothelial damage form a haemostatic plug and stop blood leakage. Circulating platelets derive from bone-marrow-resident megakaryocytes that daily produce and release approximately 100 billions of new platelets into the blood stream (Kaushansky, 2006; Semple et al., 2011). During haemostasis, tethering platelets adhere to the vascular injury through the interaction between their glycoprotein (GP) Ib/V/IX receptor complex and GP VI/GP Ia with the von Willebrand factor (vWF) and collagen provided by the lesioned environment, respectively. Adherent platelets aggregate and secrete platelet activation mediators, such as Thromboxane A2 (TXA2) and adenosine diphosphate (ADP). After activation, the platelets membrane surface becomes procoagulant enhancing the coagulation cascade ending in the formation and stabilization of the haemostatic plug and arresting blood leakage.

Platelet function is not restricted to haemostasis, as platelets also have inflammatory, angiogenic, and tissue repair properties (Nurden, 2011). Within their storage compartments (α-granules and dense granules), platelets store a plethora of bioactive molecules that, under specific circumstances, are secreted to the extracellular space targeting other cell types. Platelets-derived molecules include proteins such as chemokines, cytokines, and growth factors, as well as RNAs and microparticles (Brill et al., 2005; Chen et al., 2012; Lohmann et al., 2012; Schallmoser and Strunk, 2013; Warnke et al., 2013). Platelets granules contain several pro-inflammatory and anti-inflammatory molecules that contribute to immunity. In fact, platelets react against pathogens and regulate immune cells function (reviewed in Semple et al., 2011). For example, during inflammation, GPIbα, and P-selectin located at the surface of platelets interact with PSGL-1 and Mac-1 on monocytes/macrophages inducing their recruitment and activation (reviwed in Gawaz et al., 2005). Also, CD154 in activated platelets (Henn et al., 1998) binds to CD40 on endothelial cells (ECs) inducing the expression of cell adhesion molecules (i.e., VCAM1, ICAM1) and the endothelial release of CC-chemokine ligand 2 (CCL2) promoting the leukocyte recruitment to inflammatory sites (Andre et al., 2002). Moreover, CD154 supports B cell differentiation (Elzey et al., 2003; Von Hundelshausen and Weber, 2007) and platelet-secreted transforming growth factor beta (TGF-β) controls T_{reg} cell differentiation (Tran, 2012) indicating that the contribution of platelets to immunity is not restricted to the innate system but also involves adaptive response.

Platelets apparently also shape angiogenesis, which is a complex process that consists in the formation/sprouting of new capillaries from preexisting vessels. Platelets have a dual role.

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First, they stimulate ECs proliferation and can promote capillary formation (Pipili-Synetos et al., 1998). Indeed, α-granules contain several pro-angiogenic molecules that are secreted upon the activation of platelets, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), TGF-β, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), sphingosine-1-phosphate (S1P), etc (Kaplan et al., 1979; Heldin et al., 1981; Nakamura et al., 1985; Folkman and Klagsbrun, 1987; Ben-Ezra et al., 1990; Mohle et al., 1997; English et al., 2000; Jonnalagadda et al., 2014). Second, platelets are required to avoid leakage from angiogenic vessels and their absence inhibits the formation of new vessels *in vivo* (Kisucka et al., 2006).

Haemostasis, inflammation and angiogenesis are essential processes for tissue repair; thus, platelets are critically involved in many mechanisms that operate along the healing process (reviewed in Gurtner et al., 2008; Gawaz and Vogel, 2013). Upon tissue damage provoked by trauma or local ischemia, circulating platelets accumulate and become activated at the lesion site releasing their bioactive molecules into the damaged microenvironment and contributing to tissue repair and regeneration. For instance, stromal cell-derived factor-1 (SDF-1), hepatocyte growth factor (HGF), PDGF, serotonin, ADP, and platelets-derived microparticles regulate recruitment, proliferation, survival, and differentiation not only of immune cells (neutrophils, monocytes) necessary for the local inflammatory and the phagocytic responses, but also of cells that directly repair the lesion such as fibroblast, smooth muscle cells and tissue-specific progenitor cells (Nakamura et al., 1986; Crowley et al., 1994; Stellos et al., 2008, 2010; Mazzucco et al., 2010).

PLATELETS INFLUENCE CNS INFLAMMATION: IMPACT ON REPAIR?

Regardless of its immune privileged condition and the presence of the blood-brain-barrier (BBB), the CNS is not free from the action of platelets, particularly, in response to injury. As expected, after their adherence to endothelial cells, platelets activate, and recruit leukocytes into the damaged CNS tissue (Simon, 2012; Langer and Chavakis, 2013), thus, platelets interact with different cells in the neurovascular niche including neurons, glial cells, endothelial cells, pericytes, and other blood-derived cells (Hayon et al., 2013; Sotnikov et al., 2013). This particular feature confers platelets a substantial role in CNS inflammation in different pathological scenarios. After stroke, platelets adhere to the endothelium and get activated provoking further thrombo-inflammatory events exaggerating infarct development (Kleinschnitz et al., 2007; Nieswandt et al., 2011). In Alzheimer's disease (AD) the BBB is partially leaky and vascular inflammation occurs (Sardi et al., 2011). Interestingly, platelets might be contributing to the propagation of AD as they carry amyloid precursor protein and the amyloid beta, two peptides that are found around vessels in AD patients that constitute one of the molecular mechanisms for AD pathogenesis (Skovronsky et al., 2001; Catricala et al., 2012). In multiple sclerosis (MS), an autoimmune CNS demyelinating disease, platelets also seem to be involved to the pathology since they have been found in human chronic active MS lesions (Lock et al., 2002; Langer et al., 2012; Steinman, 2012). In an animal model for MS, platelets promote leukocyte infiltration as well as CNS inflammation (Lock et al., 2002; Langer et al., 2012). Therefore, platelets contribute to neuroinflammation and an altered platelet functions may lead to pathological conditions.

Besides their role in pathogenesis, platelets might also be involved in the regulation of regenerative processes by interacting with CNS stem/progenitor cells. Adult neural stem cells (NSCs) are undifferentiated self-renewing multipotent cells that reside in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) of the wall of the lateral ventricles (Altman, 1965; Gage, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002). Oligodendrocyte progenitor cells (OPCs) represent the major cellular source for remyelinating oligodendrocytes and are widely spread throughout the CNS (Ffrench-Constant and Raff, 1986; Woodruff and Franklin, 1999; Franklin and Ffrench-Constant, 2008). Interestingly, NSCs and OPCs drive CNS repair in response to stroke or to MS-associated demyelination (Arvidsson et al., 2002; Franklin, 2002; Kokaia and Lindvall, 2003; Gonzalez-Perez et al., 2009). While neuroinflammation was mainly considered to be an aggravating factor, several recent studies have revealed a supportive role in CNS repair (Patel et al., 2010; Jaerve and Muller, 2012; Miron and Franklin, 2014). For example, the very complex process of remyelination (Franklin and Ffrench-Constant, 2008; Rivera et al., 2010) involves a crucial inflammatory stage that precedes regeneration and occurs acutely after myelin damage. This innate immune response is, at least partially, mediated by blood-recruited macrophages and CNS-resident microglial cells. During remyelination, circulating monocytes/macrophages are recruited by chemotaxis (Charo and Ransohoff, 2006; Ruckh et al., 2012) and, depending on their inflammatory state, exert the following functional roles: (i) they are responsible for the removal (clearance) of myelin debris [which is a potent inhibitor of OPC differentiation (Kotter et al., 2006; Baer et al., 2009)] through phagocytosis, and (ii) they secrete cytokines, growth- and neurotrophic factors that stimulate OPC responses to demyelination (Setzu et al., 2006; Zhao et al., 2006; Ruckh et al., 2012; Miron et al., 2013). A regulation of macrophage recruitment and activity is essential to couple inflammation and regeneration during CNS myelin repair (Miron and Franklin, 2014). However, the cellular and molecular cues that regulate these events are still unknown. As previously mentioned, platelets promote the endothelial secretion of chemokines known to recruit circulating monocytes/macrophages (Gawaz et al., 1998, 2000). Furthermore, through the secretion of PDGF and platelet factor 4 (PF-4), activated platelets directly promote the recruitment of monocytes and modulate their activity (Deuel et al., 1982; Brandt et al., 2000; Fricke et al., 2004). Thus, it can be hypothesized that circulating platelets might influence macrophage/microglia recruitment and activity thereby linking neuroinflammation to CNS repair.

PLATELETS MODULATE CNS-RESIDENT STEM/PROGENITOR CELL FUNCTION: IMPACT ON REPAIR?

Supporting the previous hypothesis, a series of findings suggest that platelets directly exert CNS-regenerative activities and might contribute to neuroregeneration (see **Table 1**). Recently we reported that upon demyelination in the corpus callosum (CC), platelets specifically accumulate within the ipsilateral SVZ vasculature, a process associated with an enhanced survival of SVZ-resident NSCs (Kazanis et al., 2015). Importantly, we found that a mechanical non-demyelinating lesion within the CC is not enough to induce such accumulation of platelets in the SVZ vasculature, indicating that cellular degeneration is required for such an effect. Considering that SVZ-derived NSCs contribute to remyelination in the CC (Jablonska et al., 2010; Xing et al., 2014), these findings suggest that platelets might play a role in controlling the NSCs pool available for CNS repair. The mechanisms that mediate the very specific accumulation of platelets in the SVZ vasculature far from the lesion site, and that promote NSCs survival are not known. However, platelets derived molecules might be involved as in the same study we found that platelet lysate (PL) protects proliferating NSCs from apoptosis (Kazanis et al., 2015). Furthermore, it has been previously suggested that activated platelets contribute to recovery after brain injury (Hayon et al., 2012c). For example, it has been shown in an animal model for stroke that infused platelets derived microparticles (PMP) increased cell proliferation, neurogenesis and angiogenesis at the infarct boundary zone leading to improvements in behavioral outcomes (Hayon et al., 2012a). In addition to this, a different study showed that PMP promotes NSCs survival and increased their differentiation potential to glia and neurons (Hayon et al., 2012b). Also, upon intracerebroventricular administration of PL into an experimental model of stroke resulted in a significant increase in angiogenesis and in the number of proliferating SVZ-resident NSCs (Hayon et al., 2013). Besides these findings, several platelets derived molecules influence CNS progenitor function (see **Table 1**). For instance, the dense granules of platelets contain serotonin (White, 1968), which is known to control NSCs activity and adult neurogenesis (Brezun and Daszuta, 1999; Banasr et al., 2004; Goto et al., 2016). The effect of platelets derived molecules might not only target NSCs but also OPCs during remyelination. For instance, platelets' α-granules contain large quantities of PDGF and bFGF (Lohmann et al., 2012; Schallmoser and Strunk, 2013), factors that are known to promote OPCs survival, proliferation, and recruitment (Woodruff et al., 2004; Murtie et al., 2005; Zhou et al., 2006). Moreover, upon activation, platelets' α-granules secrete S1P (English et al., 2000; Jonnalagadda et al., 2014), a molecule, that is known to modulate OPCs survival, proliferation, and differentiation (Jung et al., 2007). In summary, platelets react to injury and secrete a plethora of bioactive molecules that might directly influence NSCs and OPCs function, probably, modulating CNS repair. This hypothesis could be evaluated by studying neuroregeneration in animal models that display platelet deficiencies (number and/or function). Also, by exploring gene expression databases complemented with proteomics data, further studies could identify molecules contained in platelets that may influence CNS repair.

FINAL REMARKS

There is accumulating evidence that the role of platelets is not restricted to haemostasis, but it also involves the regulation of inflammation, angiogenesis and tissue repair. The CNS contains NSCs and OPCs that contribute to cellular turnover and CNS repair. In light of the accumulating evidence that associates

TABLE 1 | Evidences suggesting possible direct contribution of platelets in CNS repair.

Platelets derived activity or factor	Experimental Model	Findings	References
Circulating Platelets	Lysolecithin-induced demyelination in the CC	Platelets aggregation in the SVZ vasculature associated to NSCs survival	Kazanis et al., 2015
Platelet lysate (PL)	*In vitro* proliferating NSCs	PL promotes NSCs survival and protects from apoptosis	Kazanis et al., 2015
Platelets-derived microparticles (PMP)	Permanent middle cerebral artery occlusion (PMCAO). PMP administrated to the brain surface	PMP increases cell proliferation, neurogenesis, and angiogenesis	Hayon et al., 2012a
PMP	*In vitro* NSCs	PMP promotes survival and increases the differentiation potential of NSCs	Hayon et al., 2012b
PL	PMCAO. PL administrated into the lateral ventricles	PL increases the number of NSC and angiogenesis in the subventricular zone (SVZ) as well as in the peri-lesion cortex	Hayon et al., 2013
Serotonin (contained in dense granules)	*In vivo* normal wild type rats. Systemic administration of serotonin	Serotonin regulate NSCs proliferation in the SVZ and hippocampus and modulate adult neurogenesis	Banasr et al., 2004
PDGF and bFGF (contained in α-granules)	Cuprizone-induced demyelination in PDGFαR+/− mice, FGF2 knockout (−/−) mice, and PDGFαR+/− FGF2−/− mice	PDGF and bFGF regulate OPCs proliferation and differentiation during CNS remyelination	Murtie et al., 2005
S1P (contained in α-granules)	*In vitro* OPCs. Use of S1P analogs: FTY720 and FTY720P, both modulators of S1P receptors	By different mechanisms S1P and its receptors regulate OPCs proliferation, survival, and differentiation	Jung et al., 2007

platelets to neuroinflammation, especially under pathological conditions, their potential role in CNS repair has to be further investigated. Recent findings indicate that neuroinflammation is also relevant for CNS repair as it contributes to debris clearance and controls CNS-resident stem/progenitor cells function, suggesting a potential role for platelets by linking inflammation to regeneration. This hypothesis is supported by the facts that circulating platelets react to CNS injury and accumulate within the adult stem cell niche and that activated platelets release a plethora of bioactive molecules that not only regulate immune cells activity but also directly modulates NSC and OPC respond to injury. It is, therefore, likely that platelets might modulate CNS repair. Prospectively, this specific lesion-induced accumulation of circulating platelets at sites of tissue damage, inflammation, and even stem/progenitor cell activity (as in Kazanis et al., 2015) opens the possibility to use genetically manipulated platelets or manufactured platelet-like particles (Risitano et al., 2012; Brown et al., 2014) when aiming for the delivery of specific molecules directly to targeted areas.

AUTHOR CONTRIBUTIONS

FR wrote the manuscript. IK, CG, and LA critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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The influence of platelet-derived products on angiogenesis and tissue repair: a concise update

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Platelet degranulation allows the release of a large amount of soluble mediators, is an essential step for wound healing initiation, and stimulates clotting, and angiogenesis. The latter process is one of the most critical biological events observed during tissue repair, increasing the growth of blood vessels in the maturing wound. Angiogenesis requires the action of a variety of growth factors that act in an appropriate physiological ratio to assure functional blood vessel restoration. Platelets release main regulators of angiogenesis: Vascular Endothelial Growth Factors (VEGFs), basic fibroblast growth factor (FGF-2), and Platelet derived growth factors (PDGFs), among others. In order to stimulate tissue repair, platelet derived fractions have been used as an autologous source of growth factors and biomolecules, namely Platelet Rich Plasma (PRP), Platelet Poor Plasma (PPP), and Platelet Rich Fibrin (PRF). The continuous release of these growth factors has been proposed to promote angiogenesis both *in vitro* and *in vivo*. Considering the existence of clinical trials currently evaluating the efficacy of autologous PRP, the present review analyses fundamental questions regarding the putative role of platelet derived fractions as regulators of angiogenesis and evaluates the possible clinical implications of these formulations.

Keywords: platelet poor plasma, platelet rich plasma, angiogenesis, tissue engineering, growth factors

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INTRODUCTION

Wound healing, a natural restorative response to tissue injury, is governed by an elaborate response driven by resident and circulating cells, homing to the injury site, that release soluble mediators or signals generated from the extracellular matrix (ECM; Guo and DiPietro, 2010). In adult humans, optimal wound healing involves a cascade of complex, orderly, and predictable events that include four overlapping phases: hemostasis, inflammation, proliferation, and remodeling. The adequate timing of these phases is decisive for ultimate restoration of the vascular system (Gosain and DiPietro, 2004; Eming et al., 2007). Platelets regulate hemostasis through vascular obliteration and fibrin clot formation (Guo and DiPietro, 2010). Platelets are anucleated cell fragments that originate from megakaryocytes in the bone marrow (Speth et al., 2015). Among the three-reservoir organelles described in platelets, namely lysosomes, alpha granules, and dense granules, the biggest compartments for protein storage are alpha granules. The latter are considered key organelles with respect to platelet function. In the clot, platelets are responsible for the activation and release of important biomolecules from their alpha granules, including platelet-specific proteins, growth

factors, coagulation factors, adhesion molecules, cytokines, angiogenic factors, proteoglycans, and cytokines/chemokines (Nurden, 2011). The release of cytokines, chemokines, and growth factors induces proliferation and activation of the cells that are involved in wound healing such as fibroblasts, neutrophils, monocytes, smooth muscle cells, and mesenchymal stem cells (MSC) (Thushara et al., 2015).

Biological products for wound treatment and surgical interventions have been an area of enormous growth in the last two decades, as our understanding of wound healing response has increased. In particular, the use of human plasmatic fractions has been proposed to locally deliver platelet-derived factors as an autologous source of biomolecules for tissue healing. In this review, we summarize (1) the importance of growth factors and biomolecules related to angiogenesis present in plasmatic fractions with different concentrations of platelets and (2) the clinical rationale for their use in cell therapies involved in the treatment of traumatic injuries as well as degenerative diseases.

CONTRIBUTION OF PLATELETS TO ANGIOGENESIS

After the inflammatory phase has been initiated, the wound healing response requires angiogenesis as a process that modulates the activation, proliferation, and migration of endothelial cells to establish new blood vessels from pre-existing vasculature (Oklu et al., 2010). Platelets play a critical role in regulating angiogenesis. Nevertheless, their contribution to blood vessel repair in the course of wound healing is still poorly understood (Eming et al., 2007; Klement et al., 2013). Alpha granules are a reservoir of biological factors for platelet physiological and pathological angiogenic responses (Peterson et al., 2010; **Table 1**). The release of these crucial angiogenic factors in platelet derived fraction preparations could be useful in tissue regeneration and wound healing.

PLATELET DERIVED FRACTIONS

The use of platelet-derived fractions in tissue repair is a developing area for clinician's and researchers. Marx et al. demonstrated a potential use of Platelet Rich Plasma (PRP) in craniofacial bone grafts in the late nineties (Marx et al., 1998), and since then, plasmatic fractions have been promoted as suitable sources of autologous growth factors. PRP may be defined as a component of plasma fraction of autologous venous blood with platelet counts in the range between 4 and 6 times above baselines considered to be of therapeutic benefit (1 million platelets/L; Chen and Liu, in press). The preparation of PRP by centrifugation was initially completed by a "two-step gradient centrifugation method." A strong first spin was used first in order to separate the erythrocytes from the clotting factors, platelets, and leukocytes. Then, the plasma was subjected to a second centrifugation step, to harvest the PRP fraction from the platelets and leukocytes. Finally, platelets in PRP were activated to release the biomolecules, using thrombin or calcium chloride. Other authors have proposed alternative methods to liberate growth factors from platelets, such as lysing the platelets by freezing them or using sonication or ultrasound (Weed et al., 2004). Once the release of biomolecules from platelets is activated, a network is formed to establish a fibrin clot that acts as scaffold for growth factors over a limited period of time (Mautner et al., 2015).

Nowadays most of the commercially accessible kits involve a one-step method to separate the plasma into three distinct layers: the erythrocytes, the buffy coat containing PRP, and the Platelet Poor Plasma (PPP) (Bausset et al., 2012; Burnouf et al., 2013). Currently, plasmatic fractions have been classified according to at least two key parameters: the presence of leukocytes and the fibrin architecture (Dohan Ehrenfest et al., 2014). Following these criteria, we can find four family fractions:

Pure Platelet-rich Plasma (P-PRP) low or without Leukocytes: Plasmatic preparations from anticoagulated venous blood "without leukocytes and with a low- density fibrin network." The white blood cell count of these samples is less than the whole blood percentage (Riboh et al., 2015). After its activation with calcium chloride or thrombin, this preparation can be used as a liquid solution or as a gel (Gobbi and Vitale, 2012; Dohan Ehrenfest et al., 2014; Mautner et al., 2015).

Platelet-rich Plasma (L-PRP) with Leukocytes: Plasmatic fractions from anticoagulated venous blood "with leukocytes and with a low-density fibrin network." The leukocytes content in these preparations is at least five times compared with the base line of whole blood counting (Filardo et al., 2013). Following activation, L-PRP might be used either as a liquid solution or in an activated gel form. Among the commercial systems available are Harvest Smart- PreP (Harvest Technologies, Plymouth, MA, USA), Biomet GPS III (Biomet Inc., Warsaw, IN, USA), Plateltex (Prague, Czech Republic), and Regen PRP (RegenLab, Le Mont-sur-Lausanne, Switzerland; Gobbi and Vitale, 2012; Dohan Ehrenfest et al., 2014; Mautner et al., 2015).

Pure Platelet-rich Fibrin (P-PRF) low or without Leukocytes: These correspond to "preparations without leukocytes and with a high-density fibrin network." Fibrin, in combination with growth factors, has been shown to effectively support cell adhesion and proliferation. P-PRF only exists in a solid activated gel form. To date, only one product of this family is commercially available, known as Fibrinet PRFM (Platelet-Rich Fibrin Matrix, Cascade Medical, Wayne, NJ, USA; Gobbi and Vitale, 2012; Dohan Ehrenfest et al., 2014; Mautner et al., 2015).

Platelet-rich Fibrin (L-PRF) with Leukocytes: Also named Choukroun's PRF. In this preparation venous blood is obtained without any anticoagulant and directly centrifuged. A cascade of calcium chloride or thrombin is used, which results in the isolation of this plasmatic fraction without any biochemical modifications. These preparations, existing only in gel form, have leukocytes and a high-density fibrin network (Dohan Ehrenfest et al., 2009, 2014; Gobbi and Vitale, 2012; Mautner et al., 2015).

For a complete and updated overview of platelet-derived fractions and the requirements for their preparation for clinical use, please refer to the following other excellent reviews: De Pascale et al. (2015), Kawase (2015), and Mautner et al. (2015).

TABLE 1 | Growth factors stored in platelets alpha granules involved in angiogenesis.

Angiogenic factor	Function	References
Vascular endothelial growth factors (VEGFs)	Regulates angiogenesis Controls proliferation, morphogenesis, migration, and survival of endothelial cells. Promotes the enlargement and branching of blood vessels.	Min Park et al., 2014 Carmeliet and Jain, 2011
Platelet Derived Growth Factors (PDGF)	PDGF-B and PDGF-C isoforms are involved in vessel maturation and recruitment of endothelial progenitor cells from the bone marrow. Recruits pericytes and vascular smooth muscle cells to maintain the blood vessel wall.	Raz et al., 2014 Dimmeler, 2005; Herbert and Stainier, 2011
Hepatocyte Growth Factor (HGF)	Mitogen for endothelial cells and stimulates secretion of VEGF.	Matsumura et al., 2013
Basic fibroblast growth factor (bFGF)	Induces proliferation and tubule formation of endothelial progenitor cells. Stimulates secretion of VEGF on endothelial progenitors *in vitro*.	Litwin et al., 2015
Connective tissue growth factor (CTGF)	Regulates vascular remodeling by controlling pericyte recruitment and inducing PDGF-B expression on endothelial cells.	Hall-Gleen et al., 2012
Angiopoietins	Maintains vessel and vascular leakiness, and induces pericyte chemotaxis	Nurden, 2011; Klement et al., 2013; Hwang et al., 2015
Stromal cell derived factor (SCGF)	Induces chemotaxis of endothelial precursors and increases formation of vascular structures.	De Falco et al., 2004
Epidermal Growth Factor (EGF)	Induces tubule formation, endothelial cell proliferation, and migration.	Klement et al., 2013

PLATELET CONCENTRATES AND ANGIOGENESIS

Diverse growth factors are involved in the process of angiogenesis, of which many are secreted by platelets (Peterson et al., 2010). Given the critical role of angiogenesis in modulating wound healing and considering that platelet-derived factors are critical for vascular activation and stabilization, it is tempting to speculate whether any of the platelet-derived formulations currently used in regenerative medicine stimulate angiogenesis. Preclinical studies, using *in vitro* assays and animal models have suggested a positive influence of platelet-derived fractions on angiogenesis.

In vitro Studies

In order to identify the mechanisms whereby platelet-derived fractions may stimulate angiogenesis, Bertrand-Duchesne et al. (2010) evaluated the presence of angiogenic growth factors in PRP samples. They detected high levels of VEGF, PDGF-BB, EGF, and basic fibroblast growth factor (bFGF). PRP supernatants were incubated with blocking antibodies to neutralize each of these growth factors and the response to these treatments was evaluated via proliferation assays in Human Umbilical Vein Endothelial Cells (HUVEC). Notably, the use of EGF neutralizing antibodies decreased significantly the proliferation of HUVEC, while the other antibodies did not affect this response. Another mechanistic study by Mammoto et al. evaluated the role of angiopoietin-1 in driving the vascular response in mouse PRP and PRF formulations. They found that mouse platelet derived fractions containing angiopoietin-1 (Ang) were responsible for increasing proliferation, migration, and differentiation of human microvascular endothelial cells (Mammoto et al., 2013). Li et al. (2014) observed that PRP might promote vascular growth andstimulate endothelial progenitor cells to form vessel-like structures. Anitua et al. (2015) recently studied the effect of a platelet concentrate displayed within a plasma suspension that forms a fibrin matrix system on angiogenesis. This product, called PRGF (plasma rich in growth factors) (P-PRP low leukoyte content), stimulated an increase in cell proliferation and a reduction in apoptosis in primary HUVEC and skeletal myoblasts.

Preclinical Animal Studies

In a recent study, Zhou et al. (2013) investigated the effect of the application of a PRP gel in open abdominal wounds performed in rats. After inducing a peritonitis lesion they performed laparotomies and animals were treated with either PPP or PRP. After 1 week of healing, the animals treated with PRP demonstrated higher blood perfusion in the original lesion as well as a more mature granulation tissue when compared to those treated with PPP. In addition, injection of the product within the injured muscle tissue of mice induced the reperfusion of blood into the lesion. Using an innovative approach to release platelet-derived growth factors in a drug-delivery system, Notodihardjo et al. (2015) used a gelatin hydrogel compound coupled to platelet rich plasma molecules to stimulate wound healing. They observed an increase in epithelialization and vascular growth when compared to other treatments, including the gelatin hydrogel system (drug delivery system) and the PRP group. In another approach to stabilize

growth factors released from platelets, a fragmine-protamine micro-nanoparticle system was used to promote healing events of skin grafts showing a positive effect on wound epithelialization and angiogenesis (Takabayashi et al., 2015). Using autologous activated platelet supernatant; Kang et al. (2014) studied their effects on vasculogenesis in peripheral blood stem cells of human origin. With this approach they observed that stem cells primed with this platelet fraction stimulated vascular growth in athymic mice (Kang et al., 2014). In skin ulcers performed in porcine, Roy et al. (2011) showed that a platelet-rich fibrin matrix was able to stimulate wound healing by enhancing angiogenesis. In order to stimulate tendon regeneration, tendon healing was studied in New Zealand rabbits in which Achilles tendons were sectioned and subsequently treated with PRP or saline. PRP treated-tendons demonstrated increased angiogenesis and better collagen fiber re-alignment when compared to saline treated specimens (Lyras et al., 2009). To this end, Mammoto et al. (2013) identified that angiopoietin-1 is highly represented in PRP. Moreover, they observed that inhibition of angiopoietin-1-Tie2 signaling was able to suppress the angiogenic induction by a platelet-rich fibrin matrix *in vivo*. These studies show that in general, platelet-derived fractions may exert a potent pro- angiogenic response in different organs or anatomical locations and clearly justify further research.

POTENTIAL APLICATIONS OF PLATELET DERIVED FRACTIONS: TISSUE ENGINEERING

As stated by Vishwakarma et al. (2015): "Tissue engineering and regenerative medicine is a rapidly growing multidisciplinary field involving the life, physical, and engineering sciences that seeks to repair, regenerate, or replace biological cell, tissue, and organ substitutes that have been lost due to congenital abnormalities, injury, disease, or aging." The development of tissue-engineered products must involve a vascular support. Cellular function and viability are highly dependent on the effective diffusive exchange of nutrients through tissue. *In vivo*, cells are found within 200 μm-away from the nearest capillary network, otherwise they may suffer from ischemia and necrosis. Most of the tissue engineering scaffolds and composites are typically avascular. Therefore, it is essential that revascularization strategies stimulate regeneration of vascular networks in order to obtain a successful clinical outcome of an implanted cell-construct (Upputuri et al., 2015). The scaffolds need to support cell proliferation and differentiation to replace specific tissue loss *in vivo*. However, they must also provide a suitable substrate that allows adequate blood vessel growth to supply nutrients and oxygen to the cells located inside this engineered composite

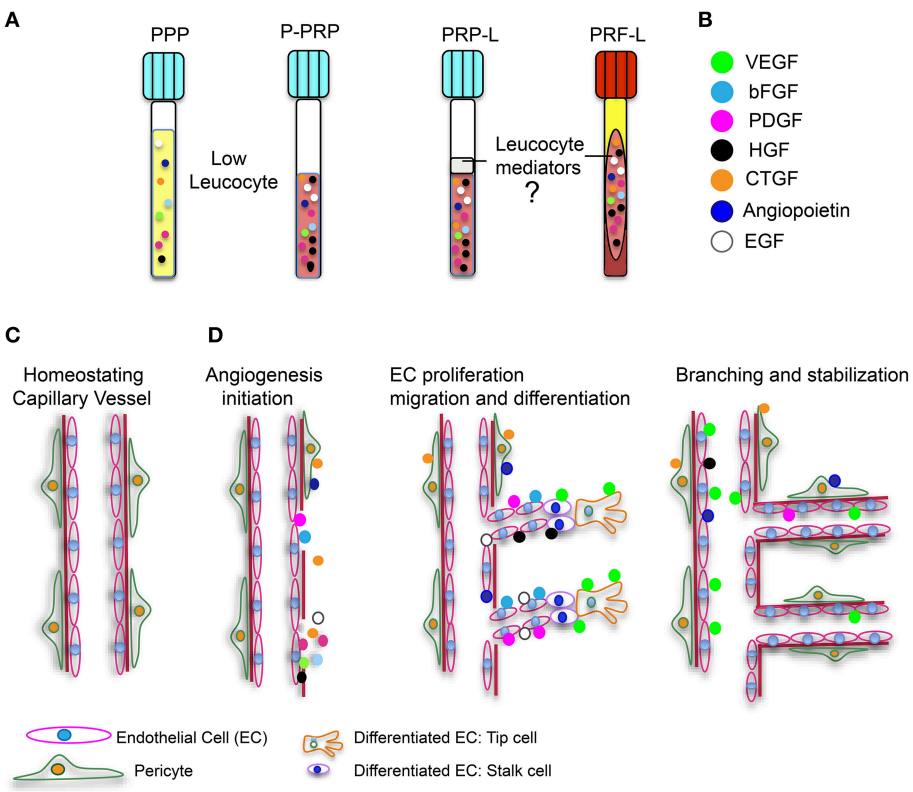

FIGURE 1 | Platelet derived fractions and involvement of angiogenic growth factors in angionesis. (A) Four platelet-derived fractions are illustrated. Two with low leucocyte content (PPP and P-PRP), and two with high leucocyte quantities (L-PRP and L-PRF). **(B)** Angiogenic growth factors are represented with the indicated color code. **(C)** Illustration of a capillary blood vessel in physiological conditions. **(D)** Influence of angiogenic growth factors during angiogenesis initiation, endothelial cell (EC) proliferation, migration, and differentiation and finally, branching and stabilization of new blood vessels during a healing event.

(Cenni et al., 2011). Considering the essential role of angiogenesis during the tissue engineering process, it is important to evaluate the scaffold features and properties to predict its vascularization potential and its possible interactions with endothelial and stem cells (Cenni et al., 2011). As already mentioned, plasmatic fractions constitute a source of main angiogenic growth factors, such as PDGF, VEGF, FGF-2, and EGF, as well as other proteins involved in the angiogenic process. Platelet angiogenic potential also resides in the presence of cytokines, integrins, hepatocyte growth factor, interleukin-8 (IL-8), IL-3, αvβ3− integrin 212, and matrix metalloproteinases (MMPs), which degrade ECM facilitating endothelial cell migration (Cenni et al., 2011; Klement et al., 2013). It is also important to note that the use of platelet-derived fractions has a considerable advantage, offering multiple pro-angiogenic factors compared to the application of high doses of recombinant growth factors to the wound site. Even the use of PPP could be a good alternative to the use of autologous growth factors. Recently, our research team evidenced the presence of angiogenic factors and biomolecules related to bone differentiation on PPP (Martínez et al., 2015). These results, combined with preclinical data provided by other research teams, suggest that (1) this PPP fraction could potentially be considered a good alternative to the use of autologous growth factors and proteins in combination with tissue engineering scaffolds and (2) that it might present an opportunity to increase the effectiveness of treatments in clinical applications (Yilmaz et al., 2011; Hateyama et al., 2014; Martínez et al., 2015). Angiogenic factors found in platelet-derived fractions can participate in cellular events involved in angiogenesis (**Figure 1**, Agren et al., 2013; Amable et al., 2013; Martínez et al., 2015). The angiogenic process requires the proliferation, migration, and adequate differentiation of endothelial cells (EC). ECs, categorized as (1) migrating leading "tip" cells to guide the direction of new blood vessel formation and (2) trailing "stalk" cells to establish the lumen of the new vessel, are indispensable to the branching and stabilization of new blood vessels (Carmeliet and Jain, 2011, **Figure 1**). However, it is still unknown whether or not "leukocyte mediators" have an effect on angiogenesis in the platelet-derived fractions. Overall, the use of platelet-derived fractions is promising in the process of angiogenesis during wound healing and regeneration. However, there is no consensus regarding the protocols utilized for their extraction, their effect on target cells, the concentration of the growth factors, and the effect of inflammatory mediators. Consensus needs to be reached in order to better exploit the clinical potential of platelet-derived fractions.

Platelet Derived Fractions and Mesenchymal Stem Cells (MSC)

Many preclinical and clinical studies have demonstrated the benefits of using MSC to promote tissue repair; MSC-based therapies are gaining ground in wound management. It has been shown that MSCs might repair damaged endothelium by secreting trophic factors that allow the recruitment of endogenous stem cells, which helps facilitate angiogenesis (Bronckaers et al., 2014). MSCs' activity depends on the instructive microenvironment or niche. To date, the positive influences of plasmatic fractions have been reported mainly in relation to PRP, proliferation, stemness, and preservation of the MSC immune-modulatory properties (Chieregato et al., 2011; Copland et al., 2013; for recent systematic review refer to Rubio-Azpeitia and Andia, 2014).

Additionally, translational medicine using MSC therapies requires protocols that can rule out the possibility of contamination or immunological reactions toward xenogeneic compounds (i.e., animal serum) used in traditional cell culture protocols (Lange et al., 2007; Goedecke et al., 2011). The use of fetal bovine serum in the maintenance of MSCs is undesirable because of viral/prion disease transmission risks that can initiate xenogeneic immune responses (Doucet et al., 2005; Even et al., 2006). Studies concerning bone marrow and periodontal ligament MSCs have shown that supplementing medium with autologous serum or platelet-derived fractions, instead of animal serum, is a good source of growth factors due to the fact that they provide sufficient *ex vivo* expansion, decrease the time required to reach confluence, increase the size of colony forming units, and maintain their osteogenic, chondrogenic, and adipogenic differentiation capability (Tonti and Mannello, 2008; Ben Azouna et al., 2012; Martínez et al., 2015). Advancement in stem cell research will help to reveal the intimate mechanisms and interactions between stem cells and platelet-derived growth factors in wounds.

FINAL REMARKS

From a therapeutic viewpoint, platelet concentrate seems to be quite promising. However, there is no consensus regarding their use. The application of platelet-derived fractions still demands standardization of its preparation, a more detailed characterization of their biomolecule composition and angiogenesis potential, as well as well-designed and controlled clinical trials. Several important questions regarding the timing of treatment and the actual impact of platelet fractions on restoring angiogenic activity remain to answer.

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Tendon Vasculature in Health and Disease

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Tendons represent a bradytrophic tissue which is poorly vascularized and, compared to bone or skin, heal poorly. Usually, a vascularized connective scar tissue with inferior functional properties forms at the injury site. Whether the increased vascularization is the root cause of tissue impairments such as loss of collagen fiber orientation, ectopic formation of bone, fat or cartilage, or is a consequence of these pathological changes remains unclear. This review provides an overview of the role of tendon vasculature in healthy and chronically diseased tendon tissue as well as its relevance for tendon repair. Further, the nature and the role of perivascular tendon stem/progenitor cells residing in the vascular niche will be discussed and compared to multipotent stromal cells in other tissues.

Keywords: tendon vasculature, tendon stem/progenitor cells, tendinopathy, lymphatics, tendon regeneration

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INTRODUCTION

Tendons are fibrous bands of connective tissue which connect muscle to bone and are able to withstand tension. Besides the mere transmission of force, their physiologic role is also the storage and recovery of energy, made possible by specific biomechanical properties. Upon damage, either due to traumatic injury and/or chronic degeneration, the macromolecular structure is disturbed, resulting in inferior tissue quality. Unlike in other, highly vascularized tissues such as skin or bone, neovascularization following tendon injury is not necessarily a hallmark of functional tissue repair. Instead, it is associated with degeneration as healthy tendons generally are poorly vascularized with relatively few cells embedded in an abundant collagen matrix. The aim of this review is to provide an overview of our current understanding of tendon vasculature and its role in healthy and developing tendon as well as the involvement of the vascular bed in tendon de- and regeneration.

VASCULATURE IN INTACT TENDONS

In early medical literature tendons are described as "*virtually dead during life*" (Edwards, 1946), based on the fact that tendons are poorly vascularized and contain only few cells compared to other tissues. Even though the vascular and lymphatic network had been visualized by dye injections, tendons were considered to be "*non-viable cables*" (Peacock, 1959). Nevertheless, although tendons resemble a sparsely vascularized tissue type they generally harbor more vessels than commonly believed.

Mechanisms and Sources of Vascularization

Generally, blood vessels emanate into tendons from the musculo—tendineous junction, from the bone insertion site, and the so called "paratenon," a loose areolar gliding tissue surrounding non-synovial tendons (Peacock, 1959; Schmidt-Rohlfing et al., 1992; Ahmed et al., 1998; Kannus, 2000). The complexity of the vascular network also depends on whether tendons are sheathed,

that is embedded in synovial tissue, or is unsheathed. In 1953, Brockis J.G. has shown that in the sheathed digital flexor tendons of the palm vessels only enter the tendon at few distinct sites, whereas in the distal part of the pals and in the forearm, where the tendon is surrounded by paratenon tissue, vessels pass through the tissue more frequently (Brockis, 1953). These two "types" of tendon were later on referred to as "avascular tendons" and "vascular tendons," with major implications for the understanding of adhesion formation following surgical repair (see below; Chaplin, 1973). Nevertheless, even though tendons clearly are not "*non-viable cables,*" they are poorly vascularized and particularly the avascular superficial zones of sheathed tendons are mainly nourished by diffusion from the synovial sheath.

Generally, the number of supplying vascular branches significantly differs between various tendons. For example, the patellar ligament is supplied by a total of three arteries and by the anastomotic arch from the Hoffa fat pad (Pang et al., 2009), whereas the Achilles tendon is supplied both by the peroneal and the posterior tibial arteries (Schmidt-Rohlfing et al., 1992). As tendons naturally are moving tissues which are extended by mechanical load, also the vasculature must be compliant to being stretched. In vascular tendons, vessels form "curves" within their embedding endotenon tissue, a loose areolar intratendineous tissue surrounding individual fascicles (Kannus, 2000). Upon loading of the tendon the vessels are stretched accordingly (Brockis, 1953). An illustrative and well described example for the anatomy of tendon vascularization is the rotator cuff, a group of four muscles and the connected tendons moving and stabilizing the glenohumeral joint. Despite the fact that all four tendons serve a relatively similar function, the supraspinatus tendon is unique in terms of its vascular bed. In this tendon initially an avascular zone, referred to as the "critical zone," usually located about 1 cm proximal from the bony insertion has been described (Lindblom, 1939). However, subsequent studies demonstrated the presence of a vascular bed and it was postulated that the filling of the blood vessels was dependent on the positioning of the arm (Rathbun and MacNab, 1970). Such relative avascular zones have also been described for other tendons, i.e., the Achilles tendon (Stein et al., 2000) or the patella tendon (Clancy et al., 1981). Clinically these zones, also referred to as the "watershed area" in the Achilles tendon, are often prone to inflammatory episodes, potentially resulting in a painful and chronic tendinopathy (Józsa and Kannus, 1997) and/or rupture of the tendon (Alfredson et al., 2001).

The role of the vasculature during tendon development and maturation is also still poorly defined. Peacock (1959) describes embryonic tendons to be "*supplied with a rich capillary network*," by analysing images of an 8 month old human embryo. A study in postnatal, immature sheep describes a massive decline in both cellularity and vessel density in the tendon of the extrinsic flexor muscles of the fingers (*musculus flexor digitorum superficialis*) (Meller et al., 2009). In line with these findings, several studies point out the (relative) decrease of cell density during tendon maturation (Ippolito et al., 1980; Józsa and Kannus, 1997; Oryan and Shoushtari, 2008). Given the fact that very little turnover of the extracellular matrix occurs in human tendons after termination of linear growth after (∼ 17–18 years of age), low vascular supply seems appropriate (Heinemeier et al., 2013). For example, the half–life of collagen in mature equine tendons was calculated to be about 200 years (Thorpe et al., 2010).

The maintenance of hypo- or avascularity certainly requires either the production of antiangiogenic factors or the inhibition of proangiogenic factors. Indeed both mechanisms have been described in e.g., the hypovascular zones of sheathed tendons (Pufe et al., 2005). The proangiogenic protein vascular endothelial growth factor (VEGF) is found to be highly expressed in cells from fetal and injured human tendons, however only low expression is evident in intact adult tendons (Pufe et al., 2001) (**Figure 1A**). The antiangiogenic factor endostatin, a proteolytic fragment of Collagen XVIII, is also involved in tendon vascularization. The distribution of endostatin in gliding tendons correlates with the grade of vascularization. Endostatin expression is strong in the gliding area and reduced in areas without pressure and the expression levels are described to be influenced by mechanical load (Pufe et al., 2003) (**Figure 1B**). Taken together, our understanding of the molecular machinery controlling the complex vascularization process in tendons however remains fragmentary.

Lymphatic Drainage of Tendons

So far, the lymphatic drainage of intact tendons has gained very little attention. Early literature reports lymphatic vessels to be associated with blood vessels in calf tendons as demonstrated by injection of India ink (Edwards, 1946). However, by now it is commonly accepted that the identification of lymph vessels simply by morphologic parameters is insufficient and even by means of immunohistochemistry it is challenging to separate blood from lymph vessels. A minimum of three lymph associated markers is recommended to distinguish lymph from blood vessels (Schroedl et al., 2014). Lymphatics play a crucial role in tissue repair mechanisms, due to injury or inflammatory processes and it has long been known that lymph vessels proliferate during inflammation. However, the role of lymphatic vessels in tendon disease has been neglected so far. To our knowledge there is no literature available on the lymphatic drainage in common tendon disorders such as tendinopathy, calcific tendinitis, or chronic tendon inflammation due to mechanic overuse. Recently, we have shown that intact rat Achilles tendons are void of lymphatics, which start to grow into the tendon repair tissue upon injury (Tempfer et al., 2015). Whether lymphatic ingrowth is a cause for impaired tissue quality and scar formation or merely a side effect requires further investigation. This may pave the way for future attempts to target lymphatic vessels to improve tendon regeneration, as it is successfully performed in other, non-musculoskeletal diseases, such as corneal and ocular surface inflammation (Bock et al., 2013).

Tendon Vessels as a Niche for Stem/ Progenitor Cells

As with many other tissues, vertebrate tendons harbor a population of stem/ progenitor cells (Salingcarnboriboon et al., 2003; Bi et al., 2007; Tempfer et al., 2009). These cells display

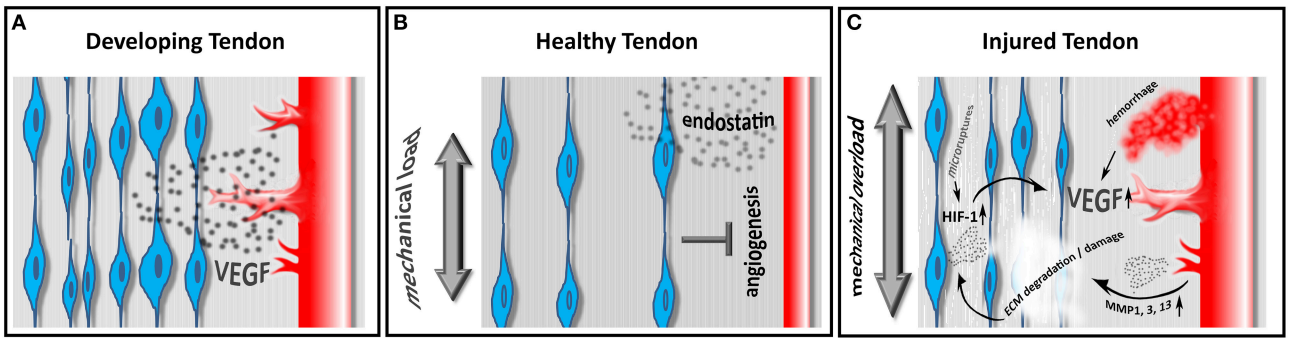

FIGURE 1 | Embryonic tendons show a high density of tendon cells producing VEGF, ultimately resulting in a pronounced angiogenic response in developing tendons (A). In healthy adult tendons the relative cell density decreases and tenocytes produce the antiangiogenic factor endostatin in response to physiological mechanical load, thus limiting neo-angiogenesis **(B)**. In diseased tendons, tenocytes produce HIF-1 in response to mechanical overload and/ or hypoxia. HIF-1 in turn induces the expression of VEGF, promoting neoangiogenesis. Hemorrhage due to vascular injury also leads to an increase in VEGF and to the production of matrix metalloproteinases (MMPs), resulting in a further weakening and degradation of the tendon matrix **(C)**.

mesenchymal stem cell (MSC)—like properties, displaying plastic adherence and a differentiation potential toward the osteoblast, adipocyte, and chondrocyte lineage. Further, they express classical MSC—associated surface markers such as Stro-1, CD44, CD90, and CD146 (Salingcarnboriboon et al., 2003; Bi et al., 2007; Rui et al., 2013) and reside within a fibromodulin- and biglycan-rich niche (Bi et al., 2007). We have shown that perivascular cells of the human supraspinatus tendon harbor a population of cells expressing both tendon- and stem cell associated markers (Tempfer et al., 2009). This is well in line with the finding that perivascular cells derived from a variety of tissues, such as skeletal muscle, pancreas, adipose tissue, and placenta show MSC–properties (Crisan et al., 2008). Interestingly, Mienaltowski et al. (2013) describe the presence of two distinct stem/progenitor cell populations within tendons, either located in the paratenon or the tendon proper. Both cell sources were negative for the perivascular surface marker CD133 and a differential expression pattern for the vascular marker endomucin as well as for tenomodulin and scleraxis. These data suggest that different stem/progenitor cell populations exist within distinct niches at the tendon proper and peritenon and the stem/progenitor cells in the peritenon might be more vascular in origin.

Generally, the role of perivascular MSCs in regeneration remains a matter of debate. Caplan A.I. proposes these cells to be activated upon local injury and to act as immunomodulatory agents guiding inflammation and regeneration (Caplan, 2008). Particularly in tendon, the function of resident stem/ progenitor cells remains unclear. As tendons naturally only have a poor regenerative capacity, leading to inferior tissue quality following injury or chronic degeneration, the pool of endogenous stem/progenitor cells apparently fails to functionally restore the damaged tissue within the milieu of an injured tendon (Voleti et al., 2012). Actually, tendon stem/progenitor cells are suspected to contribute to tendon degeneration by differentiation toward the osteoblast/chondrocyte lineage (Magne and Bougault, 2015). In diseases such as calcific tendinitis or in the formation of bony enthesophytes in spondyloarthritis, ectopic bone is formed within tendons. As tendon stem/progenitor cells have an even higher potential to undergo osteogenic differentiation than bone marrow derived MSC (Bi et al., 2007), it is speculated that they at least contribute to the pathogenesis of calcific tendinitis (Rui et al., 2011). Taken together, the *in vivo* role of tendon stem cells remains largely unknown and they potentially contribute to both tendon homeostasis and tendon pathologies by direct cell differentiation and/or production of trophic factors.

Nevertheless, transplantation of bone-marrow stromal cells and tendon-derived stem/progenitor cells has been proven to be beneficial for the functional repair of tendon tissue in various animal models (reviewed by Docheva et al., 2015). Recently, Lee CH et al. have shown that a rare tendon resident population of perivascular cells expressing CD146 can be expanded and stimulated *in vivo* by connective tissue growth factor (CTGF) in order to regenerate a tendon defect in a rat model (Lee et al., 2015). However, in order to fully harness the regenerative capacity of tendon stem cells we need to gain further insight into the *in vivo* identity of these cells and how they are modulated by the local niche. So far, this remains experimentally challenging due to the lack of tendon-specific markers.

VASCULATURE IN TENDON DISEASE

Tendon Adhesion Formation

Peritendinous adhesions often lead to significant functional impairment after tendon surgery. Particularly sheathed tendons, such as the flexor tendon of the hand, frequently lose their gliding capacity after surgical repair, with a prevalence of ∼ 4% being reported (Dy et al., 2012). In a rabbit study, three main factors have been identified, which in combination support the formation of adhesions: (i) suture of the partially damaged tendon, (ii) excision of the synovial sheath, and (iii) immobilization. If only one of these factors is avoided, adhesion formation can be significantly reduced (Matthews and Richards, 1976).

As nutrition of sheathed tendons is mainly provided by diffusion from the synovial membrane, the local loss of this tissue combined with a fibrin clot on the avascular outer layer

of the tendon causes invasion of microvessels resulting in the formation of fibrous adhesions (Pennington, 1979). More recently, tendon adhesion formation using a mouse model for flexor tendon injury has been demonstrated to follow a typical wound healing response, with overlapping phases of inflammation, vessel ingrowth, and an increase in apoptotic cells over a follow-up time-period of 120 days (Wong et al., 2009).

Attempts to block adhesion formation by merely "wrapping" the tendon with organic or inorganic materials failed as the tendon proper became necrotic in many cases, indicating the importance of vascular supply (Weckesser and Shaw, 1949; Chaplin, 1973). Current research-based strategies include the use of multilayer membranes loaded with non-steroidal anti-inflammatory drugs (NSAIDs) to prevent fibrosis, mimicking the synovial membrane (Jiang et al., 2015) as well as the implantation of bioengineered synovia—like membranes (Baymurat et al., 2015).

Vasculature in Achilles Tendinopathy

Tendinopathy is a painful, chronic disease commonly affecting various tendons such as the Achilles tendon or the tendons of the lateral elbow ("Tennis elbow"). As Achilles tendinopathy (AT) is the most frequent and best studied form of this disease, we will focus on this particular tendon. AT often affects people with high levels of sports activities. For example, 52% of elite long-distance runners are at risk for sustaining an Achilles tendon injury during their career (Kujala et al., 2014). AT is characterized by pain in the tendon during initial loading, subsiding with continued activity; as the condition becomes chronic, pain can be persistent. Overuse is considered to be the underlying cause; however the etiology and pathogenesis have not yet been fully clarified. Similarly, the source of the pain and the underlying mechanisms of pain remain unclear. Histologically, matrix disruption is commonly observed in AT, but is not necessarily involved in the pathogenesis as it also occurs in asymptomatic tendons (Magnan et al., 2014). Neovascularization is commonly seen in AT, as shown by Doppler sonography (Ohberg et al., 2001; Zanetti et al., 2003) and along with the ingrowth of vessels, also innervation is enhanced in tendinopathic zones, which may be causative for the pain associated with AT (Alfredson et al., 2001).

As mentioned above, during tendon development, high levels of VEGF are expressed. Molecules that are developmentally regulated are often re-expressed during the disease state. Indeed, the expression of VEGF in degenerative and spontaneously ruptured Achilles tendons is detectable at high concentrations when compared with adult, healthy Achilles tendons (Pufe et al., 2001). *In vitro*, cyclic mechanical load induces the expression of VEGF and hypoxia inducible factor 1 (HIF-1) in a frequency dependent fashion, indicating this mechanism being involved in tendon cell response to overload (Petersen et al., 2006) (**Figure 1C**).

Some authors report AT to be a result of an inadequate repair process following microtrauma, i.e., due to overuse. Because of the lack of blood vessels within the mid portion of the tendon a neurogenic inflammatory process is activated to repair these microruptures. This neurogenic inflammation occurs in the tissue surrounding the Achilles tendon and matrix and induces the expression of metalloproteinases (MMPs) responsible for the degradation of extracellular matrix. Concomitantly, cytokines such as VEGF, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) are overexpressed. VEGF not only promotes angiogenesis, but also upregulates the expression of MMPs and downregulates tissue inhibitors of metalloproteinases (TIMP-3), further progressing the remodeling of the tendon tissue (van Sterkenburg and van Dijk, 2011).

Tendon stem/ progenitor cells are also suspected to contribute to the pathophysiology of tendinopathies. In a rabbit tendinopathy model it was shown that tendon stem/ progenitor cells display an altered cell fate *in vitro*. They proliferate less and have a greater potential do undergo osteogenic and chondrogenic differentiation (Rui et al., 2011). Indeed, mineralization processes are also found in human AT and patella tendinopathy. One study on human tendinopathic tissue demonstrated that some mineralized deposits in Achilles and patella tendons are formed by a process resembling endochondral ossification, with bone formation and remodeling mediated by populations of osteoblasts and osteoclasts (Fenwick et al., 2002).

Regarding treatment strategies for AT, eccentric loading (e.g., muscle movements leading to muscle and tendon elongation) has shown to be a safe, cheap, and effective method to reduce pain and to improve tendon structure (Beyer et al., 2015). Interestingly, this method also reduces the number of neovessels in the affected area, which is considered to be causative for the beneficial outcome (Ohberg and Alfredson, 2004). Similarly, a combination of cryotherapy and compression of the tendinopathic area was shown to be an effective treatment, leading to a significant reduction of tendon blood flow (Knobloch et al., 2006). Also the use of topical nitroglycerin and low level laser irradiation are discussed to exert their positive effects by affecting tendon microcirculation. Nitroglycerin is a vasodilator and the positive effects reported are likely due to improved clearance of metabolic products, whereas low level laser irradiation may cause microthrombosis and/ or partial destruction of neovessels. However, for both therapies the underlying mechanisms of action remain poorly understood (Knobloch, 2008).

Another area often affected is the adult enthesis organ that connects tendons with bone, allowing the transmission of force from muscle to bone. The Achilles enthesis is frequently affected by non-inflammatory enthesopathies due to overuse or microtraumas and inflammation may occur resulting in major pain and disability. However, the underlying intrinsic and extrinsic factors also remain poorly understood.

Generally, mineralized and non-mineralized entheses can be differentiated. The Achilles tendon enthesis organ is mineralized and four zones can be distinguished: Zone 1 is built of dense fibrous connective tissue (the tendon proper), and the extracellular matrix (ECM) is mainly composed of collagen type I and III. Zone 2 consists of non-mineralized fibrocartilage and fibrochondrocytes. Here the ECM is formed by aggrecan and the collagen types I, II, and III. Zone 3 is formed of mineralized fibrocartilage with fibrochondrocytes, the predominant collagen being Col II next to I and X as well as calcium phosphate crystals. Between Zone 2 and Zone 3 the so called tidemark forms the boundary between soft, non-mineralized, and hard, mineralized tissue. Zone 4 finally is made up of the bone itself (Benjamin

and McGonagle, 2009; Apostolakos et al., 2014). This gradual transition from compliant soft tissue to rigid bone absorbs local stress concentrations minimizing the risk of injury. Further, it has been reported that there is no direct cellular communication between bone and tendon tissue and the fibrocartilage at the enthesis acts as a barrier between cells in the two tissues (Ralphs et al., 1998). Whereas osteocytes and tendon cells directly communicate via gap junctions, the fibrochondrocytes in the calcified fibrocartilage zone were shown to lack similar structures.

Other than tendon, in a healthy state the enthesis is avascular (Dörfl, 1969). The lack of blood vessels reflects the compressive forces to which fibrocartilage is subject: vessel lumina would be occluded by compression. However, intratendinous vessels can anastomose directly with those of the bone at fibrous entheses (Benjamin and McGonagle, 2001). Similar to tendon, in development the enthesis is vascularized. During the growing period, bone grows into the tendon by endochondral ossification, where fibrocartilage is replaced by bone. Generally, cartilage erosion must be preceded by vascular invasion, yet it remains unclear how the blood vessels in the fibrous zone regress as the fibrous tissue is replaced by fibrocartilage (Gao et al., 1996; Benjamin and McGonagle, 2001).

CONCLUSIONS

Neovascularization is critical to tissue repair and wound healing. Therefore, strategies to enhance vascularization to promote regeneration are considered promising treatment modalities, i.e., the use of platelet rich plasma (PRP) to restore functional bone (Zhang et al., 2013) or skin (Kakudo et al., 2011). However, in acute or chronic tendon injuries hypervascularity often does not pave the way to functional recovery of the tissue. Therefore, to overcome the limited intrinsic regeneration capacity of tendon and to achieve scarless healing will most likely require a balanced manipulation of the angiogenic response in tendon tissue. For a variety of treatment methods, such as the use of PRP, the availability of clinical data is limited, due to heterogeneity in application (Khan and Bedi, 2015). In order to develop rational strategies to achieve a well-balanced angiogenic response following tendon injury, we need a thorough understanding of the molecular and cellular networks driving tendon vascularization and regeneration—a challenge for years to come.

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HT and AT wrote the article.

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REVIEW
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The Granulocyte-colony stimulating factor has a dual role in neuronal and vascular plasticity

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Granulocyte-colony stimulating factor (G-CSF) is a growth factor that has originally been identified several decades ago as a hematopoietic factor required mainly for the generation of neutrophilic granulocytes, and is in clinical use for that. More recently, it has been discovered that G-CSF also plays a role in the brain as a growth factor for neurons and neural stem cells, and as a factor involved in the plasticity of the vasculature. We review and discuss these dual properties in view of the neuroregenerative potential of this growth factor.

Keywords: SCI, ALS, G-CSF, neurogenesis, arteriogenesis, angiogenesis, neuroregeneration, plasticity

Introduction

In the last two decades the neuroscience community experienced a change of thought on the regeneration capacity of the central nervous system (CNS). The earlier dogma of incapability of CNS regeneration was overthrown by findings of astounding plasticity derived from neuron- or neuronal network- inherent adaptations, fostered by neurogenesis and by supporting vasculogenesis. These findings have raised hopes for developing new treatment approaches for neurological diseases.

Granulocyte-colony stimulating factor (G-CSF) is a 19.6-kDa glycoprotein which has been well-characterized as a growth factor for hematopoietic progenitor cells. It is an FDA- and EMA-approved drug commonly used to treat neutropenia and to mobilize bone marrow hematopoietic stem cells for transplantation (Nicola et al., 1983). For many years G-CSF has been thought of as a highly specific growth factor in the hematopoietic system. However, more recent studies have shown the presence of the G-CSF/G-CSF-receptor (G-CSFR) system in the brain and roles in neuroprotection, neural tissue repair as well as improvement in functional recovery

Abbreviations: ALS, amyotrophic lateral sclerosis; ANG, angiogenin; ANGPT2, angiopoietin 2; BrdU, bromodeoxyuridine; CNS, central nervous system; EMA, european medicines agency; FDA, food, and drug administration; G-CSF, Granulocyte-Colony Stimulating Factor; G-CSFR, Granulocyte-Colony Stimulating Factor Receptor; GM-CSF, Granulocyte-macrophage colony stimulating factor; HUVEC, human umbilical vein endothelial cells; IL, interleukin; kDa, kilo Dalton; MAP-2, microtuble-associated protein 2; NeuN, neuronal nuclei; NSC, neuronal stem cell; PCA, posterior communicating artery; SCI, Spinal Cord Injury; SOD1, superoxide dismutase 1; SVZ, subventricular zone; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; 3-VO, 3-vessel occlusion.

have been described (Schneider et al., 2005b). The elevated expression of G-CSF/G-CSFR on neurons subjected to ischemia suggested that G-CSF serves as an autocrine protective signaling mechanism in response to neural injury. Furthermore, G-CSF receptors may also be present on glial cells, however at much lower levels (Schneider et al., 2005a). G-CSF exerts potent anti-apoptotic activity in neurons that appears largely responsible for its neuroprotective effects in acute injury models. In addition, G-CSF stimulates neurogenesis (Schneider et al., 2005b; Schmidt et al., 2015), arteriogenesis in the CNS (Sugiyama et al., 2011), and markedly improves long-term behavioral outcome after cortical ischemia (Schneider et al., 2005a) or spinal cord injury (SCI) (Dittgen et al., 2012). There is debate on the role of G-CSF-stimulated hematopoietic stem cells that may migrate to the injured CNS. Due to those effects and the ability of G-CSF to cross the intact blood brain barrier (Schneider et al., 2005b), facilitating peripheral administration, G-CSF got in the focus for treating acute and chronic neurodegenerative disorders, with protective and recovery enhancing effects in animal models of stroke, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, traumatic brain injury, and SCI (Diederich et al., 2012). In this review, we concentrate on direct and indirect effects of the cytokine G-CSF on CNS spinal regeneration especially by neurogenic and vasculogenic mechanisms and critically discuss the available pre-clinical and clinical evidence in SCI and ALS.

G-CSF-mediated Neural Progenitor Activation

The concept of adult neurogenesis is a relatively new one and has added one principally new option to influence plasticity and regeneration in the CNS. Neurogenesis can occur in the hippocampus (dentate gyrus), the olfactory bulb, and the subventricular zone (SVZ) (Winner and Winkler, 2015). There is debate whether adult neurogenesis occurs in the spinal cord near the central canal. One physiological role for hippocampal neurogenesis is pattern separation. There has been debate on whether neurogenesis is even relevant for humans, but detailed post-mortem studies on humans using radiocarbon dating in the brain have revealed that neurogenesis is particularly strong in the human hippocampus. Pathophysiologically, neurogenesis has been invoked as a possible causative or modifying factor in depression and schizophrenia. The case is stronger for depression, since several antidepressants stimulate neurogenesis (Hanson et al., 2011). Further, physical exercise which is anti-depressant, stimulates neurogenesis in rodents (van Praag et al., 1999; Yau et al., 2011) as well as humans (Déry et al., 2013). Interestingly, antidepressant medication for example fluoxetine, typically requires several weeks in order to be therapeutically active, which corresponds with the duration of newly generated nerves to be functional integrated. Moreover, impairment of neurogenesis has been shown to block the antidepressive activity of antidepressants such as fluoxetine (Santarelli et al., 2003). Also, imaging studies have shown a decrease in hippocampal volume in some cases. A function for G-CSF in stimulating neurogenesis was first described in 2005 in mice, treated subcutaneously with G-CSF (Schneider et al., 2005a). Neurogenesis was quantified using a standard bromodeoxyuridine (BrdU) incorporation protocol. While healthy mice showed a doubling of BrdU/neuronal nuclei (NeuN) double positive cells in the hippocampus 6 weeks after treatment initiation, mice subjected to cortical ischemia further increased that rate, pointing to a particular relevance of this stimulation also under pathological conditions to potentially enhance CNS plasticity. Most likely, stimulation of progenitor cells occurs via the G-CSF receptor present on these cells. Interestingly, the G-CSFR is already expressed at the embryonic stage in radial glia, which also later form the adult neural precursor cells (Kirsch et al., 2008). In G-CSF knock-out mice, hippocampal neurogenesis is strongly diminished, and the mice show deficits in behavioral plasticity (Diederich et al., 2009). For SCI and ALS the question whether neurogenesis occurs in the spinal cord and could be enhanced by G-CSF is of particular importance as this is the site of the main pathology. A careful review of the data on progenitor cells in the spinal cord comes to the conclusion that these cells exist in the ependymal zone of the central canal, but are restricted to a gliogenic fate (Sabelström et al., 2014). However, this restriction can be overcome by transplanting those cells into the hippocampus (Shihabuddin et al., 2000) and possibly growth factors like G-CSF could help to overcome this restriction in the spinal cord, but experimental proof for this is lacking so far.

G-CSF-mediated Vasculogenic Effects

Prior to discussing effects on the vasculature it is important to clarify some conceptual issues. Overall, arteriogenesis refers to the remodeling of pre-existing capillary connections into conducting vessels by mechanisms involving shear stress and monocyte recruitment (Van Royen et al., 2001). Angiogenesis, on the other hand, describes the process of capillary sprouting driven by hypoxia and vascular endothelial growth factor (VEGF) (Heil et al., 2006). While angiogenesis is a key mechanism supporting tumor growth by improving local oxygen diffusion distances, it does not contribute to substantial increases in blood conductance, since only end-stage capillaries in the circulation are created (Buschmann and Schaper, 1999, 2000). Arteriogenesis is therefore a mechanism that is relevant for ischemic conditions such as vessel occlusions and decreased blood flow. The first hematopoietic factor for which evidence was found for a role in arteriogenesis was not G-CSF, but Granulocyte-macrophage colony stimulating factor (GM-CSF), as early as 2001 by Buschmann et al. (2001). GM-CSF was selected based on its propensity to stimulate monocyte and thrombocyte generation and the hypothesis of a key role of monocytes and thrombocytes in the arteriogenic process and stem cell niche. While these experiments made use of the femoral occlusion model in rabbits to observe arteriogenesis from pre-existing anastomosing capillaries, a publication from 2003 demonstrated also induction of arteriogenesis in the CNS (Buschmann et al., 2003). In this work, the authors made

use of the 3-vessel occlusion (3-VO) model. In this model, both vertebral arteries and the left carotid artery are occluded. Treatment of rats over 7 or 21 days with 40 μg/kg/day GM-CSF led to a significantly larger increase in the diameter of the left PCA (posterior communicating artery) (72 vs. 39% increase in control animals after 7 days of treatment). Moreover, GM-CSF treatment also preserved good CO_2-reactivity, indicating that the vessel was functionally intact with regard to blood flow regulation. Moreover, this treatment also resulted in an improved energy situation (ATP-depletion) in the hypoperfused brain in this 3-VO model (Schneeloch et al., 2004). In 2006, Deindl and colleagues were the first who also detect arteriogenic effects of G-CSF in a myocardial infarction model in mice. Treatment with 100 μg/kg/day G-CSF for 5 consecutive days after myocardial infarction led after 30 days observation to decreased mortality (68.8 vs. 46.2%), reduced final infarct size, and reduced thinning of the left ventricular wall. Moreover, hemodynamic parameters were improved such as ejection fraction, contractility, and relaxation of the ventricle. The authors could show G-CSF induced arteriogenesis from collateral vessels with proliferation of endothelial cells and smooth muscle cells (Deindl et al., 2006). Also, a decreased potential for arrhythmia generation in the infarcted heart was noted (Kuhlmann et al., 2006). A number of smaller clinical trials have indeed been conducted in patients with myocardial infarction with mixed results, the latest by Hibbert and colleagues in 86 patients (Hibbert et al., 2014). Later, arteriogenic effects of G-CSF were also noted in cerebral ischemia models (Sugiyama et al., 2011) and in the 3-VO model described above (Duelsner et al., 2012). G-CSF showed a very similar profile to GM-CSF in the 3-VO model in terms of doses used and treatment effects. In addition to the beneficial effects of arteriogenesis for blood flow enhancement, locally increased angiogenesis in the CNS could provide the critical neurovascular niche for neurogenesis and neuronal remodeling. Indeed, a number of articles suggest angiogenic activity of G-CSF in the brain. Bussolino and colleagues were the first to describe angiogenesis evoked by G-CSF (Bussolino et al., 1991). They used G-CSF secreting pellets implanted into the cornea of rabbits and observed formation of new capillaries within 8 days after implantation. Boiled G-CSF did not achieve this effect. *In vitro*, G-CSF enhanced proliferation and migratory behavior of HUVEC cells. In the brain, Lee et al. reported much later that the vascular surface area, vascular branch points, vascular length, number of BrdU-labeled endothelial cells, and endothelial nitric oxide synthase and angiopoietin 2 (ANGPT2) expression were all significantly increased in G-CSF–treated rats in the focal cerebral ischemia model (Lee et al., 2005). Ohki and colleagues found that G-CSF increased plasma levels of VEGF from neutrophils *in vivo* (Ohki et al., 2005). Furthermore, blockade of the VEGF pathway eliminated G-CSF–induced angiogenesis in a hindlimb ischemia model (measured as capillary density in the gastrocnemius muscle), suggesting that G-CSF–induced vasculogenesis is VEGF-dependent and could be indirectly mediated by this mechanism (Ohki et al., 2005). The finally responsible mechanism for these vasculogenic effects of G-CSF has not been unambiguously established. Direct effects on endothelial cells via the G-CSF receptor, increase in monocyte counts, and indirect effects via induction of VEGF release have all been discussed. In summary, G-CSF has both arteriogenic and angiogenic effects, thereby both enabling increased blood flow via generation of conductance vessels, and improving local oxygen diffusion and providing a neurovascular environment for repair mechanisms in the CNS.

G-CSF in SCI

Acute SCI is associated with a significant burden of illness. Worldwide the estimated range of the incidence of SCI lies between 15 and 30 per million inhabitants per year (Wyndaele and Wyndaele, 2006). Therapeutic approaches in the acute phase including early resuscitation, steroid application, decompression/stabilization, have been reported to be associated with somewhat better outcomes in incomplete SCI. Thus, far, there is still no really effective treatment for SCI available, and the degree of neurological recovery is largely dependent on the magnitude of the initial injury. A considerable number of animal studies using various models of SCI have demonstrated convincing beneficial effects of G-CSF therapy (**Table 1**). Mechanisms triggered by G-CSF include anti-apoptotic effects and improved neurite outgrowth (Pitzer et al., 2010). Vasculogenesis certainly has a beneficial role for SCI repair and in limiting secondary damage after the initial traumatic event (Oudega, 2012). The degree of angiogenesis during the subacute phase after SCI correlates with regenerative responses, and the newly formed vascular bridge might provide scaffolding to accelerate axonal regeneration across the injury site. Angiogenesis might contribute to the regenerative response of neural tissue and enhance recovery of locomotor function after injury. Recent reports demonstrated the pro-regenerative effects of G-CSF in SCI which could be due to the enhancement of angiogenesis (Kawabe et al., 2011; Dela Peña et al., 2015). Kawabe and colleagues report significantly increased vessel numbers and increased expression of angiogenic cytokines after treatment with G-CSF in an experimental SCI model (Kawabe et al., 2011). G-CSF significantly promoted angiogenesis in both the white and gray matter of the spinal cord after injury. The total number of vessels with a diameter >20 μm was significantly greater and expression of angiogenic cytokines was significantly higher than in the control group. The G-CSF-treated group showed greater recovery of hindlimb function than the control group. As for a contribution of neurogenesis to the beneficial effects of G-CSF treatment, the problem with restricted neurogenesis in the spinal cord applies here. However, for SCI there is a way around this restriction and this is by making use of stem cell transplantation. An example of this approach was provided by Pan et al. (2008). Neural stem cells (NSC) were embedded in fibrin glue in combination with or without G-CSF and were transplanted into the gap in the injured spinal cord. Higher expression levels of NeuN and MAP-2 and advanced regeneration according to the clinical motor score, motor evoked potential and conduction latency was observed in the group treated the fibrin glue, G-CSF and NSC compared to the group

TABLE 1 | Published preclinical studies in the use of G-CSF for SCI.

Drug/dosage	Application/treatment duration	Outcome	SCI model/recipients	References
G-CSF 200 μg/kg/day ± BMDC (4 weeks prior SCI)	sc. injected immediately after injury for 5 consecutive days	• Increased number of neutrophil antigen-negative BMDC in the lesioned site 24 h after injury • Increased number of BMDC 6 weeks after injury • Most of the BMDC in the lesioned site were Mac−1+ • Spared white matter was significantly larger • Recovery of hindlimb function	Compression model at T8 level, C57BL/6 mice	Koda et al., 2007
G-CSF 200 μg/kg/day	sc. injected immediately after injury for 5 consecutive days	• G-CSF Receptor expression by neurons in the spinal cord • Prevented glutamate-induced neuronal death during the acute phase • Increased number of surviving neurons after chronic phase • Increased recovery of hindlimb motor function	Compression model at T7-T8 level, BALBc/Cr mice	Nishio et al., 2007
G-CSF 50 μg/kg compared to MPSS	Single dose, injected sc. immediately after injury	• Decreased MPO, LPO activity and MDA concentration in the first 24 h after SCI, which may reduce tissue damage • No protective effect on the organelles after trauma	Contusion model at level T7-T9, Wistar albino rats	Sanli et al., 2010
G-CSF 50 μg/kg/day	sc. injection, within the first 72 h after injury for 3 consecutive days	• G-CSF Receptor expression on microglia cells • Microglia recruitment to the injury site in the first 72 h • Inhibited expression of pro-inflammatory factors promoted the expression of neurotrophic factors • Affect activation status of microglia • Inhibited the expression of markers of M1 macrophage in BV2 microglial cell line *in vitro* • Promoted microglia to adopt M2 phenotype • NFκB was involved in G-CSF induced M2 polarization • IBA1+ cells within the lesion site after G-CSF treatment • Attenuated accumulation of IBA1+ cells in the gray and white matter • Increased cell viability of BV2 microglial cell line • Reduced expression of NFκB in BV2 microglial cell line	Hemisection model at level T9-T11, Kunming mice	Guo et al., 2013
G-CSF 50 μg/kg/day ± NSC in the lesion	sc. injected immediately after injury for 5 consecutive days	• Improved motor function (BBB score) after 8 and 12 weeks • High expression levels of neuronal markers • Increased expression of GFAP • Increased BrdU positive cells • Regenerating tissue bridging with NSC and G-CSF	Transection model at level T8–T9, Sprague-Dawley rats	Pan et al., 2008
G-CSF 50 μg/kg/day ± BMC, MSC	sc. injection on day 7 after SCI for 5 consecutive days	• Improved functional recovery (BBB score, plantar test) • Improved behavioral parameters • Increased cross sectional areas of the white matter	Compression model at T8–T9 level, Wistar rats	Urdzíkova et al., 2006
G-CSF 300 μg/kg/day ± SCF	sc. injection on day 11 after SCI for 10 consecutive days	• Improved recovery of hindlimb motor function (BBB score) • Increased number of intrinsic BrdU+ cells • Increased number of intrinsic F4/80+ cells • Proliferation of OPC was activated • Activation of intrinsic spinal cord cell proliferation • Increase in intrinsic microglia cells was observed in lesion • No effects of SCF or G-CSF on the migration of transplanted BMDC to the lesion sites	Static contusion model at level T11-T12, C57BL/6 mice	Osada et al., 2010
G-CSF iv. 60 μg/kg sc. 30 μg/kg/ day	Single dose injected iv. immediately after injury (5 min after surgery) followed by continuous sc. application for 2 weeks	• G-CSF Receptor is upregulated in the CNS upon SCI • Counteracts apoptotic cell death in the injured spinal cord • Increased expression of the anti-apoptotic Bcl-XL gene • Promotes neurite outgrowth *in vitro*	Transection model at level T8–T9 (transected to 75%), C57BL/6 mice	Pitzer et al., 2010

(Continued)

TABLE 1 | Continued

Drug/dosage	Application/treatment duration	Outcome	SCI model/recipients	References
		• Enhanced branching capacity of hippocampal neurons • Effects on both the CST and serotonergic tracts • Increased number of large motoneurons • Improved functional connectivity post-injury • Improved functional outcome • G-CSF overexpression in CNS correlated with an improved functional motor outcome		
G-CSF iv. 60 μg/kg sc. 30 μg/kg/	iv. injection immediately after injury, followed by continuous sc. application for 14 days	• Improved functional recovery (BBB score, gridwalk test, and swim test)	Contusion model level T9-T10, Wistar rats	Dittgen et al., 2012
G-CSF 15 μg/kg/day	iv. injection, 1 h after injury and daily for 5 consecutive days	• Increased number of vessels with diameters > 20 μm • Increased expression of angiogenic cytokine mRNA (VEGF, HGF, FGF 2) • Promotion of functional recovery permeability	Contusion model at level T8-T9, Sprague-Dawley rats	Kawabe et al., 2011
G-CSF 15 μg/kg/day	iv. injection, 1 h after surgery and daily for 4 consecutive days	• G-CSF Receptor expression on neurons, astrocytes and oligodendrocytes in normal spinal cord • G-CSF Receptor expression on GFAP+ astrocytes and MOSP+ oligodendrocytes • Increased number of MOSP+ oligodendrocytes • Suppressed inflammatory cytokine expression 72 h after injury • Smaller percentage of apoptotic oligodendrocytes 72 h and 1 week after surgery • Larger number of MAP-2+ neurons • Normal appearing myelin was higher than in control group • Better myelin integrity and preservation • Decreased Iba-1+ cell number • Improved hindlimb recovery	Contusion model at level T8–T9, Sprague-Dawley rats	Kadota et al., 2012
G-CSF 20 μg/ml ± GM-CSF	Single dose, injected ip. immediately after SCI	• improved functional recovery (BBB score) • Reduced cavity sizes • Marginal white matter seemed to be more intact • Repressed GFAP expression 1 and 4 weeks after injury • Repressed CSPG expression • Maintenance of the integrity of axon fibers • Suppressive ED-1+ cells 3 days after injury	Compression model at level T9, Sprague-Dawley rats	Chung et al., 2014

BBB, Basso, Beatti, and Bresnahan locomotor rating scale; BMC, bone marrow cells; BMDC, bone marrow derived cells; BV2, micro glia cell line; CNS, central nerve system; CSPG, chondroitin sulfate proteoglycan; MSC, mesenchymal stem cells; CST, corticospinal tract; FGF2, fibroblast growth factor 2; GFAP, Glial fibrillary acidic protein; GM-CSF, granulocyte-macrophage colony stimulating factor; HGF, hepatocyte growth factor; IP, intraperitoneal; IV, intravenous; LPO, lipidperoxidation; M1, Macrophage type 1; M2, Macrophage type 2; MAP-2, microtubule associated protein; MDA, Malondialdehyde; MOSP, myelin oligodendrocyte-specific protein; MPO, Myeloperoxidase; MPSS, Methylprednisolone sodium succinate; NSC, neuronal stem cells; OPC, oligodendrocyte precursor cell; SC, subcutaneous; SCF, stem cell factor; SCI, spinal cord injury; VEGF, vascular endothelial growth factor.

without G-CSF (Pan et al., 2008). Based on the considerable preclinical evidence a number of smaller clinical trials have been initiated (**Table 2**). Several of these clinical studies demonstrated a neurological and functional improvement in SCI patients treated with G-CSF (Takahashi et al., 2012; Derakhshanrad et al., 2013; Inada et al., 2014; Saberi et al., 2014). Sakuma et al examined patients with a compression myelopathy and observed reduced progression of myelopathy in all 15 treated patients. Neurological improvements in motor and sensory functions were obtained in all patients after the administration, although the degree of improvement differed among the patients (Sakuma et al., 2012).

G-CSF and ALS: Preclinical and Preliminary Clinical Research

ALS represents a progressive and fatal neurodegenerative disease affecting motor neurons with a typical median disease course of 2–5 years and a lifetime risk of 1:400 (Ingre et al., 2015). A large number of clinical trials have been undertaken for this terrible disease, but the only approved drug so far is riluzole with limited treatment effects (Miller et al., 2012). Preclinical studies demonstrated that chronic or repeated subcutaneous administrations of G-CSF exert positive effects on survival rate, disease progression and immune status of SOD1-G93 mice

TABLE 2 | Published clinical studies on the use of G-CSF for SCI treatment.

Drug/dosage	Application/treatment duration	Outcome	Pat. Nr.	Patient characteristics	Side effects	References
G-CSF (a) 5 μg/kg/day + surgery ($n = 5$) or (b) 10 μg/kg/day + surgery ($n = 10$)	iv. injection for 5 consecutive days	• Suppressed the progression of myelopathy in all 15 patients • Neurological improvements in both motor and sensory function	15	Cervical myelopathy patients, JOA score decreased during recent 1-month period	No serious adverse events occurred during or after treatment	Sakuma et al., 2012
G-CSF (Gran®) (a) 5 μg/kg/day ($n = 5$) or (b) 10 μg/kg/day ($n = 11$)	iv. injection, within 48 h after injury for 5 consecutive days	• AIS grade increased by one step in 80% of patients treated like a) and 50% of patients treated like b) • Increase in AIS motor scores detected 1 day after G-CSF administration in 10 μg group • Improvement in light touch and pin prick scores in the 10 μg group	16	Traumatic SCI patients recruited within 48 h of the primary injury	No severe adverse effects after G-CSF injection	Takahashi et al., 2012
G-CSF (Gran®) 10 μg/kg/day ($n = 17$) control group ($n = 24$)	iv. injection, within 48 h after injury for 5 consecutive days	• AIS grade improvement at least 1 step in 88.2% in the G-CSF group and 33.3% in the control group • Increased AIS improvement within patients with incomplete paralysis • No differences in pinprick score • Significant differences maintained 1 year after treatment	41	Traumatic SCI patients recruited within 48 h of the primary injury	No serious adverse effects (One patient in the G-CSF group developed fever proved to be an urinary tract infection One patient developed mild hepatic dysfunction)	Inada et al., 2014
G-CSF Filgrastim (Neupogen®) 5 μg/kg/day	sc. injection for 5 days	• Upper extremity but not lower extremity motor scores improved • Improved light touch sensory scores • Pinprick sensory scores improved • Increase in SCIM III score • Improvements in bladder and bowel management	19	Patients with chronic motor complete spinal cord injury, at least 3 months of active rehabilitation, at least 3 months duration of SCI	Mild side effects such as bone pain, rash, fever, neuropathic pain, and spasticity, all of them resolved after 1 week	Derakhshanrad et al., 2013
Filgrastim (Neupogen®) 5 μg/kg/day	sc. injection for 7 consecutive days	• Motor incomplete patients had increased improvement in AIS motor score, light touch, and pinprick sensory scores compared to motor complete patients • Less improvement in SCIM III scores of motor incomplete patients compared to motor complete patients	74	Traumatic SCI of at least 6 months duration, with stable neurological status in the last 3 months, undergone at least 3 months of standard rehabilitation, 52 motor complete and 22 motor incomplete patients	Not mentioned	Saberi et al., 2014

AIS, ASIA Impairment Scale; ASIA, American Spinal Injury Association; JOA, Japanese Orthopedic Association score; SCIM Spinal Cord Independence Measure.

(for details see **Table 3**). Some reports describe more gender-specific effects in ALS mouse models with positive effects on survival rate and motor performance only present in male but not female mice (Naumenko et al., 2011). Mechanisms that have been described include suppression of neuronal apoptosis and oligodendrocyte cell death by regulating the expression of apoptosis related proteins (Nishio et al., 2007; Pitzer et al., 2008). Another claimed mechanism of action is via mobilizing bone marrow cells into the spinal cord (Koda et al., 2007), or enhancing the availability of circulating hematopoietic stem cells in neuronal lesion sites and their ability for neurogenesis and angiogenesis (Nishio et al., 2007), although this is a very debated field. Moreover, reducing demyelination and expression of inflammatory cytokines such as TNF-α and IL-1β could be contributing to improved outcome after G-CSF treatment (Koda et al., 2007; Nishio et al., 2007; Kawabe et al., 2011; Kadota et al., 2012).

Several angiogenic key players may be related to ALS with VEGF being the first described factor to contribute to ALS (Oosthuyse et al., 2001) and angiogenin (ANG) recently identified as a second angiogenic element related to that disease (Gao and Xu, 2008). VEGF represents a pro-angiogenic factor with neuroprotective properties. *In vitro* as well as *in vivo* studies showed that VEGF promotes neuronal survival (Silverman et al., 1999; Jin et al., 2000a,b) and prolongs the life span of ALS animal models (Lambrechts et al., 2003; Storkebaum and Carmeliet, 2004; Storkebaum et al., 2004). Several clinical studies investigate the VEGF-system as a possible treatment option for patients suffering from ALS (Pronto-Laborinho et al., 2014). An enhanced angiogenesis might protect motoneurons from degradation by increasing neurovascular perfusion. Studies demonstrate a correlative reduction in the human umbilical vein endothelial cell proliferative and angiogenic activities, which may contribute to the induction of ALS (Crabtree et al., 2007; Wu et al., 2007). Therefore, G-CSF might act as potent medication for the treatment of ALS by activating pro-angiogenic systems.

With regard to neurogenesis as a possible mechanism there are no data available from animal models that demonstrate a direct regenerative effect of G-CSF treatment on the first or second motoneuron. However, hippocampal and SVZ neurogenesis are certainly triggered and enhanced under the chronic G-CSF treatment protocols used in the animal studies that show beneficial effects of G-CSF treatment (Pitzer et al., 2008; Henriques et al., 2011) and one could speculate that this could indirectly functionally ameliorate the ALS phenotype via enhanced motor learning. There are papers that report enhanced progenitor cell proliferation and migration in the ependymal zone of the central canal in the lumbar spinal cord of ALS mouse models (Chi et al., 2006a,b, 2007), but true neurogenesis has not been reported in these studies. Based on these pre-clinical findings several smaller studies in ALS patients have been initiated with promising outcomes (**Table 4**) and no severe adverse effects even following long-term administration (Grassinger et al., 2014; Khomenko et al., 2015) Some studies reported no beneficial effects of G-CSF for patients suffering from ALS (Nefussy et al., 2010; Chiò et al., 2011).

Conclusion and Clinical Implications

In this review we have described the neuroregenerative potential of the hematopoietic growth factor G-CSF for SCI and ALS with a focus on vasculogenic and neurogenic mechanisms of action. Apart from all other arguments in favor of G-CSF as a novel type of neuroprotective drug, particularly due to its multiple mechanisms of action, and broad preclinical proof of principle one major advantage of this protein is that we are dealing with a drug with a well-known pharmacological profile, and an excellent safety record. Appropriate intervention in SCI depends on the nature, extent, and duration of the disease state, as pathophysiology can dramatically evolve over time. The initial mechanical damage occurs immediately after SCI followed by a cascade of potentially harmful secondary events that include the formation of free radicals, detrimental inflammatory responses and death of neurons and glia. A drug counteracting the induction of the secondary damage and promoting neuroregeneration is for major importance in the treatment of patients suffering from acute or chronic CNS diseases. Acute SCI causes immediate mechanical damage to the microvasculature of the cord followed by a secondary injury to the vessels, this combination produces spinal cord ischemia. Thus, angiogenesis is critically important to reduce secondary damage to the spinal cord vasculature. G-CSF plays a major function in the induction of angiogenesis and arteriogenesis which may promote neuroregeneration via the induction of a regeneration-friendly environment. How endogenous neurogenesis can contribute to SCI regeneration is unclear at present, spinal cord neurogenesis does not appear to play a significant role—central neurogenesis may however well-contribute to recovery processes due to an increased level of plasticity for central "rewiring" of descending motor pathways. Local transplantation approaches together with G-CSF appear as an attractive way toward exploiting neurogenesis as a repair mechanism.

Clinical trials conducted so far look promising, and at least G-CSF treatment appears feasible and safe. However, proper controlled and randomized trials are lacking to draw sound conclusions. Many questions are on the table regarding the timing of treatment post-injury: (acute vs. delayed), G-CSF dosing (high dose, low dose), the treatment mode (chronic continuous vs. intermittent; intravenous vs. subcutaneous or even locally applied). For ALS, the antiapoptotic properties, protection of the neuromuscular junction, immune modulating, as well as angiogenic properties appear key in counteracting the pathology. As in SCI, a multitude of pathophysiological processes need to be addressed together, which is an argument for a growth factor as therapeutic agent (Henriques et al., 2010). The direct impact of the neurogenic potential of G-CSF for modifying the disease course is not so clear at the present state of knowledge. As is the situation with SCI, there are interesting clinical studies that indicate feasibility and safety of treatment,

TABLE 3 | Published preclinical studies in the use of G-CSF for ALS.

Drug/dosage	Application/treatment duration	Outcome	SCI Model	References
Filgrastim 30 μg/kg/day	sc. injection for 8 weeks via osmotic minipumps, mice at 11 weeks of age	• Prolonged survival of SOD1 mice • Delayed disease onset • Flattened disease progression • Flattened loss of grip strength • Reduced muscle atrophy • Larger muscle diameter • Decreased fibrillations • Ameliorated loss of motor neurons • No effect on microglial (Iba1) and astroglial (GFAP) markers • Reduced decrease of oligodendroglial markers (PLP) • Trend toward increased pan-neuronal markers (NSE)	SOD1-G93A mice	Pitzer et al., 2008
Filgrastim 30 μg/kg/day	sc. injection for 4 weeks via osmotic minipumps, mice at 11 weeks of age	• Restored transcriptomic deregulations of SOD1 mice • Transcriptome close to presymptomatic SOD1 mice or wild type animals • Modulation of genes closely related to neuromuscular functions (CCR4-NOT, Prss12)	SOD1-G93A mice	Henriques et al., 2014
Filgrastim 100 μg/kg/day	Week 10 of age until death via single sc. injections for 5 consecutive days per week	• Increased survival of SOD1 mice • Higher amount of surviving α-motoneurons • Increased amount of large myelinated axons • Increased microglia recruitment around neurons • Splenomegaly	SOD1-G93A mice	Yamasaki et al., 2010
Filgrastim 200 μg/kg/day ± surgery	Week 12 of age, single sc. injections from 5 days before hypoglossal axotomy until po. day 20 or day 40	• Increased viability rate of hypoglosal neurons • Increased number of Iba1-positive microglia	SOD1-G93A mice	Yamasaki et al., 2010
Pegfilgrastim 300 μg/kg/week	Week 12–16 of age, until clinical end stage via sc. single injections	• Prolonged survival of SOD1 mice • More sustained motoric capacity • No effect on spinal cord neuronal survival • Attenuated astrogliosis and microgliosis in the spinal cord • Attenuated production of inflammatory cytokines in microglia and peripheral monocytes • Reduced severity of muscle denervation	SOD1-G93A mice	Pollari et al., 2011
Pegfilgrastim 300 μg/kg/week	Week 12 of age until scarification via single sc. injections once per week	• Gender specific alterations • Reduced levels of reactive oxygen species in males but not females • No effect on survival rate or motor performance in females	SOD1-G93A mice	Naumenko et al., 2011
Adeno associated virus upregulation of endogenous G-CSF expression	One viral im. or is. injection (bilaterally at the L1 level)	• Delayed body mass decrease • Delayed paresis • Increased survival of SOD1 mice • Delayed clinical end point • Improved neuromuscular junction integrity and enhanced motor axon regeneration following nerve crush injury	SOD1-G93A mice	Henriques et al., 2011

Iba1, ionized calcium-binding adapter molecule; IM, intramuscular; IS, intraspinal; GFAP, glial fibrillary acidic protein; NSE, neuron specific enolase; PLP, proteolipid protein; PO, post operation; SC, subcutaneous.

TABLE 4 | Published clinical studies on the use of G-CSF for ALS treatment.

Drug/dosage	Application/treatment duration	Outcome	Pat. Nr.	Patient characteristics	Side effects	References
Filgrastim (Neupogen®) 300 μg/kg/day ± CD133+ stem cells	3 days via single sc. injections/implantation of isolated CD133+ stem cells into the motor cortex	• No difference in FVC • Significant improvement of the ALSFRS score	23	Patients with confirmed ALS according to the clinical and neurophysiological criteria; no familiar ALS patients present	Not mentioned	Martinez et al., 2009
Filgrastim 300 μg/kg/day ± CD133+ stem cells	3 days via single sc. injections/implantation of isolated CD133+ stem cells into the motor cortex	• Positive tendency toward disease stabilization	67	Patients with confirmed ALS according to El-Escorial clinical and neurophysiological criteria	No severe adverse effects, mild and transient adverse effects; Major adverse effects after catheter insertion and stem cell transplantation including headache, insomnia, generalized malaise, back pain, vomiting and abdominal pain; One patient died due to acute subdural hematoma which occurred after treatment for myocardial infarction	Martínez et al., 2012
Filgrastim 10 μg/kg/day	day 1–10 and 20–25 via single sc. injections	• No differences in clinical tests (JTT, ALSFRS score, z-scores) reduced decline in fractional anisotropy	10	Patients with confirmed ALS according to the clinical and neurophysiological criteria	Mild to moderate adverse effects including well-described side effect profile of G-CSF including headache, bone pain and malaise	Duning et al., 2011
Filgrastim 300–600 μg/day	5 days via single sc. injections, 6 days via single injections	• No significant changes in disease progression	8	5 patients with confirmed ALS according to the clinical and neurophysiological criteria and 3 patients with probable ALS	No adverse effects	Cashman et al., 2008
Filgrastim 2 μg/kg/day	5 days via single sc. injection	• Reduced decline of ALSFRS score during first 3 month after treatment • Reduced decline of CAMP amplitude during first 3 month after treatment • Faster change of the ALSFRS score in the second 3 months after treatment	13	Patients with diagnosis of definite or probable ALS with duration less than 3.5 years	Mild adverse effects with 2 patients developing symptoms of infection and 3 other patients having mild fever	Zhang et al., 2009
Ratiograstim 5 μg/kg/day up to 10 μg/kg/day	One/two sc. injections for 5 consecutive days every 4 weeks	• Longtime safety and feasibility with no major side effects up to 3 years • Sustained mobilization of CFC • Loss of immature progenitors following long-term administration	6	Patients with diagnosis of definite or probable ALS according to the El-Escorial clinical and neurophysiological criteria	Mild, treatment-related adverse effects including bone pain, muscle pain, headache, fever, dyspnea and pre-syncope	Grassinger et al., 2014

(Continued)

TABLE 4 | Continued

Drug/dosage	Application/treatment duration	Outcome	Pat. Nr.	Patient characteristics	Side effects	References
Lenograstim (Myelostim®) 2 × 5 μg/kg/day	4 days every 3rd month via single sc. injection	• No effects on the decline of the ALSFRS-R score • No modification of quality of life • Reduced serum/CSF levels of MCP-1 • Reduced CSF levels of IL-17 • Enhanced serum levels of IP-10	24	Patients with diagnosis of definite, probable or probable-laboratory-supported ALS and less than 12 months disease duration	Few and transitory adverse effects including flu-like symptoms, nausea and asthenia; 2 severe adverse with 1 patient developing hyper prolactinemia and 1 patient developing thrombosis but both seem to be unrelated to study drug	Chiò et al., 2011
Filgrastim (Neupogen®) 5 μg/kg/day	4 days every 3rd via single sc. injections	• Trend of slowing disease progression	19	Patients with diagnosis of definite or probable ALS according to the El-Escorial clinical and neurophysiological criteria	Mild drug-related adverse effects including bone and muscle pain; no major adverse effects	Nefussy et al., 2010
Combination of PDgrastim, filgrastim, 300 μg recombinant G-CSF, equal to 30,000,000 IU of filgrastim, mannitol and sodium acetate 5 μg/kg/q12 h	5 days via single sc. injection	• No effect on the monthly ALSFRS-R score decline • No effect on the change of the ALS questionnaire and manual muscle testing scores • No effect on the change of the nerve conduction velocity • Lower elevation of CD34+ cell counts in female compared to male patients • Trend toward a greater ALSFRS-R score reduction in female G-CSF-treated patients compared to male placebo-treated counterparts	40	Patients with diagnosis of definite or probable ALS according to the El-Escorial clinical and neurophysiological criteria	Not mentioned	Amirzagar et al., 2015

FVC, forced vital capacity; ALSFRS, ALS Functional Rating Scale; ALSFRS-R, ALS Functional Rating Scale Revised; JTT, Jebsen Taylor Test; CAMP, compound muscle action potential; CFC, colony forming cells; CSF, cerebrospinal fluid; MCP-1, monocyte chemoattractant protein-1; IL-17, interleukin-17; IP-10, interferon-induced protein 10; IU, international unit; SC subcutaneous.

and show promising hints for efficacy, but larger randomized controlled studies are needed to get definite answers. Imaging (Duning et al., 2011) and potentially other biomarkers may help in clinical development in ALS. Several questions for clinical development remain open, in particular feasibility and safety of a potentially yearlong treatment. Very recent and promising data support this (Grassinger et al., 2014; Khomenko et al., 2015).

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REVIEW
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Exosomes as Novel Regulators of Adult Neurogenic Niches

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Adult neurogenesis has been convincingly demonstrated in two regions of the mammalian brain: the sub-granular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, and the sub-ventricular zone (SVZ) of the lateral ventricles (LV). SGZ newborn neurons are destined to the granular cell layer (GCL) of the DG, while new neurons from the SVZ neurons migrate rostrally into the olfactory bulb (OB). The process of adult neurogenesis persists throughout life and is supported by a pool of neural stem cells (NSCs), which reside in a unique and specialized microenvironment known as "neurogenic niche". Neurogenic niches are structured by a complex organization of different cell types, including the NSC-neuron lineage, glial cells and vascular cells. Thus, cell-to-cell communication plays a key role in the dynamic modulation of homeostasis and plasticity of the adult neurogenic process. Specific cell-cell contacts and extracellular signals originated locally provide the necessary support and regulate the balance between self-renewal and differentiation of NSCs. Furthermore, extracellular signals originated at distant locations, including other brain regions or systemic organs, may reach the niche through the cerebrospinal fluid (CSF) or the vasculature and influence its nature. The role of several secreted molecules, such as cytokines, growth factors, neurotransmitters, and hormones, in the biology of adult NSCs, has been systematically addressed. Interestingly, in addition to these well-recognized signals, a novel type of intercellular messengers has been identified recently: the extracellular vesicles (EVs). EVs, and particularly exosomes, are implicated in the transfer of mRNAs, microRNAs (miRNAs), proteins and lipids between cells and thus are able to modify the function of recipient cells. Exosomes appear to play a significant role in different stem cell niches such as the mesenchymal stem cell niche, cancer stem cell niche and pre-metastatic niche; however, their roles in adult neurogenic niches remain virtually unexplored. This review focuses on the current knowledge regarding the functional relationship between cellular and extracellular components of the adult SVZ and SGZ neurogenic niches, and the growing evidence that supports the potential role of exosomes in the physiology and pathology of adult neurogenesis.

Keywords: exosomes, adult neurogenesis, niche, stem cells, extracellular vesicles, psychiatric disorders, neurological disorders, biomarkers

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INTRODUCTION

It was long believed that mammalian neurogenesis, i.e., the process of generating functional neurons from neural precursors, occurred only during embryonic and perinatal stages (Ming and Song, 2005). Altman's pioneering studies in the 1960's provided the first histological evidence for the presence of newborn neurons in the adult brain of rodents (Altman and Das, 1965, 1966; Altman, 1969). However, the fact that young neurons are continuously incorporated into the adult brain circuitry was not widely accepted until the mid-1990's (Gross, 2000; Kaplan, 2001). Advances in immunohistological techniques and particularly the introduction of bromodeoxyuridine (BrdU), a thymidine analog that can be incorporated *in vivo*, allowed to recognize proliferative populations of cells within the central nervous system (CNS) and to identify the destination of adult-born neurons. To date it is well known that neural stem cells (NSCs) reside in the brain of most adult mammals, including humans, and that neurogenesis does occur throughout life (Eriksson et al., 1998; Gage, 2000; Lie et al., 2004; Abrous et al., 2005; Ming and Song, 2005; Lledo et al., 2006; Merkle and Alvarez-Buylla, 2006; Bergmann et al., 2015; Kempermann et al., 2015). Interestingly, regions harboring active adult neurogenesis are located in discrete but specific areas of the brain. These areas, known as "neurogenic niches", are composed of different cell types, specific cell-cell contacts, and particular extracellular cues originated both locally and distantly. Thus, the function of the different cellular and molecular components of the niche supports the physiology of NSCs, balancing quiescence with proliferation, and regulating cell differentiation (Conover and Notti, 2008).

In this context, not only the cytoarchitectonic organization of the niche but also the ways of communication between the cellular components, are critical to understand the adult neurogenic process under both physiological and pathological conditions. Cells communicate reciprocally with other cells by (i) intercellular contacts, and (ii) secreted molecules, such as growth factors, cytokines, hormones, etc. (paracrine or endocrine communication). However, a novel way of cell-to-cell communication mediated by extracellular vesicles (EVs) has attracted the attention of several researchers in different fields. EVs, such as exosomes, carry a specific cargo of proteins, lipids and nucleic acids and are currently consider one of the most complex and physiologically relevant messengers between cells. This review focuses on the cellular components of the adult neurogenic niches, the mechanisms involved in intercellular communication, and the potential role of exosomes as regulators of the neurogenic process, and as mediators and novel biomarkers of neuropsychiatric and neurological disorders associated with defective adult neurogenesis.

ADULT NEUROGENIC NICHES

In the adult mammalian brain, neurogenesis is well documented to continue throughout life in two regions: the subventricular zone (SVZ) of the lateral ventricles (LV) and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (**Figure 1A**). A lineage model from NSCs to mature neurons has been characterized in both, the SVZ and the SGZ adult neurogenic niches (**Figure 1B**). In this model, NSCs give rise to neural progenitor cells (NPCs), also known as transit amplifying cells because of their limited division potential, which differentiate into migrating neuroblasts and then, into neurons that integrate into pre-existing circuits (Gage, 2000). Neuroblasts in the SGZ migrate short distances and integrate into the existing circuitry of the granular cell layer (GCL) of the DG (**Figure 1C**); those from the SVZ migrate along the rostral migratory stream (RMS) and supply newborn neurons for the olfactory bulb (OB; **Figures 1A,D**). Interestingly, glial and vascular cells are also major contributors to the configuration of functionally structured neurogenic niches (**Figures 1B–D**). Growing evidence suggests that the adult mammalian brain contains other neurogenic niches that are capable of generating new neurons and glial cells, particularly after injury or after some inductive stimuli (Lin and Iacovitti, 2015). The neocortex (Gould et al., 1999; Gould, 2007; Cameron and Dayer, 2008) and the hypothalamus (Kokoeva et al., 2005) have also been reported to support adult neurogenesis, but the magnitude of the neurogenic process in these regions is still under debate. Although the functional significance of adult-born neurons under physiological and/or pathological conditions has not been completely clarified and is being actively pursued, it is well-defined that neurogenesis in the SVZ and SGZ of the adult brain depends on the presence and maintenance of NSCs, which is tightly regulated by their highly specialized microenvironments or neurogenic niches (Palmer et al., 2000; Alvarez-Buylla and Lim, 2004; Ma et al., 2005; Merkle and Alvarez-Buylla, 2006).

Niches are defined by their ability to anatomically house stem cells and functionally control their development *in vivo* (Ma et al., 2008). The concept that stem cells reside within specific niches was first suggested in the 1970's (Schofield, 1978), but it was not until the 2000's, when substantial progress was made in describing both the cellular components of the niches and their functional interactions, in several mammalian tissues, including skin, intestine and bone marrow (Spradling et al., 2001; Li and Xie, 2005; Scadden, 2006). In the adult brain, much is known about the cellular composition and organization that characterize the SVZ and SGZ neurogenic niches (Ma et al., 2008; Mirzadeh et al., 2008; Aimone et al., 2014; Bjornsson et al., 2015; Licht and Keshet, 2015). Furthermore, the interaction and functional coordination of these components as well as the heterogeneity and complexity of neurogenic niches and their emerging roles under pathological conditions is being pictured (Jordan et al., 2007; Alvarez-Buylla et al., 2008).

The Subventricular Zone (SVZ) Niche

Adult NSCs persist in a narrow niche along the walls of the LV, bordered on one side by the ependymal surface lining the

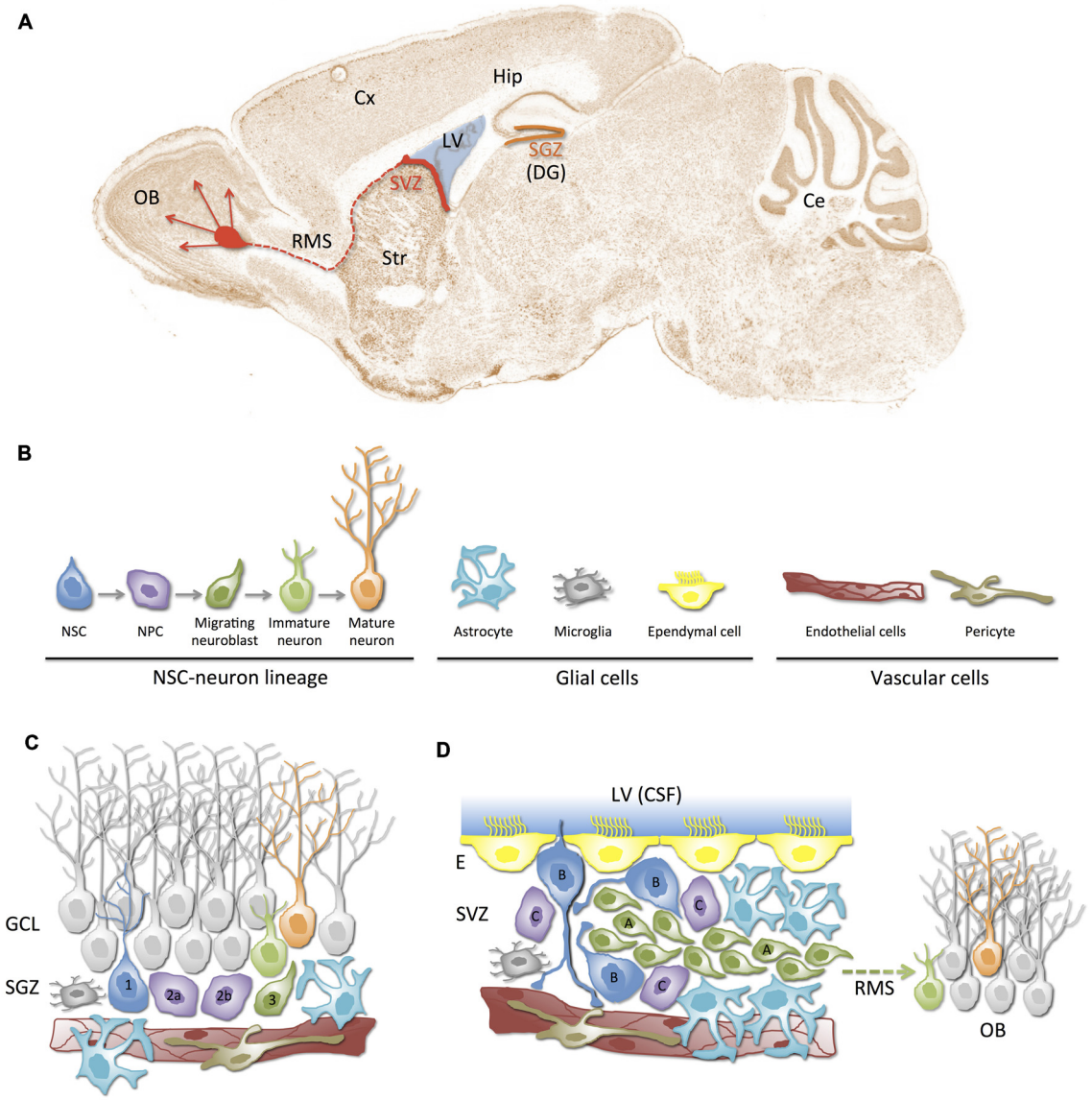

FIGURE 1 | Neurogenic niches in the adult mammalian brain. (A) Schematic representation of the neurogenic regions (niches) in a sagittal section of the adult mouse brain: the subgranular zone (SGZ, orange) in dentate gyrus (DG) of the hippocampus (Hip), and the subventricular zone (SVZ, red) in the lateral wall of the lateral ventricles (LV). SVZ-derived newborn neurons migrate towards the olfactory bulb (OB) through the rostral migratory stream (RMS). **(B)** Cellular components of neurogenic niches. In addition to the neural stem cell (NSC)-neuron lineage, neurogenic niches are composed of glial cells (astrocytes, microglia and ependymal cells) and vascular cells (endothelial cells, pericytes). **(C,D)** Illustration of SGZ and SVZ neurogenic niches. The cytoarchitecture and relationships between cellular components of the niche are represented. Different cell types (color and shape) correspond to those depicted in **(B)**. Note the close proximity between blood vessels and NSCs/NPCs in both niches. **(C)** The SGZ neurogenic niche. Radial type 1 cells correspond to the NSCs that give rise to type 2a/b NPCs, which differentiate into type 3 neuroblasts. Neuroblasts migrate guided by astrocytes and become maturing neurons that finally mature and integrate into the granular cell layer (GCL). **(D)** SVZ neurogenic niche. This niche is located underneath the ependymal lining (E) of the LV. It is composed of type B quiescent cells (NSCs), which can activate and generate type C NPCs that rapidly proliferate and generate type A neuroblasts. Neuroblasts migrate long distances through the rostral migratory stream (RMS) to the OB where they mature into interneurons. Note that a tunnel of astrocytes and a scaffold of blood vessels guide migration of neuroblasts. Also note that monociliated type B cells can directly contact cerebrospinal fluid (CSF) and blood vessels. Ce, cerebelum; Cx, cortex; Str, striatum.

cerebrospinal fluid (CSF)-filled ventricles and on the other by a complex arrangement of parallel blood vessels (Mirzadeh et al., 2008; Shen et al., 2008; **Figure 1D**). NSCs that reside in the SVZ, also known as Type B cells, exhibit hybrid characteristics of astrocytes (GFAP+) and immature progenitors (S100β+, Nestin+, Sox2+; Kriegstein and Alvarez-Buylla, 2009). Type B cell bodies are typically located under the ependymal lining of the LV and some of them have a short apical process with a single primary cilium that projects through the ependymal cell layer to contact the CSF directly, and a basal process that ends on the blood vessels of the SVZ plexus (Mirzadeh et al., 2008). Interestingly, apical processes of various type B

cells form bundles at the center of a "pinwheel" of ependymal cells (Mirzadeh et al., 2008). As a result of their position and polarized phenotype, type B cells are strategically located to receive cues from both the vascular and the CSF compartments (**Figure 1D**). Quiescent type B cells can eventually divide asymmetrically to give rise to type C (Mash1+) transit-amplifying progenitor cells (Doetsch et al., 1997; Merkle and Alvarez-Buylla, 2006). Most of type C cells, in turn, divide to give rise to PSA-NCAM+ neuroblasts (type A cells). Type A cells form clusters and chains that migrate toward the OB guided by a channel of astrocytes and by a parallel scaffold of blood vessels. The anatomical structure formed by migrating (type A) neuroblasts is known as the RMS. Within the OB, these immature neurons differentiate into two types of GABAergic interneurons: the granular neurons and the periglomerular neurons, which integrate into the existing neuronal circuitry (Merkle and Alvarez-Buylla, 2006; Curtis et al., 2007; Kriegstein and Alvarez-Buylla, 2009; **Figures 1A,D**). Interestingly, type B/C cells can also originate glia (oligodendrocytes or astrocytes; Menn et al., 2006).

The Subgranular Zone (SGZ) Niche

The SGZ is a region located beneath the GCL of the DG of the hippocampus (**Figure 1C**). The NSCs to mature neurons lineage model described in the SVZ can be comparably applied in the SGZ. A similar subset of GFAP+/Sox2+/Nestin+ radial glia-like cells are also believed to be quiescent NSCs of the SGZ (Seri et al., 2001). These NSCs, also known as type 1 cells, give rise through asymmetric division to transit-amplifying non-radial progenitors (Nestin+/GFAP−) or type 2a cells. Type 2a cells subsequently originate what appears to be a more fate-committed (Tbr2+) intermediate progenitor (type 2b) cell population, which give rise to doublecortin (Dcx) + (type 3) neuroblasts. Finally, neuroblasts differentiate into maturing glutamatergic granule cells that migrate, guided by astrocytic processes, and integrate in the GCL of the DG (Aimone et al., 2014; **Figure 1C**).

CELLULAR COMPONENTS OF THE NEUROGENIC NICHE AND THEIR ROLE IN THE NEUROGENIC PROCESS

The highly hierarchical NSC-neuron lineage in both the SVZ and SGZ requires the precise regulation of NSCs self-renewal, fate specification, maturation and integration of new neurons in the existing neural circuitry. The convergence of several cellular and extracellular factors contributes to building a unique and specialized niche or microenvironment that regulates the physiology of NSCs during the course of adult life. Therefore, the identification and functional characterization of these factors emerges as a key aspect not only to better understand the biology of adult NSCs but also to develop novel therapies for a number of neurological and psychiatric disorders associated with defects in adult neurogenesis. Significant advances have been made in the description of the cellular components of the neurogenic niches and the mechanisms by which they can individually or coordinately contribute in regulating adult neurogenesis (reviewed in Ma et al., 2008; Aimone et al., 2014; Bjornsson et al., 2015; **Figure 2**).

Astrocytes

Astrocytes represent one of the major contributors to the neurogenic niche (Song et al., 2002). Co-culture experiments with astrocytes isolated from the adult hippocampus and cortex, but not from the spinal cord, induce neuronal differentiation of NPCs (Barkho et al., 2006; Oh, 2010). Furthermore, hippocampus-derived astrocytes are more efficient than cortical astrocytes in promoting neuronal fate of NPCs (Oh, 2010), suggesting that the functional heterogeneity of astrocytes may be reflecting unique characteristics of different brain regions or environments. Interestingly, they may exert either positive or negative regulatory roles in the neurogenic process (**Figure 2**). Astrocytes negatively control neuronal differentiation of neural stem/progenitor cells through cell–cell contacts in which Jagged1-mediated Notch pathway and intermediate filament proteins GFAP and vimentin play a significant role (Wilhelmsson et al., 2012). Similarly, some astrocyte-secreted factors such as insulin-like growth factor binding protein 6 (IGFBP6) and decorin inhibit neuronal differentiation of adult NSCs/NPCs (Barkho et al., 2006). On the other hand, positive effects on neuronal differentiation of adult SGZ NSCs involve cell-cell contact by ephrin-B2(+) astrocytes (Ashton et al., 2012). Likewise, astrocytic ATP release positively regulates proliferation of NSCs in the SGZ (Cao et al., 2013) and astrocyte-derived soluble factors such as Wnt3 (Seri et al., 2001; Lie et al., 2005), neurogenesin-1 (Ueki et al., 2003), thrombospondin-1 (Lu and Kipnis, 2010) and interleukins such as IL-1β, IL-6 (Barkho et al., 2006), promote hippocampal neurogenesis. In agreement with a positive regulatory action of astrocytes, age-related changes in astrocyte population, including the decline of FGF-2 + astrocytes, correlate with low levels of neurogenesis in the aged hippocampus (Shetty et al., 2005).

Astrocytes also contribute to the migration of neuroblasts along the RMS by creating a physical route or tube where they (i) communicate with migrating neuroblasts (Bolteus and Bordey, 2004), and (ii) help to maintain the architecture of the vasculature scaffold in a VEGF- and thrombospondin-4-dependent fashion (Bozoyan et al., 2012; Girard et al., 2014). Similarly, in the adult SGZ, astrocytic radial processes facilitate the short-distance migration of newly generated neurons from the SGZ to the GCL of the DG (Shapiro et al., 2005). In addition, adult hippocampal astrocytes promote maturation and synaptic integration of the neural progeny of NSCs in co-culture experiments (Song et al., 2002).

Ependymal Cells

Mature ependymal cells form a simple cuboidal to low columnar epithelium-like structure lining ventricular cavities. These cells have microvilli and tufts of motile cilia that contribute to the CSF hydrodynamic flow (Nelson and Wright, 1974; Ibañez-Tallon et al., 2004; Spassky et al., 2005). Disruption of ependymal lining or ciliary defects can provoke CSF flow disturbances and hydrocephalus (Jiménez et al., 2001; Wagner et al., 2003;

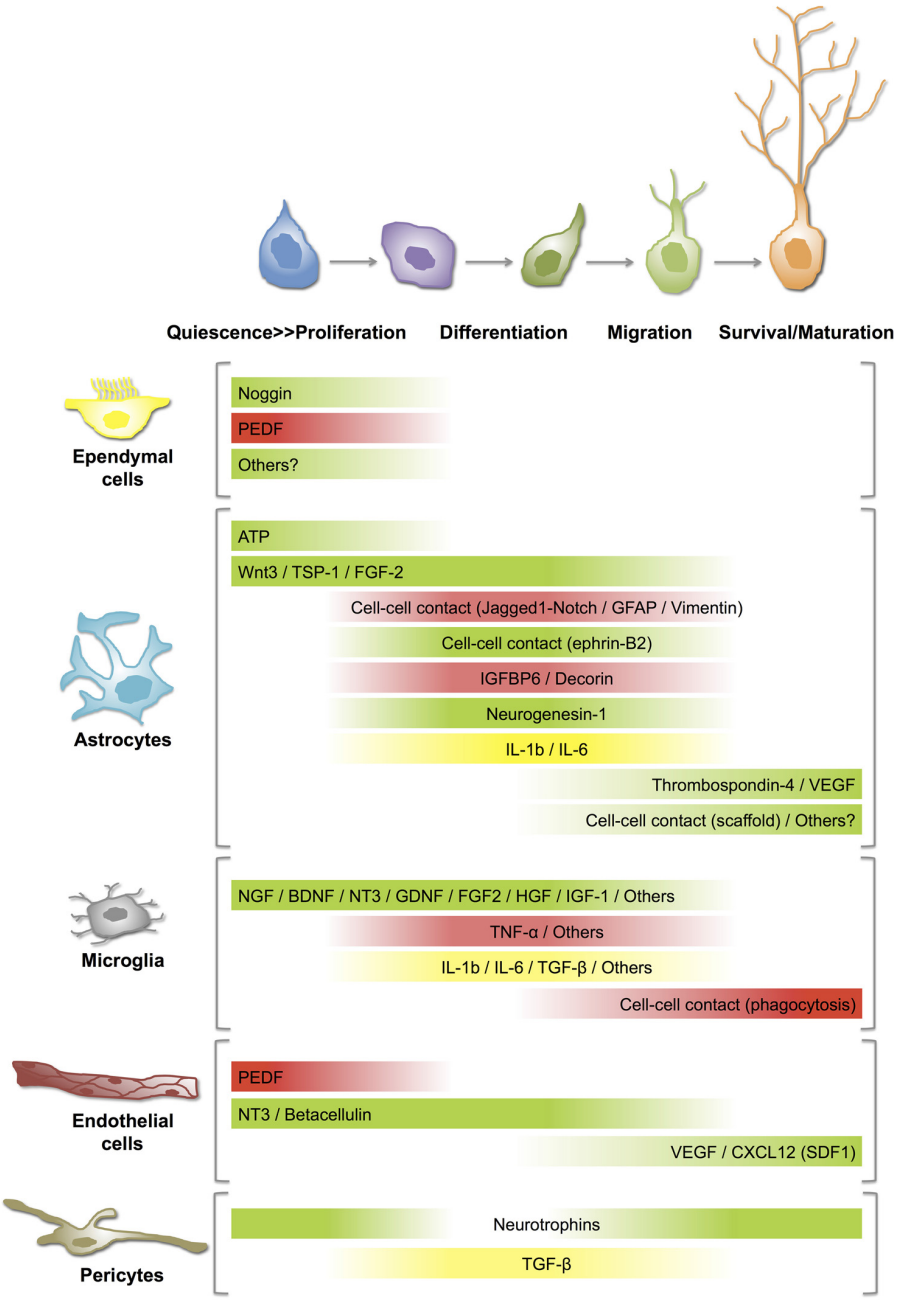

FIGURE 2 | Distinct roles of niche cells on NSC-neuron lineage at different stages/steps of adult neurogenesis. Best-described cell-cell communication mechanisms in neurogenic niches rely on soluble mediators or direct contact between the signaling and the targeting cells. The gradient bars represent varying influence of secreted factors or cell-cell contacts on (i) activation of quiescent NSCs (proliferation); (ii) differentiation or fate specification; (iii) migration; and (iv) survival/maturation of newborn neurons. Green: stimulation or activation; Red: inhibition; Yellow: activation or inhibition according to the circumstances. For details and references, see the text. ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CXCL, chemokine (C-X-C motif) ligand; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IL, interleukin; NGF, nerve growth factor; NT, neurotrophin; PEDF, pigment epithelium-derived factors; SDF, stromal cell-derived factor; TGF, transforming growth factor; TNF, tumor necrosis factor; TSP, thrombospondin; VEGF, vascular endothelial growth factor.

Ibañez-Tallon et al., 2004; Banizs et al., 2005; Town et al., 2008). Because of their location, ependymal cells display certain barrier and signaling functions between CSF and neural tissue (Sarnat, 1992; Del Bigio, 1995). Ependymal cells are joined together at their apical regions by N-cadherin-based adherens junctions (Brightman and Reese, 1969; Del Bigio, 1995; Rodríguez and Bouchaud, 1996) and blocking N-cadherin function *in vitro* induces apoptosis of ependymal cells

and denudation of ventricular walls (Oliver et al., 2013). Ependymal denudation of ventricular walls increases with aging (Luo and Craik, 2008) and is associated with the pathogenesis of neurodevelopmental disorders such as periventricular heterotopia (Ferland et al., 2009), spina bifida aperta (Sival et al., 2011) and hydrocephalus (Jiménez et al., 2009; Wagner et al., 2003; Domínguez-Pinos et al., 2005). Comprehensive analyses by Spassky et al. (2005) indicate that most ependymal cells are born at embryonic stages and that differentiated ependymal cells are postmitotic. However, it has been suggested that ependymal cells can be generated postnatally in certain brain regions (Bátiz et al., 2011). Furthermore, it has been argued that ependymal cells lining the SVZ can act as NSCs (Chojnacki et al., 2009). Interestingly, pathological stimuli such as stroke can stimulate forebrain ependymal cells to proliferate and generate neurons and astrocytes (Carlén et al., 2009).

Beyond this controversy, it is well defined that ependymal cells are a key component of the SVZ neurogenic niche. They are a source of noggin and pigment epithelium-derived factor (PEDF; **Figure 2**). Noggin is a bone morphogenetic protein (BMP) antagonist involved in the maintenance of a neurogenic niche (Lim et al., 2000). Opposite results have suggested that BMP signaling can inhibit adult neurogenesis (Lim et al., 2000), or promote it (Colak et al., 2008). Thus, the role of ependymal-derived noggin on the SVZ physiology is still not clear. PEDF is secreted by both ependymal and endothelial cells, and specifically participates in the self-renewal capacity of type B cells of the SVZ, thereby maintaining a pool of undifferentiated NSCs in the neurogenic niche (Ramírez-Castillejo et al., 2006). Furthermore, it is known that PEDF modulates stemness of NSCs by activating Notch-dependent transcription in these cells (Ramírez-Castillejo et al., 2006; Andreu-Agullo et al., 2009). In this context, the loss or disruption of the ependyma in the SVZ neurogenic niche would impair ependymal cell-mediated signaling pathways and alter its neurogenic potential. This appears to be the case in the SVZ niche of the hyh mutant mice where ependymal cells of the LV are completely lost during early postnatal stages and proliferation of SVZ progenitors is dramatically reduced (Jiménez et al., 2009). Interestingly, ependymal-denuded surfaces become progressively covered by a layer of astrocytes that acquire certain morphological and antigenic ependymal cell-like properties and probably contribute to the repair of the ependymal lining (Páez et al., 2007; Luo et al., 2008; Roales-Buján et al., 2012). However, the presence of a reactive astroglial "scar" can reduce the proliferative activity of SVZ neural progenitors, blocking neuronal regeneration and revascularization and thus, interfering with the recovery of an injured area (Fawcett and Asher, 1999; Kernie et al., 2001).

Microglia

Most microglial cells of the CNS are already generated by the end of the second postnatal week (Ginhoux et al., 2013). In the adult brain, resting microglial cells represent the resident macrophages and they survey the brain parenchyma (Nimmerjahn et al., 2005). In the SGZ of the hippocampus, microglial cells have a prominent surveillant and phagocytic role. It has been demonstrated that most newborn granule neurons of the adult SGZ undergo apoptosis and are phagocytosed by microglial cells (Sierra et al., 2010, 2013). Beyond their role as brain ś professional phagocytes, microglial cells can also serve neuroprotective or neurotoxic roles depending on the physiological and pathological circumstances (Luo and Chen, 2012; Hellwig et al., 2013; Brites and Vaz, 2014). In addition, microglial cells can interact with NSCs/NPCs (Su et al., 2014) and regulate adult neurogenesis by secreting several soluble mediators, such as growth factors and cytokines, that influence either positively or negatively the neurogenic process (reviewed in Kim and de Vellis, 2005; Harry, 2013; **Figure 2**). *In vitro* experiments suggest that microglia secret factors that promote neuronal differentiation of SVZ-derived NSCs but not their maintenance or self-renewal (Walton et al., 2006). On the other hand, activation of microglial cells can result in negative regulatory actions on the SGZ neurogenic process (Sierra et al., 2014). For example, inflammatory or LPS-mediated activation of microglia can inhibit neurogenesis and favor gliogenesis and this effect is partially mediated by the secretion of proinflammatory cytokines, such as TNF-α (Monje et al., 2003; Butovsky et al., 2006; Carpentier and Palmer, 2009). On the other hand, when microglial cells are stimulated with interleukin-4 (IL-4) and interferon-γ, they secrete insulin-like growth factor-1 (IGF-1) and induce neuronal differentiation of neural progenitors (Butovsky et al., 2006). Additionally, the environmental context of the neurogenic niche determines not only the mode of activation of microglial cells and the positive or negative regulatory actions of these cells as a population but also defines the pro- or anti-neurogenic properties of specific microglial-secreted cytokines, such as the transforming growth factor β (TGF-β; Battista et al., 2006; Douglas-Akinwande et al., 2006). Interestingly, physical exercise (running)-induced cell proliferation and neurogenesis in the SGZ (van Praag et al., 1999b; Aimone et al., 2014) is associated to a decrease in microglial function (Olah et al., 2009; Vukovic et al., 2012; Gebara et al., 2013). Inversely, age-related changes in microglia activity, including an increase in their reactive profile with higher secretion of pro-inflammatory cytokines (Njie et al., 2012), potentially contribute to the decline of neurogenesis seen with aging (Gemma et al., 2010; Gebara et al., 2013).

Endothelial Cells and Pericytes

The close physical proximity of NSCs/NPCs with blood vessels within both adult neurogenic niches suggests that critical factors derived from the vasculature may act as major modulators of the neurogenic process and consequently, several authors consider the SVZ and the SGZ as "vascular niches" (Palmer et al., 2000; Shen et al., 2008; Tavazoie et al., 2008; Licht and Keshet, 2015). Interestingly, vascular-derived signals or messengers can be originated distantly, in peripheral organs (blood-borne substances), and/or locally from endothelial cells and pericytes (paracrine communication). Endothelial cells are able to secrete several factors that modulate adult neurogenesis (Palmer et al., 2000; Shen et al., 2004; **Figure 2**). Several studies suggest that endothelial cell-

produced brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) stimulates SGZ neurogenesis both *in vivo* and *in vitro* (Jin et al., 2002; Cao et al., 2004; Kim et al., 2004). Interestingly, endothelial cells and NSCs reciprocally influence each other to couple SGZ neurogenesis to angiogenesis, and it is suggested that VEGF is critical to coordinate these processes (Palmer et al., 2000; Riquelme et al., 2008; Udo et al., 2008; Ruiz de Almodovar et al., 2009). Furthermore, VEGF appears to be necessary for exercise-induced SGZ neurogenesis (Fabel et al., 2003). Together with ependymal cells, endothelial cells secrete PEDF and thus, stimulate self-renewal of NSCs (Ramírez-Castillejo et al., 2006; Andreu-Agullo et al., 2009). Neurotrophin-3 (NT3) and betacellulin, secreted by vascular endothelial cells of both the SVZ plexus and the choroid plexus, regulate SVZ neurogenesis by different mechanisms. NT3 helps to maintain the quiescence of NSCs (Delgado et al., 2014), whereas betacellulin promotes proliferation of NPCs and neuroblasts (Gomez-Gaviro et al., 2012). Vascular endothelial cells also secrete a chemokine known as CXCL12 or stromal derived factor-1 (SDF1) that differentially modulates SVZ NSC-neuron lineage (Kokovay et al., 2010). In type B and type C cells, SDF1 upregulates EGFR and alpha-6 integrin, activating and attracting them to the blood vessels while SDF1 stimulates motility of type A neuroblasts (Kokovay et al., 2010).

The role of pericytes in adult neurogenic niches is less well characterized. Located in intimate contact with endothelial cells, they can act as regulators or transducers of both blood-circulating signals and endothelial-derived factors (Armulik et al., 2011). Pericytes secrete TGF-β, a known modulator of adult neurogenesis and blood-brain barrier (BBB; Dohgu et al., 2005). They also secrete neurotrophins in response to hypoxic conditions (Ishitsuka et al., 2012). In addition to their potential paracrine function, it has been demonstrated that pericytes remain relatively undifferentiated, retaining the capacity to differentiate into several cell types, including neural-related progeny. Consequently, it has been proposed that pericytes may act as NSCs under certain circumstances (Dore-Duffy et al., 2006; Dore-Duffy and Cleary, 2011). On the other hand, pericytes might be involved in the pathogenesis of different CNS diseases and have been proposed as a potential therapeutic target (Lange et al., 2013).

DISTANT REGULATION OF THE NEUROGENIC NICHE: NEUROTRANSMITTERS, CSF-DERIVED FACTORS, AND BLOOD-BORNE SUBSTANCES

Neurotransmitters (Innervation)

Numerous neuromodulatory systems have been shown to affect proliferation and differentiation of NSCs/NPCs and the maturation of adult-born neurons. Adult SVZ receives inputs from dopaminergic projections from the ventral tegmental area (VTA) and the substantia nigra (Baker et al., 2004). It has also been shown that SVZ type C cells express D2-like dopaminergic receptors (Höglinger et al., 2004; Kippin et al., 2005). However, the effects of dopamine on adult NPCs are controversial and it has been suggested that dopamine differentially affects type B and C cells via a distinctive mix of receptors in each cell type (Berg et al., 2013). On the other hand, dopamine D2 receptor-induced neurogenesis appears to be mediated by ciliary neurotrophic factor (CNTF), a mitogen expressed by astrocytes that is upregulated by D2 agonists (Yang et al., 2008). The role of dopamine in the SGZ neurogenic process is less well understood (Veena et al., 2011) but it has been suggested that, similarly to the SVZ, D2 receptors are involved in hippocampal neurogenesis *in vivo* (Yang et al., 2008). Other major neuromodulatory systems include the serotoninergic and cholinergic systems. The role of serotonin in SGZ neurogenesis has been deeply studied because of its link to depressive-like behaviors (Gould, 2007). The DG receives serotoninergic inputs from the median and dorsal raphe nuclei (Leranth and Hajszan, 2007). Interestingly, it is well documented that fluoxetine, a serotonin-reuptake inhibitor widely used as antidepressant, increases the proliferation rates in the hippocampal SGZ (Malberg et al., 2000). Similarly, serotonergic axons originated in the raphe nuclei form a widespread network at the ventricular surface of the LV and contact type B NSCs of the adult mouse SVZ. Activation of serotonin receptors of Type B cells increases proliferation (Tong et al., 2014). Mouse SVZ NSCs/NPCs also received inputs from choline acetyltransferase-positive axons originating in the SVZ itself. The release of acetylcholine by these neurons depends on their activity and can directly control SVZ proliferation (Paez-Gonzalez et al., 2014).

In addition, local or regional network neurotransmitters also play a significant role in adult neurogenesis. It has been demonstrated that non-synaptic GABA signaling (released by type A neuroblasts) can regulate proliferation of type B NSCs in the SVZ by activating $GABA_A$ receptors. This pathway provides a feedback mechanism to control the balance between self-renewal and differentiation of NSCs (Liu et al., 2005). Interestingly, this mechanism can also be reinforced by other SVZ GABAergic inputs such as the striatal GABAergic neurons (Young et al., 2014). In the SGZ niche, GABA helps to maintain the quiescence of radial glia-like (type 1) cells through activation of γ2-subunit containing $GABA_A$ receptors (Song et al., 2012). Similarly to the SVZ, these GABAergic inputs appear to be non-synaptic and due to diffusion from nearby parvalbumin+ basket cells synapses (Song et al., 2012). On the other hand, type 2 NPCs and young neurons receive more direct GABAergic inputs (Ge et al., 2006; Markwardt et al., 2009). In the adult DG, maturing neurons also receive glutamatergic inputs and it has been shown that NMDA receptor activation is critical for the survival and integration of young neurons (Tashiro et al., 2006).

CSF-Derived Factors

The SVZ is uniquely situated to experience the effects of the flow and the composition of the CSF. Intriguingly, the direction

of migrating neuroblasts in the RMS, parallels that of CSF flow (Sawamoto et al., 2006). Furthermore, when ependymal ciliary movements and CSF flow are disrupted, neuroblasts become disoriented (Sawamoto et al., 2006). On the other hand, the CSF contains several compounds that participate in the neurogenic process (Lafon-Cazal et al., 2003; Bunn et al., 2005; Redzic et al., 2005; Zappaterra and Lehtinen, 2012). Transcriptome analyses of adult and embryonic choroid plexus (ChP) have revealed that ChP epithelial cells express several growth factors and signaling molecules that can act either as positive (TGF-α, amphiregulin, IGF2, and FGF2) or negative (TGF-β superfamily members) regulators of adult neurogenesis (Marques et al., 2011; Liddelow et al., 2012). They also secrete SLIT1/2, known chemorepulsive signals that help type A neuroblasts to migrate in the RMS (Nguyen-Ba-Charvet et al., 2004). Other factors, such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), play important roles in promoting proliferative activity and maintenance of undifferentiated neural progenitors (Shimazaki et al., 2001; Gregg and Weiss, 2005). Recently, it was found that two G-protein coupled receptor (GPCR) ligands present in the adult CSF, namely sphingosine-1-phosphate (S1P) and prostaglandin-D2 (PGD2), promotes quiescence of SVZ NSCs (Sato et al., 2007; Kondabolu et al., 2011; Codega et al., 2014). The role of the ChP/CSF-mediated signaling pathway has been highlighted in aged and hydrocephalic animal models. Aging-related changes in the ChP/CSF system negatively affect adult neurogenesis (Baruch et al., 2014). Additionally, it has been demonstrated that the composition of the CSF is altered in hydrocephalus (Mashayekhi et al., 2002; Owen-Lynch et al., 2003) and hydrocephalic CSF inhibits cell proliferation cortical cells cultures, suggesting that CSF composition modifies some of the properties of NPCs (Owen-Lynch et al., 2003). In fact, the altered composition of CSF affects the normal cortical development in hydrocephalic H-Tx embryos (Miyan et al., 2003). However, the specific CSF changes that mediate these effects are actually not known, and the influences of the hydrocephalic CSF on the SVZ neurogenic niche have not yet been reported.

Blood-Borne Substances

As stated before, the vasculature can influence NSCs proliferation and differentiation by providing signaling molecules secreted locally by endothelial cells and pericytes, as well as by providing systemic blood-circulating factors (Tavazoie et al., 2008; Egeland et al., 2015; Licht and Keshet, 2015). Blood-borne factors that influence adult neurogenesis, including hormones, cytokines, metabolites, and gases can gain access to the SVZ neurogenic niche either via the ChP-CSF pathway route or more directly via the SVZ vasculature (Egeland et al., 2015; Licht and Keshet, 2015). In both cases, the presence of barriers must be considered. Indeed, ChP epithelial cells are joined together by tight junctions and constitute the blood-CSF barrier (BCB) while the presence of tight junctions between brain parenchymal endothelial cells constitute the BBB (Engelhardt and Sorokin, 2009). It has been suggested that SVZ vessels have a looser BBB than other brain vessels, allowing circulating substances to modulate adult neurogenesis. In this context, Tavazoie et al. (2008) have demonstrated that some circulating fluorescent tracers have better access to the brain parenchyma through SVZ vessels than through vessels located in other cerebral regions. Furthermore, NSCs/NPCs can directly contact blood vessels at specialized sites that lack pericyte coverage and glial end feet, a feature unique to SVZ vascular plexus (Tavazoie et al., 2008).

Several hormones seem to modulate adult neurogenesis under particular conditions. For example, prolactin can enhance SVZ neurogenesis in pregnant mice (Shingo et al., 2003), while increased levels of glucocorticoids associated with stress have the opposite effect (Snyder et al., 2011). In turn, dietary restriction and exercise/enriched environment positively modulate neurogenesis and it is proposed these effects are, at least in part, consequence of changes in the composition of blood-borne substances (van Praag et al., 1999a; Wu et al., 2008). Interestingly, the age-related decline of the neurogenic niche could be restored by extrinsic young signals. Using a mouse heterochronic parabiosis model, Katsimpardi et al. (2014) showed that (i) aged cerebral vasculature is remodeled, and (ii) SVZ neurogenesis is re-activated in response to young systemic factors. Furthermore, the authors revealed that GDF11, a circulating TGF-β family member, participate in this process (Katsimpardi et al., 2014). These examples emphasize the fact that the adult neurogenic niche communicates with the systemic circulation and highlight the role of blood-borne substances in the modulation of adult neurogenesis.

Interestingly, in addition to the well-recognized soluble signals originated both locally by the cellular components of the adult neurogenic niches (reviewed in "Cellular components of the neurogenic niche and their role in the neurogenic process" Section), or distantly (reviewed in "Distant regulation of the neurogenic niche: neurotransmitters, cerebrospinal fluid-derived factors, and blood-borne substances" Section), novel types of intercellular messengers, such as EVs, have emerged. EVs, and particularly exosomes, are considered one of the most complex ways of cell-to-cell communication, allowing the transfer of mRNAs, microRNAs (miRNAs), proteins and lipids between cells and thus, being able to modify the physiology of recipient cells. However, their precise role in adult neurogenic niches remains virtually unexplored.

EXOSOMES: UNIQUE MESSENGERS FOR CELL-TO-CELL COMMUNICATION

EVs, and particularly exosomes, are emerging as one of the major mediators of intercellular communication. It is known since the late 1960's/early 1970's that membrane-enclosed vesicles are present outside cells in different tissues and biological fluids (Colombo et al., 2014). Originally, these EVs were thought to be released only by outward budding or shedding of the plasma membrane (PM) but 10 years later, it was described that vesicles contained within the lumen of so-called multivesicular endosomes or multivesicular bodies (MVBs) could be secreted (Harding and Stahl, 1983; Pan and Johnstone, 1983). Thus, the

term "exosome" was introduced to identify those EVs that have been originated from the endosomal system (Johnstone et al., 1987) and since then, the field of exosome research has expanded exponentially. Now it is known that various types of EVs coexist in the extracellular milieu. Some of them are originated from shedding of the PM (microvesicles and apoptotic bodies) and others (exosomes) are secreted by exocytosis after fusion of MVBs with the PM. Thus, the various intracellular origins and modes of formation can probably lead to different compositions and functions of EVs (Kowal et al., 2014). Exosomes represent one sub-type of these EVs and a lot of progress has been made in the last few years to understand the basic mechanisms by which exosomes are formed and secreted, and their function in cell-cell communication under physiological and pathological conditions.

Biogenesis of Exosomes

Exosomes are small membrane vesicles, 30–100 nm in size, that are secreted by almost all cell types. They originate in the endocytic pathway, which is involved in the trafficking of several proteins that are internalized and can either recycle back to the PM or get sorted to degradation (Gould and Lippincott-Schwartz, 2009; Klumperman and Raposo, 2014) In these pathways, early endosomes mature into late endosomes (LE), and during this process, they suffer an inward budding process of their membrane and accumulate intraluminal vesicles (ILVs; **Figure 3A**). Because of their mature morphological features, LEs are referred to as MVBs. In most cells, the main fate of MVBs is to fuse with lysosomes, ensuring the degradation of their content. However, organelles with hallmarks of MVBs, can also fuse directly with the PM, releasing ILVs into the extracellular milieu (Raposo et al., 1996; Jaiswal et al., 2002; **Figure 3A**). To date is still unknown how different subpopulations of MVBs co-exist within cells and whether these different populations follow the same or different routes such as fusion with lysosomes for degradation or fusion with PM for exocytosis (Edgar et al., 2014). The best-described mechanism for the formation of MVBs and ILVs is driven by the endosomal sorting complexes required for transport (ESCRT) machinery. This machinery is composed of approximately thirty proteins that assemble into four complexes (ESCRT-0, -I, -II and -III) with associated proteins (VPS4, VTA1, ALIX also called PDCD6IP) conserved from yeast to mammals (Hanson and Cashikar, 2012). The ESCRT-0 complex recognizes and sequesters ubiquitinated transmembrane proteins in the endosomal membrane, segregates them into microdomains and binds the ESCRTI complex. ESCRTI, in turn, recruits ESCRTII subunits, and then both complexes initiate membrane deformation into buds with sorted cargo, allowing cytosolic proteins and RNAs (including mRNAs and miRNAs) to get into the forming vesicles. Following, the ESCRTII complex recruits ESCRTIII subunits inside nascent vesicles and together with the ATPase VPS4, provoke the membrane cleavage required to free ILVs while ubiquitin molecules and ESCRT subunits are released into the cytosol for their recycling (Hanson and Cashikar, 2012). Recently, alternative ESCRT-independent mechanisms have been proposed for the formation and sorting of specific cargo into exosomes, including a lipid-driven mechanism in which the synthesis of ceramide by a neutral sphingomyelinase is involved (Trajkovic et al., 2008) and a sphingosine 1-phosphate receptor-dependent mechanism (Kajimoto et al., 2013). Alternatively, ALIX, an ESCRTIII binding protein, by interacting with proteins containing late-domain motif LYPXnL, such as syntenin or the GPCR Par1, can be sorted into exosomes by a mechanism that requires only the recruitment of the ESCRTIII subunit CHMP4 (Sette et al., 2010; Baietti et al., 2012; Hurley and Odorizzi, 2012).

Content and Extracellular Fate of Exosomes

Considering the mechanisms involved in the biogenesis of ILVs, a complex mix of proteins, lipids and other cytosolic molecules is specifically sorted and incorporated into ILVs/exosomes.The content of exosomes is currently compiled in databases named Exocarta[1], Vesiclepedia[2] and EVpedia[3] which are continuously updated by the scientific community (Mathivanan et al., 2012). According to the current version of Exocarta, 9769 proteins, 1116 lipids, 3408 mRNAs, and 2838 miRNAs have been identified in exosomes from many different cell types and organisms. Much effort has been made to establish the proteomics of exosomes. Initially, it was found that exosomes contain proteins also present in endosomes, the PM, and the cytosol, but few components from other organelles such as nucleus, mitochondria or Golgi, confirming that they are not composed of a random set of proteins (Théry et al., 2001). The most frequently identified proteins in exosomes includes (i) proteins involved in MVB biogenesis; (ii) membrane associated proteins (lipid rafts proteins, GTPases and other membrane trafficking proteins); (iii) transmembrane proteins (targeting/adhesion molecules, tetraspanins (CD9, CD63, CD81), membrane fusion proteins); (iv) cytoskeletal proteins (actin, syntenin, moesin); (v) signal transduction proteins (annexin, 14–3–3 proteins); (vi) chaperones (Hspa8, Hsp90); and (vii) metabolic enzymes (GAPDH, LDHA, PGK1, aldolase, PKM), among others (Chaput and Théry, 2011; Mathivanan et al., 2012; **Figure 3B**). Interestingly, and even though there is an important overlap in terms of protein composition with other EVs, many of these proteins, such as tetraspanins, CD63 and CD81, are considered exosome markers.

In addition, exosomes contain a specific lipid composition (Kowal et al., 2014): they are enriched in ceramide, cholesterol, sphingomyelin, and phosphatidyl-serine. Interestingly, they are not enriched in lysobisphosphatidic acid, a lipid described in ILVs (Matsuo et al., 2004). As stated before, some of these lipids can be involved in the formation of ILVs or their release outside the cell. One very striking aspect of the content of exosomes is that they are highly enriched in small (no longer than 200 nucleotides) non-coding RNAs (ncRNAs). The most widely described exosome-associated ncRNAs are the miRNAs

[1]http://www.exocarta.org
[2]http://www.microvesicles.org/
[3]http://student4.postech.ac.kr/evpedia2_xe/xe/

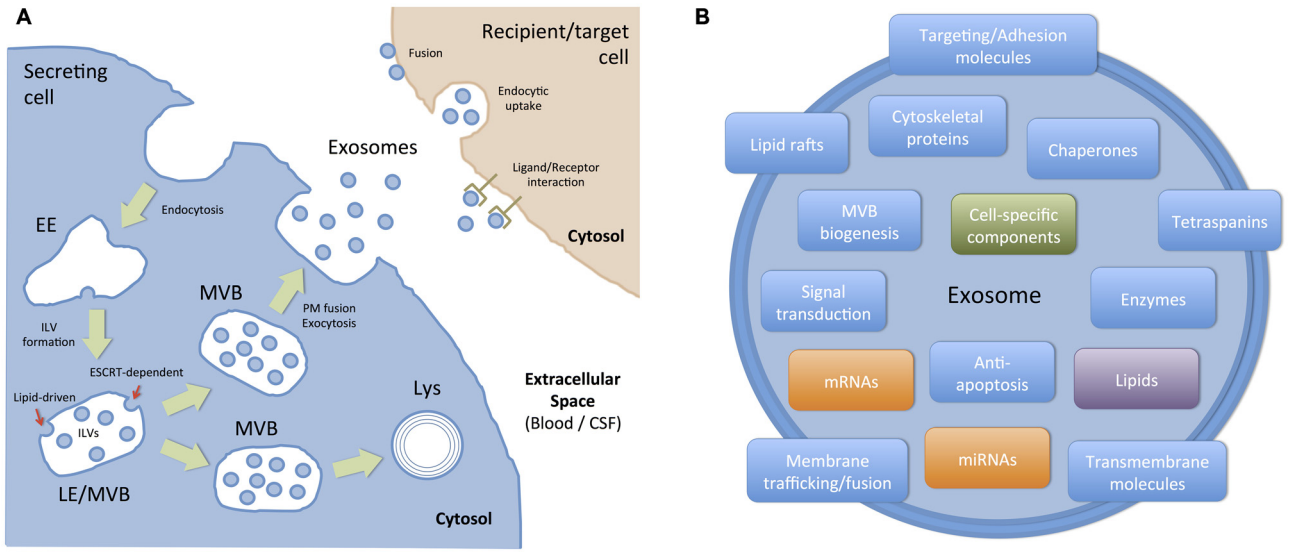

FIGURE 3 | Biogenesis, targeting and composition of exosomes. **(A)** Exosomes are presumed to be a relatively homogeneous population of vesicles originated in the endocytic pathway as intraluminal vesicles (ILVs) of late endosomes (LE) or multivesicular bodies (MVBs). Basically, ILVs are formed by the inward budding of the membrane of maturing early endosomes (EE) to MVBs. Cargo sorting into ILVs include: (i) ESCRT-dependent mechanisms, where the endosomal sorting complex required for transport (ESCRT) and other associated proteins are involved, and (ii) ESCRT-independent (lipid-driven) mechanisms, which depends on the presence of ceramide and neutral sphingomyelinase. Then, a population of MVBs is destined to degradation into lysosomes (Lys) and another population of MVBs fuse with the plasma membrane (PM), thus allowing ILVs to be exocytosed as exosomes. Once in the extracellular space, secreted exosomes can act as local signals (paracrine communication) or travel through biological fluids (e.g., blood, CSF) to reach body sites located distant from their cell/tissue of origin. Exosomes interact with recipient/target cells in several ways: (i) they can activate surface receptors (ligand/receptor interaction); (ii) they can transfer exosomal cargo to recipient cells by membrane fusion or by a connexin-dependent mechanism; and (iii) they can be endocytosed by a macropinocytic mechanism and then fuse with endosomal membranes to transfer their cargo. **(B)** Exosomes are small (30–100 nm) membrane bound vesicles with a complex and functionally relevant composition. They contain nucleic acids (mRNA and miRNA), and a vast array of different proteins and lipids depending on their host cell. However, they are generally enriched in proteins involved in MVB formation, tetraspanins, membrane trafficking and fusion, and a number of cytosolic proteins. In addition to these generic components, molecules associated with particular cell types and/or pathological situations have also been identified in exosomes.

of 20–22 nucleotides that target the 3′ untranslated region (UTR) of specific mRNAs to inhibit, in most cases, their translation. As a consequence of this, miRNAs can modify the phenotype and/or the physiology of the recipient cell, modulating cellular processes as relevant as proliferation, differentiation, and survival, among others (Cocucci and Meldolesi, 2015). The mechanisms that control the incorporation of the cargo into exosomes are currently under intense investigation (Villarroya-Beltri et al., 2014). Importantly, besides a "constitutive" array of proteins, lipids and RNAs, the content of exosomes varies according to the cellular source and to the physiological or pathological situation of the cell and its environment. Thereby, exosomes serve as interpreters of the cellular state where they came from. The role of exosomes as intercellular messengers has been studied extensively using cells of the immune system (Bobrie et al., 2011), where it has been demonstrated that they modulate antigen presentation and the immune response (Théry et al., 2009). Similarly, the role of exosomes in several cancer types has been well documented. For instance, glioblastoma-derived exosomes deliver genetic information to recipient endothelial cells, promoting tumor growth and invasion (Skog et al., 2008).

Once secreted, exosomes can interact with target cells to modify their function (Mittelbrunn and Sánchez-Madrid, 2012) and thus, they can act as local or paracrine messengers or communicators. Also, they can reach biological fluids, such as blood, CSF, urine, etc., and act as distant messengers (**Figure 3A**). The targeting of exosomes to their recipient cells involves different mechanisms (Colombo et al., 2014; Mulcahy et al., 2014). In some cases, ligand/receptor interaction of EVs to target cells might be sufficient to activate intracellular pathways and induce physiological changes in target cells. In other cases, for instance when miRNAs contained in exosomes induce gene silencing in the recipient cell, the content of EVs must be transferred inside that cell. In those cases, fusion of exosomes with the PM or endocytosis followed by fusion of exosomes with the membrane of endocytic compartments must take place (**Figure 3A**). Microenvironmental pH appears to be relevant for the fusion of exosomes with endosomal membranes (Parolini et al., 2009). Alternatively, a novel connexin 43-mediated mechanism to transfer information between exosomes and recipient cells has recently been described (Soares et al., 2015). According to this paper, the content of exosomes can be transferred to recipient cells by a gap junction-like communication, thus opening new possibilities about the mechanisms that might operate in specific environments or situations.

CAN EXOSOMES SERVE AS (PHYSIOLOGICAL OR PATHOLOGICAL) MESSENGERS BETWEEN CELLULAR COMPONENTS OF THE ADULT NEUROGENIC NICHE?

An efficient and well-regulated communication between cells is vital to ensure brain homeostasis and plasticity throughout life, particularly in the adult neurogenic niches. Thus, information transfer through exosomes (and other EVs) appears as a unique mechanism compared with other forms of intercellular communication. Even though the physiological and/or pathological role of locally- or distantly-generated exosomes in the adult neurogenic niches is currently virtually unknown, growing indirect evidence strongly suggest that exosomes might be one the major elements communicating and coordinating the function of the adult neurogenic niches. First, neural stem/progenitor cells and most of the cell types present in the CNS, including those cells that constitute and regulate the neurogenic niche, secrete and/or are target of exosomes and other EVs (**Table 1**). Furthermore, some of the biomolecules expressed (and sercreted) by niche cells have been reported to be present in exosomes under physiological or pathological conditions (**Table 1**). Interestingly, several examples highlight the role of exosomes as (i) messengers between neural cells (neurons and glial cells) either locally or distantly (via CSF or volume transmission); (ii) blood-CNS communicators (including their potential as therapeutic vehicles); and (iii) modulators of several stem cell niches.

Exosomes in Neuron-Neuron Communication

Classical inter-neuronal communication involves synaptic transmission, a dynamic and plastic process that is tightly regulated by neuronal activity (Regehr et al., 2009). Exosome-mediated communication between pre and postsynaptic cells participates in synaptic plasticity, as it has been shown in the *Drosophila* neuromuscular junction (Korkut et al., 2013). Using cultures of mixed hippocampal cells with exosomes derived from the neuroblastoma cell line N2a and labeled with GFP-CD63 and GFP-TTC, it was found that they interact either with neurons, astrocytes or oligodendrocytes. On the other hand, exosomes released by cortical neurons upon synaptic activation interact with neurons but not with GFAP+ astrocytes. Furthermore, some exosomes co-localize with synaptophysin indicating that they bind to pre-synaptic sites (Chivet et al., 2014).

Exosomes in Neuron-Glia Communication

The communication between neurons and glia is important for brain physiology during both development and adulthood. The different glial cell types help to maintain neuronal activity. Oligodendrocytes protect axons with the myelin sheath and also provide trophic support to neurons (Nave and Trapp, 2008). To maintain these functions over time there is a constant communication between neurons and oligodendrocytes, but the mechanisms underlying this phenomenon are not well understood. Frühbeis et al. demonstrated that upon glutamate stimulation, oligodendrocytes secrete exosomes, which are endocytosed by neurons. Furthermore, exosomal cargoes improve neuronal metabolism and viability in situations of nutrient deprivation or oxidative stress exposure (Frühbeis et al., 2013b). It is also noteworthy that this work demonstrated that the internalization of exosomes by neurons occurs through a clathrin and dynamin-dependent mechanism, shedding light on the mechanisms that may be involved in exosome internalization. On the other hand, selective elimination of synaptic connections comprises the engulfment of neurites. In a recent study, it was shown that neuron-derived exosomes stimulate microglial phagocytosis of neurites via upregulation of complement factors (Bahrini et al., 2015).

Exosomes in Glia-Glia Communication

The communication between glial cells through exosomes has been studied to a lesser extent. Exosomes secreted by oligodendrocytes are selectively internalized through macropinocytosis by microglia, both *in vitro* and *in vivo* (Fitzner et al., 2011). Remarkably, only those microglial cells that do not show antigen-presenting capacity endocytose exosomes, thus supporting the idea that different types of microglial cells co-exist and are differentially involved in immune functions.

Exosomes in the CSF as Volume Transmission Vehicles

It has been proposed that the CSF compartment plays an essential role in volume transmission within the CNS; thus, molecules or messengers secreted in one brain region may reach the CSF and exert their function in sites located far from its secretion site (Agnati and Fuxe, 2014; Borroto-Escuela et al., 2015). Given the close contact between the CSF and the interstitial fluid of several brain areas, including the SVZ, it is conceivable that exosomes originated in the brain parenchyma can be found in the CSF and *vice versa*. Actually, isolation of membrane vesicle-enriched fractions and further proteomic studies have demonstrated the presence of exosomes in the human CSF (Street et al., 2012; Grapp et al., 2013; Chiasserini et al., 2014). Furthermore, the exosome content of the CSF is supposed to reflect ongoing brain processes, and especially those related to plasticity, disease or repair. Proteins related to the onset or progression of some CNS diseases such as APP (Alzheimer's disease), PrPsc (prion disease), and α-synuclein (Parkinson's disease), among others, have been found in the exosomal fraction of CSF-samples (Pegtel et al., 2014). Exosomes in the CSF decrease with age while those derived from the embryonic CSF positively act on the stem cell niche (Street et al., 2012; Feliciano et al., 2014), revealing their influence on recipient cells. A clear demonstration of exosomal secretion into the CSF has been recently obtained in ChP epithelial cells. Using cell-culture assays, human CSF analyses and *in vivo* tracing experiments, the authors describe a novel pathway of exosome-mediated folate delivery into the CSF and subsequently, into the brain parenchyma (Grapp et al., 2013).

TABLE 1 | Niche cells (and other CNS cells) secrete exosomes with potential physiological and pathological functions.

Niche cells	Examples of biomolecules expressed by niche cells and found in exosomes (*)	Physiological function	Pathological function	Reference
NSCs/NPCs	T-cell immunoglobulin mucin protein 4 (TIM-4) Interferon gamma (IFN-γ)/IFN-γ receptor 1 (Ifngr1) Heat shock protein 70 KDa (Hsp70) VCAM1 **Connexin 43 (Cx43)** **VEGF** **miR-let7b** **miR-9**	Regulate NSC proliferation and differentiation (miR-let7b and miR-9) (Zhao et al., 2009, 2010) Favor exosome targeting and transfer of information to acceptor cells (Cx43) (Soares et al., 2015) Regulate the SGZ neurogenic niche (VEGF) (Kirby et al., 2015) Maintain protein homeostasis and regulates cell survival (Hsp70) (Takeuchi et al., 2015) Modulate gene expression in target cells via Stat1 activation (IFN-γ) (Cossetti et al., 2014)	Mediate viral entry (TIM-4) (Sims et al., 2014) Modulates immune response (Cossetti et al., 2014) Autoimmunity (Kang et al., 2008) Neurophatological development of NSCs/NPCs (Feliciano et al., 2014)	Marzesco et al. (2005) Huttner et al. (2008) Kang et al. (2008) Kunze et al. (2009) Akerblom and Jakobsson (2013) Bian et al. (2013) Drago et al. (2013)
Neurons	p75 (neurotrophin receptor) Nedd4 family proteins and Nedd4 family-interacting protein 1 (Ndfip1) miR-124a Cystantin C L1 cell adhesion molecule (L1-CAM) GPI-anchored prion protein GluR2/3 (glutamate receptors subunits) Hsp70 α-synuclein PrPsc APP **miR-34a**	Regulate neuronal physiology: neurite outgrowth, cell death/survival balance (p75). Escudero et al. (2014) Neuroprotection (e.g., removal of proteins during stress; Nedd4) (Putz et al., 2008; Low et al., 2015) Regulate translation of glutamate transporter GLT1 in astrocytes (Morel et al., 2013) and neuronal fate in SVZ NSCs (miR-124a) Cheng et al. (2009) and Akerblom et al. (2012) Regulate proliferation and differentiation of adult NSCs (Cystatin C) (Ghidoni et al., 2011) Regulatory function at synapses (Faure et al., 2006) Stimulate microglial phagocytosis (synaptic pruning) (Bahrini et al., 2015) Regulate NSCs differentiation, neuroblasts migration and neuron maturation (miR-34a) (Mollinari et al., 2015) Volume transmission (Agnati and Fuxe, 2014)	Vehicles for the transfer of toxic proteins (PrPsc: Prion disease; APP: Alzheimer's disease; superoxide dismutase: amyotrophic lateral sclerosis (ALS); alpha-synuclein: Parkinson's disease) Fevrier et al. (2004), Alvarez-Erviti et al. (2011b) and Bellingham et al. (2012b) miRNAs can stimulate inflammatory response (activating Toll-like receptors) in stroke, ALS and other neurodegenerative diseases (Paschon et al., 2015) miR-124 dysregulation is associated to several CNS disorders Sun et al. (2015) Cystatin C dysregulation is associated to Alzheimer's disease Ghidoni et al. (2011)	Smalheiser (2007) Lachenal et al. (2011) Von Bartheld and Altick (2011) Smythies and Edelstein (2013) Chivet et al. (2012, 2013, 2014) Ryan et al. (2015)
Astrocytes	Prostate Apoptosis Response 4 (PAR-4) Ceramide Synapsin I FGF-2 VEGF PEDF Hsp70	Promote neurite outgrowth and neuronal survival (synapsin I) (Wang et al., 2011) Induce glial cell proliferation (miR-125b) (Pogue et al., 2010) Favor exosome targeting and transfer of information to acceptor cells (Cx43) (Soares et al., 2015)	Amyloid-induced exosomes are enriched in ceramide and PAR-4. Apoptosis induction in Alzheimer's disease (Wang et al., 2012) Astrogliosis. Alzheimer's disease, Down's syndrome (miR-125b) (Pogue et al., 2010)	Taylor et al. (2007) Guescini et al. (2010) Frühbeis et al. (2012) Hajrasouliha et al. (2013) Agnati and Fuxe (2014)

(Continued)

TABLE 1 | (Continued).

Niche cells	Examples of biomolecules expressed by niche cells and found in exosomes (*)	Physiological function	Pathological function	Reference
	IGFBP6 **miR-125b** **Cx43**	Angiogenesis. Proliferation and differentiation of varios cell types (FGF-2, VEGF) (Proia et al., 2008) Regulate stemness of NSCs (PEDF) (Ramírez-Castillejo et al., 2006) Volume transmission (Agnati and Fuxe, 2014)		
Oligodendrocytes (**)	Superoxide dismutase, catalase Myelin proteins (CNP, MBP, MOG, PLP) Lipids (galactocerebroside, sulfatide, cholesterol)	Enhance neuronal stress tolerance, promote neuronal survival during oxygen/glucose deprivation, and regulate neuronal physiology (increase firing rate, modulate gene expression and signal transduction pathways) (Frühbeis et al., 2013a; Fröhlich et al., 2014) Trophic support for axons (Krämer-Albers et al., 2007) Oligodendrocyte-microglia communication (Fitzner et al., 2011)	Neuroinflammation (Gupta and Pulliam, 2014) Autoantigen in multiple sclerosis (PLP) (Krämer-Albers et al., 2007)	Hsu et al. (2010) Lopez-Verrilli and Court (2013) Peferoen et al. (2014)
Microglia	Surface-bound aminopeptidase N (CD13) Monocarboxylate (lactate) transporter 1 (MCT1) Metabolic enzymes (Gliceraldehyde-3-phosphate dehydrogenase) **miR-155** **Growth Factors (FGF2, IGF1, BDNF)**	Neuropeptide (enkephalins) catabolism (CD13) (Potolicchio et al., 2005) Supportive, neuroprotective role (Hooper et al., 2012; Prada et al., 2013) Exosome release is modulated by serotonin and Wnt3a (Hooper et al., 2012; Glebov et al., 2015) Modulate proliferation and differentiation of NSCs /NPCs (growth factors) (Grote and Hannan, 2007; Ma et al., 2009)	Propagation of inflammation signals. Neurodegenerative diseases (Prada et al., 2013) Inflammation-induced hippocampal neurogenic deficits (Woodbury et al., 2015)	Bianco et al. (2005) Potolicchio et al. (2005) Bianco et al. (2009) Tamboli et al. (2010) Turola et al. (2012) Su et al. (2014) Gomez-Nicola and Perry (2015)
Endothelial Cells	Delta-like 4 (Dll4), (membrane-bound Notch ligand). miR-126 miR-214 miR-296 (angiomirs) **Growth Factors (VEGF, PEDF)**	Induce angiogenesis (miR-126, miR-214 and Dll4) (Sheldon et al., 2010; van Balkom et al., 2013; Sharghi-Namini et al., 2014; van Balkom et al., 2015) Increase levels of pro-angiogenic receptors (miR-296) (Würdinger et al., 2008) Modulate proliferation and differentiation of NSCs/NPCs (growth factors) (Grote and Hannan, 2007)	Cellular stress changes RNA and protein composition of endothelial cell-derived exosomes. Transfer of stress signals (hypoxia, inflammation, hyperglycemia) to target cells (de Jong et al., 2012) Inflammation-induced EVs (exosomes?) induce changes in protein expression pattern of cerebrovascular pericytes (Yamamoto et al., 2015)	Shen et al. (2004) Simak et al. (2006) Tavazoie et al. (2008) Jung et al. (2009) Haqqani et al. (2013) Crouch et al. (2015) van Balkom et al. (2015)

(*) In bold are presented those biomolecules expressed by the referred cell type and found in exosomes of other cell types. (**) Oligodendrocytes are not typically considered niche cells but are included here as another example of CNS cell-to-cell communication via exosomes.

Exosomes in the Blood to CNS Communication and as Therapeutic Vehicles

Exosomes constitute one of the most attractive vehicles to communicate peripheral organs with the CNS and *vice versa*. The fact that blood-circulating exosomes may reach and be incorporated into different organs has stimulated scientists from diverse disciplines to explore the use of exosomes as therapeutic vehicles able to deliver specific drugs (Suntres et al., 2013). This widespread interest includes the use of exosomes as therapeutic agents in cancer (Pitt et al., 2014; Greening et al., 2015; Tran et al., 2015), and in infectious and allergic diseases (Admyre et al., 2008; Prado et al., 2008; Hosseini et al., 2013), to give just a few examples. Exosomes are considered attractive vehicles of blood to CNS communication due to (i) their stability (their cargo is protected from RNAses and proteases); (ii) their lack of immunogenicity (when derived from the same patient); (iii) the possibility of adding surface proteins or antibodies to target specific cell types; (iv) the possibility of loading a specific molecular cargo with therapeutic actions; and (v) importantly, their capacity to cross the BBB (Aryani and Denecke, 2014; Ridder et al., 2014; György et al., 2015; Kawikova and Askenase, 2015). siRNAs, for example, have been successfully targeted to specific brain regions in mice following systemic injection of siRNA-electroporated exosomes (Alvarez-Erviti et al., 2011c; El-Andaloussi et al., 2012). This strategy has been reported to be effective as a way to decrease α-synuclein aggregates in wild type as well as in transgenic mice expressing the phospho-mimic S129D α-synuclein, which is prone to aggregation (Cooper et al., 2014). Exosomes have also been used to deliver curcumin and JSI124 (activator of transcription 3 inhibitor) to brain microglia of mice via an intranasal route, protecting from inflammation and delaying tumor growth (Zhuang et al., 2011). These examples emphasize the potential of exosomes, not only as therapeutic vehicles, but also as physiological and pathological messengers between peripheral organs and the CNS. Even though the mechanisms by which peripheral exosomes have access to the brain tissue are unknown, the fact that adult NSCs reside in vascular niches, leads to the interesting proposal that blood-borne exosomes may influence adult neurogenic niches.

Exosomes as Modulators of Diverse Stem Cell Niches

Cancer stem cells (CSCs) or cancer initiating cells (CICs) are tumor cells that have properties of self-renewal, clonal tumor initiation, and metastatic potential (Zhang et al., 2015b). As other stem cells, CSCs reside in distinct regions within the tumor called niches, which protect CSCs from immune responses and preserve their phenotypic plasticity (Plaks et al., 2015). Furthermore, the niche is believed to play a pivotal role in the resistance of CSCs to some cancer therapies (Kuhlmann et al., 2015; Plaks et al., 2015). It is known that exosomes released locally from tumor cells are able to modify the niche, promoting angiogenesis and tumor cell proliferation (Tickner et al., 2014). In addition, cancer-derived exosomes can travel to sites located outside the tumor to induce cellular changes associated with the promotion of a "pre-metastatic" niche, a special microenvironment that is able to receive and harbor cancer cells, thus favoring metastasis. This has been demonstrated in the case of melanoma metastasis in the bone marrow (Peinado et al., 2012) and liver metastasis of pancreatic ductal adenocarcinoma (Costa-Silva et al., 2015). Even though the mechanisms by which exosomes modulate primary tumor and pre-metastatic niches are not fully understood, it has been shown that isolated exosomes from body fluids of cancer patients (blood, ascites fluid and urine, CSF) contain several growth factors and cytokines able to modulate the environment of the metastatic niche (Ung et al., 2014).

On the other hand, non-tumoral mesenchymal stem cells (MSCs) are extensively used in different cell therapy-based clinical trials today. It is known that functional improvement with MSCs therapies is not mainly due to cell engraftment or differentiation at the site of injury but they exert their effects through their secreted products, including exosomes (Lai et al., 2015). Exosomes harvested from the conditioned media of MSCs cultures increase angiogenesis and neurogenesis, and promote functional recovery in animal models of stroke and traumatic brain injury (Xin et al., 2013a,b; Zhang et al., 2015c). Thus, in therapies for brain disorders, it has been proposed that exosomes derived from MSCs function as an extension of MSCs, and like their cell-source, exosomes can target biological processes that stimulate functional repair of the damaged nervous system (Lai et al., 2015). However, it is thought that similar exosomes isolated from MSCs of different origins (i.e., bone marrow, menstrual, chorion or umbilical cord) might differentially affect target cells, possibly due to a differential molecular cargo (Lopez-Verrilli and Court, 2013). In this context, albeit a direct participation of exosomes in the adult neurogenic niches has not yet been addressed, it is conceivable that exosomes derived from the different cell types of the neurogenic niche as well as the heterogeneous exosome mixture that may reach the niche via CSF-volume transmission or through the vasculature, affect the neurogenic process in a differential and highly specific manner. Given the relevance of these actions to a large array of diseases of the nervous system, the cellular and molecular mechanisms involved in the regulatory functions of exosomes in the neurogenic niche are an attractive field for future investigations.

MOLECULAR COMPONENTS OF EXOSOMES WITH A POTENTIAL ROLE IN THE REGULATION OF THE NEUROGENIC NICHE

MicroRNAs

Several steps of adult neurogenesis are mediated or regulated by miRNAs. For example, miR-let7b and miR-9 regulate proliferation and differentiation of NSCs (Zhao et al., 2009, 2010). Similarly, miR-34a regulates NSCs differentiation, neuroblast migration and neuron maturation (Mollinari et al., 2015). miR-124a is a key determinant of neuronal fate of SVZ NSCs by targeting Sox9 (Cheng et al., 2009; Akerblom et al., 2012) or by targeting the JAG-Notch signaling pathway

(Liu et al., 2011). On the other hand, miR-128 overexpression reduces the levels of doublecortin (Dcx) in differentiating NPCs, indicating that miR-128 can target and potentially take part in the regulation of Dcx levels in adult neurogenesis (Cernilogar et al., 2015). Furthermore, it has been recently shown that miR-124, miR-128 and miR-137, can act cooperatively and synergistically to promote neuronal differentiation of NSCs by targeting overlapping gene sets containing a highly interconnected transcription factor network (Santos et al., 2015). miRNAs are also involved in glial cell proliferation/differentiation and angiogenesis, phenomena closely related and associated with adult neurogenesis. For example, miR-125b is involved in glial cell proliferation under physiological and pathological conditions (Pogue et al., 2010). On the other hand, miR-126 and miR-214 induce angiogenesis (Sheldon et al., 2010; van Balkom et al., 2013, 2015; Sharghi-Namini et al., 2014), while miR-296 increase levels of pro-angiogenic receptors (Würdinger et al., 2008). Several CNS disorders associated with defective neurogenesis has been linked to miRNAs function. For example, dysregulation of miR-124 is associated with neurodegenerative and stress-related disorders (Sun et al., 2015). Similarly, miR-155 is essential for inflammation-induced hippocampal neurogenic dysfunction via microglial activation (Woodbury et al., 2015).

Despite their relevant and determinant role on the neurogenic process, the precise target cells and mechanisms by which miRNAs are transferred to target cells in the neurogenic niche are virtually unknown. However, all the miRNAs mentioned above have been found in exosomes from different cellular origins (**Table 1**). Furthermore, most of them are expressed by different niche cells; thus, it is conceivable that some of these miRNA are transported within exosomes to exert their function on NSCs/NPCs and regulate the neurogenic process. In addition, a recent systemic characterization of exosomal RNA profiles in human plasma samples by RNA sequencing analyses showed that miRNAs were the most abundant (Huang et al., 2013). Interestingly, the same study showed that five (miR-128, miR-124, miR-125b, miR-9, and miR-let7b) out of the twenty most abundant exosomal miRNAs are involved in the neurogenic/angiogenic process. Thus, these results highlight not only the fact the neurogenesis-modulating miRNAs are incorporated into exosomes, but also stress the possibility of exosome-mediated communication between the systemic circulation and the CNS.

Proteins and Signaling Peptides

Some of the biomolecules that have already been functionally characterized as modulators of adult neurogenesis, have also been described as components of exosomes. For example, TGF-β, a negative regulator of the adult neurogenic niche, has been documented to be present in exosomes of diverse cellular origins. As such, TGF-β-carrying exosomes circulate in the blood stream under diverse pathological conditions ranging from renal to pregnancy-related diseases and in consequence, might indirectly affect neurogenesis if crossing the BBB (Szajnik et al., 2013; Hong et al., 2014; Tan et al., 2014; Torreggiani et al., 2014; Raimondo et al., 2015; Solé et al., 2015). In addition to the protein, the mRNA coding for TGF-β has been found in exosomes from glioblastoma multiforme patients (Muller et al., 2015). On the other hand, positive regulators of adult neurogenesis such as Ephrin-B2 or components able to activate EGFR signaling have also been found in exosomes (Mathivanan et al., 2010; Higginbotham et al., 2011). Similarly, VEGF has been found in exosomes derived from several cell types (Thompson et al., 2013; Ekström et al., 2014; Torreggiani et al., 2014). Interestingly, it has been recently shown that adult hippocampal NSCs/NPCs secrete large amounts of VEGF *in vitro* and *in vivo* and this self-derived VEGF is functionally relevant for maintaining the neurogenic niche (Kirby et al., 2015). Several other signaling peptides known to determine cell fate in the neurogenic niche and described in the present work, such as PEDF, IGFBP6, EGF, FGF-2, Hedgehog, Notch, as well as proteins of the Wnt signaling pathway, just to mention a few, have been found in the exosomal fractions from different cell types and conditions (Graner et al., 2009; Nazarenko et al., 2010; Lai et al., 2011; Hajrasouliha et al., 2013; Wendler et al., 2013). Remarkably, most of the studies describing the presence of these proteins in exosomes correspond to proteomic analyses. In this regard, several intriguing issues remain to be addressed. The mechanisms involved in the delivery of several growth factors and signaling peptides to MVBs and their incorporation into ILVs are still speculative. Moreover, the molecular machinery responsible of transferring exosomal cargo into target cells are unknown. Further studies conducted to elucidate these molecular mechanisms will give clues about how adult neurogenesis is regulated not only under physiological conditions but also under certain CNS disorders.

CNS DISORDERS ASSOCIATED TO IMPAIRED ADULT NEUROGENESIS: POTENTIAL ROLE OF EXOSOMES IN THEIR PATHOGENESIS AND AS BIOMARKERS

Several neurodegenerative disorders have been associated to defects in the adult neurogenic process in the DG and/or subventricular zone/OB system (Steiner et al., 2006; Shruster et al., 2010; Mu and Gage, 2011; Winner et al., 2011; Regensburger et al., 2014; Foltynie, 2015; He and Nakayama, 2015; Le Grand et al., 2015; Winner and Winkler, 2015). On the other hand, significant progress has been done to unravel the function and regulation of adult neurogenesis in psychiatric diseases (Eisch et al., 2008; Jun et al., 2012). Depression is associated to a reduction in SGZ neural progenitor proliferation in the DG (Gould et al., 1998; Jacobs et al., 2000). Similarly, experimentally-induced inhibition of neurogenesis favors depressive-like behaviors in animal models (Wang et al., 2015; Xiang et al., 2015). On the other hand, different therapies that relieve depressive symptoms, such as antidepressant drug treatments (Malberg et al., 2000; Santarelli et al., 2003) or physical exercise (van Praag et al., 1999a; Lugert et al., 2010), increase SGZ neurogenesis. However, the mechanisms that stimulate adult hippocampal neurogenesis

in those treatments are currently not clear (Jun et al., 2012).

Even though we are yet to obtain a conclusive causal relationship between adult neurogenesis and neurodegenerative or psychiatric diseases, growing evidence reveals how these disorders may proceed through impairment in the regulation of adult neurogenic niches. Thereby, despite their particular genetic or environmental origin, defects in adult neurogenesis appears as common hallmark functionally associated to the pathogenesis of different CNS diseases. Recent studies emphasize a putative role of exosomes in the pathogenesis of neurodegenerative and psychiatric disorders. Furthermore, considering that (i) exosomes may condition the microenvironment of a particular region such as the neurogenic niche; (ii) the content of exosomes in a particular cell type may change in different physiological and pathological conditions; and (iii) that brain parenchyma-derived exosomes may have access to the CSF and the peripheral blood circulation, neurogenic niche-derived exosomes may represent not only powerful regulators of adult neurogenesis, but also attractive therapeutic targets and useful biomarkers for different CNS disorders.

Exosomes in Neurodegenerative Disorders

In neurodegenerative disorders such as Alzheimer's disease (Rajendran et al., 2006; Saman et al., 2012), Parkinson's disease (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011a,b) and Prion diseases (Fevrier et al., 2004), the content of pathological forms of the toxic proteins associated to exosomes is increased and as such, it has been proposed that exosomes may favor the amplification and spread of protein misfolding diseases (Bellingham et al., 2012b; Danzer et al., 2012; Grad et al., 2014). In fact, when the production of exosomes was inhibited in a mouse model of Alzheimer's disease by decreasing the synthesis of ceramide, a lipid enriched in exosomes, total amyloid as well as plaque levels were reduced (Dinkins et al., 2014). In brain cells, the abnormal protein aggregates present in neurodegenerative disorders activate the proteasome and autophagy pathways, tending to restore proteostasis (protein homeostasis; Hetz and Mollereau, 2014). A possible explanation of exosomal loading and secretion of cytotoxic proteins is that the overload of misfolded proteins may saturate the mentioned cytoprotective pathways, leading to their elimination via exosomes, although the mechanistic links of these processes are yet unknown (Baixauli et al., 2014). Interestingly, exosomes might also act by sequestering toxic proteins (Kalani et al., 2014). In Alzheimer's Disease, in which exosomes are loaded with amyloid-β (Aβ) peptides and with molecules involved in its synthesis, degradation and aggregation, exogenous exosomes expressing surface proteins such as the cellular prion protein (PrPc), a receptor for Aβ, can sequester Aβ and counteract its negative effects on plasticity (An et al., 2013).

Exosomes in Psychiatric Disorders

Almost nothing is known about changes in exosome content or release and their action on recipient cells in psychiatric disorders, such as schizophrenia, major depressive disorder, bipolar disorder or anxiety disorders, among others (Tsilioni et al., 2014). Interestingly, defects in the adult neurogenic process have been associated to the pathogenesis and/or progression of most of these disorders. In schizophrenia and bipolar disorders, specific exosome-related miRNAs (miR-497 and miR-29c, respectively) were upregulated in the prefrontal cortex of post-mortem brains (Banigan et al., 2013). We have shown that treatment with the antidepressant drug fluoxetine upregulates the content of the forebrain astrocyte-derived enzyme Aldolase C in CSF exosomes. The content of this enzyme is further upregulated in exosomes after chronic restraint stress, but not after stress induced by complete immobilization (Sandoval et al., 2013; Ampuero et al., 2015). A better knowledge of molecular changes associated to brain-derived exosomes in psychiatric disorders will allow a better comprehension of the neurobiological features of these complex diseases and in turn, would lead to the proposition of new treatment strategies.

Exosomes as Potential Biomarkers of CNS Disorders

One of the yet unmet goals in CNS diseases, especially in the psychiatric sphere, is the establishment of biological markers, especially those obtained by non-invasive strategies, which could be used as diagnostic tools or to monitor disease progression, treatment effects and prognosis. Thus, the miRNA and protein cargo of exosomes obtained from peripheral body fluids, such as plasma or urine, constitute remarkable candidates as biomarkers (Cheng et al., 2014; Zhang et al., 2015a). With the exception of an intracranial cancer type, glioblastoma multiforme (Skog et al., 2008; Shao et al., 2012), it has not yet been possible to show in a convincing way that exosomes produced by neurons or glial cells might reach the blood circulation. Serum exosomes from glioblastoma multiforme patients carry tumor specific epidermal growth factor receptor vIII (EGFRvIII), and transforming growth factor beta 1 (TGF-β1; Skog et al., 2008; Graner et al., 2009). The proposed pathways are a defective BBB (Sáenz-Cuesta et al., 2014), a CSF-blood pathway or a transport process by transcytosis across endothelial cells in an intact BBB, as it has recently been suggested (Haqqani et al., 2013).

The fact that neurodegenerative diseases are associated with increases in plasma exosomes containing misfolded, pathological forms of proteins strongly suggest a central origin of them. Indeed, exosomes can carry proteins that serve as common biomarkers of a disease. A neuroblastoma cell line (SH-SY5Y) expressing α-synuclein release exosomes that contain this protein and are capable of transporting α-synuclein to SH-SY5Y cells that do not express it (Alvarez-Erviti et al., 2011a). *In vivo* experiments have shown that a small proportion of radioactively labeled α-synuclein delivered to the CSF can be recovered in plasma exosomes (Shi et al., 2014) pointing to a central origin of the protein, although its cellular origin remains obscure. Neurons infected with prion protein in its cellular (PrP^{c}) as well as with its pathogenic form (PrP^{sc})

release exosomes containing prion proteins as well as a specific miRNA signature (Fevrier et al., 2004; Vella et al., 2007; Bellingham et al., 2012a). In intracerebrally infected animals, exosomes carrying PrP^{sc}, accounting for about 20% of the plasma infectivity, could be harvested from plasma samples (Properzi et al., 2015). In this study, PrP^{sc} was identified by Western blots with the caution of using sucrose gradient-purified exosomes to avoid the presence of blood-derived immunoglobulin contaminations that would obscure the usefulness of protein exosome markers in the molecular weight range of the IgG and IgM light and heavy chains (Properzi et al., 2015).

To identify EVs of neuronal origin, affinity purification using antibodies against neuronal membrane proteins, such as neural cell adhesion molecule-1 (NCAM-1) or neural cell adhesion molecule L1 (L1CAM), have been used. With this experimental strategy, the group of Kapogiannis has been able to detect plasma or serum exosomes with increased content of Aβ1–42, and of phosphorylated forms of tau and type 1 insulin receptor substrate (IRS-1) specifically associated to patients suffering Alzheimer's Disease. Interestingly, these findings are proposed as useful predictive tools up to 10 years before the clinical onset of the disease (Fiandaca et al., 2015; Kapogiannis et al., 2015). In summary, the molecular content, i.e., miRNAs or proteins, in exosomes are emerging as strong candidates for providing CNS disease-specific biomarkers. The consistency of the results depends on the isolation method and thus, the purity of the fraction under analysis (exosomes vs. microparticles or microvesicles), or contamination with extravesicular blood-borne molecules while their origin in neuronal or glial cells is awaiting an indisputable proof. Furthermore, despite their purity, there are other shortcomings that need be to be addressed when attempting to use exosomes as biomarkers: (i) the pattern of exosomal RNA may change according to the extraction methods, due to differential susceptibility to exosomal lysis according to their membrane composition (Eldh et al., 2012; Van Aelst and Heymans, 2013); (ii) problems when trying to replicate previously published microarray analysis have been reported, mainly due to journals not enforcing more strict guidelines to interpret and report microarray data (Shields, 2006; Ioannidis et al., 2009); (iii) a further source of confusion is the possibility that medications taken by patients may alter the composition of exosomes, thus adding another level of complexity in the analysis and interpretation of the data (Aryani and Denecke, 2014).

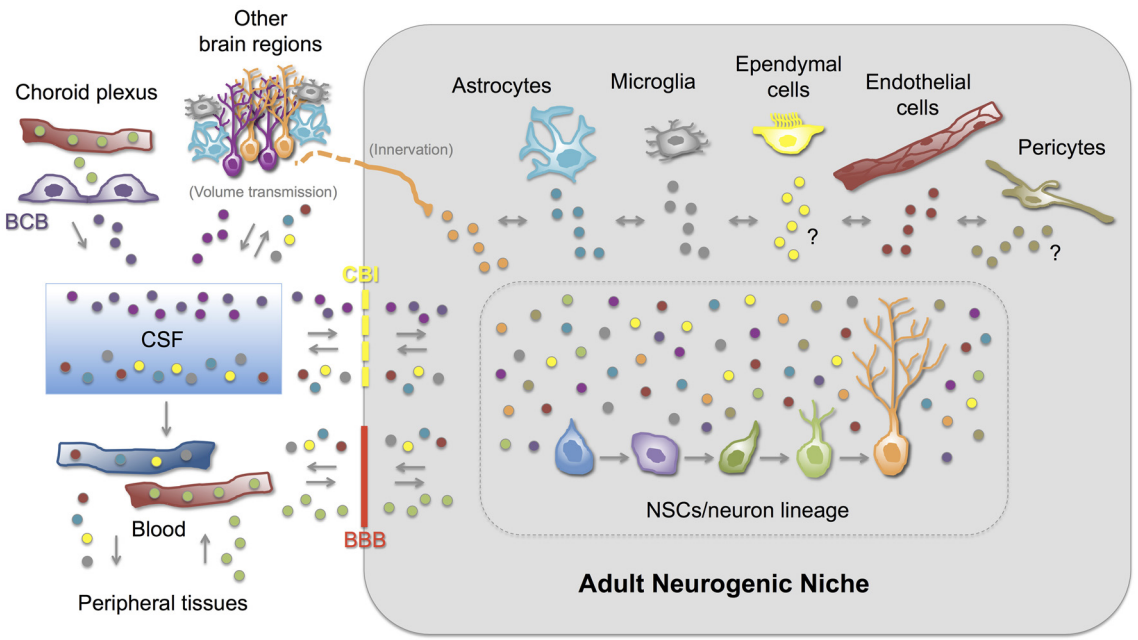

FIGURE 4 | Exosomes as regulators of adult neurogenesis. The NSC-neuron lineage is exposed to a complex mix of exosomes within the neurogenic niche. Exosomes secreted locally by different niche cells can influence the physiology of other niche cells and the progression of different neurogenic stages. It is well demonstrated that neurons, astrocytes, microglia and endothelial cells secrete exosomes. NSCs/NPCs are also able to secrete exosomes (not depicted). Exosomes release by ependymal cells and pericytes has not been reported up to now (?). Additionally, exosomes originated in cells located far from the neurogenic niches can influence its nature. Exosomes derived from cells located in other brain regions can reach neurogenic niches through innervation of the niche or via CSF-mediated volume transmission. Indeed, besides being a source of soluble molecules for the SVZ neurogenic niche, the presence of exosomes in the CSF has been demonstrated in several mammalian species including humans. Furthermore, choroid plexus epithelial cells secrete exosomes into the CSF. Interestingly, exosomes produced in cells and tissues outside the central nervous system (CNS; peripheral tissues) can potentially reach neurogenic niches either directly, through the vasculature (blood) of the niche, or indirectly, through the choroid plexus. Conversely, exosomes originated in the neurogenic niche might have access to the CSF and to the peripheral blood circulation. The ability of exosomes to cross-communicate the CNS (neurogenic niches) with peripheral tissues highlights their potential role as physiological/pathological mediators of different CNS disorders (explanation for CNS-peripheral tissues co-morbidities, for example) and as biomarkers (CSF/blood samples). BBB, blood-brain barrier; BCB, blood-CSF barrier (choroid plexus epithelial cells); CBI, CSF-brain interface.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we focus on the potential role of EVs, and in particular, of exosomes in the regulation of the neurogenic niche (**Figure 4**). Exosomes, as messengers able to modulate the physiology of the niche, might originate from cells residing within the niche or from distant cells/tissues, thus having access to the neurogenic niche through the vasculature (blood-circulating exosomes) or by volume transmission via the CSF. The possible effects of molecular components already known to be present within exosomes on adult neurogenic process are also addressed. This scenario opens the possibility of a novel form of communication between niche cells and regulation of adult neurogenesis, able to both, regulate locally the extent of neurogenesis, and sense and integrate physiological conditions and pathological disturbances in diverse body systems.

On the other hand, exosomes originated in the CNS and in the neurogenic niche (i) can vary according to the circumstances, and (ii) might reach the peripheral blood circulation, thus linking or communicating directly the physiological or pathological status of the CNS to peripheral organs or tissues. This could be relevant (1) to understand the high prevalence of co-morbidity of pathologies associated to impaired neurogenesis and peripheral disorders, such as major depression and diabetes or inflammatory diseases (Kessler et al., 2003; Empana et al., 2005; Evans et al., 2005; Katon et al., 2008; Katon, 2008; Bonaz and Bernstein, 2013; Filipovic and Filipovic, 2014), and (2) to use blood-circulating CNS-derived exosomes as biomarkers of brain disorders.

The contribution of specific cell- and tissue-derived exosomes on adult neurogenesis should be further investigated with the use of proper animal models in which exosomes should be labeled with the use of molecular biology techniques, and later should be validated in health and disease with the use of a panel of biomarkers able to define specific exosome populations. We envisage that, in the near future, many of this work will be addressed by a growing community of researchers interested in the role of exosomes in disease-related processes that, among others, affect the neurogenic niche in a yet unsuspected manner.

AUTHOR CONTRIBUTIONS

All the authors have contributed substantially to the writing and revising of the manuscript. LFB and UW have participated in the conception and design of the work. LFB have designed the figures. RIM, ZDV and LFB have designed and completed the table. MAC, RIM, PVB, ZDV, CAL, P-ET have participated in writing, drafting and revising of different sections of the manuscript. All the authors have approved the final version of the manuscript.

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Hypoxic pre-conditioning increases the infiltration of endothelial cells into scaffolds for dermal regeneration pre-seeded with mesenchymal stem cells

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Many therapies using mesenchymal stem cells (MSC) rely on their ability to produce and release paracrine signals with chemotactic and pro-angiogenic activity. These characteristics, however, are mostly studied under standard *in vitro* culture conditions. In contrast, various novel cell-based therapies imply pre-seeding MSC into bio-artificial scaffolds. Here we describe human bone marrow-derived MSC seeded in Integra matrices, a common type of scaffold for dermal regeneration (SDR). We show and measured the distribution of MSC within the SDR, where cells clearly establish physical interactions with the scaffold, exhibiting constant metabolic activity for at least 15 days. In the SDR, MSC secrete VEGF and SDF-1α and induce transwell migration of $CD34^+$ hematopoietic/endothelial progenitor cells, which is inhibited in the presence of a CXCR4/SDF-1α antagonist. MSC in SDR respond to hypoxia by altering levels of angiogenic signals such as Angiogenin, Serpin-1, uPA, and IL-8. Finally, we show that MSC-containing SDR that have been pre-incubated in hypoxia show higher infiltration of endothelial cells after implantation into immune deficient mice. Our data show that MSC are fully functional *ex vivo* when implanted into SDR. In addition, our results strongly support the notion of hypoxic pre-conditioning MSC-containing SDR, in order to promote angiogenesis in the wounds.

Keywords: mesenchymal stem cells, scaffolds, wound healing, angiogenesis, hypoxia

INTRODUCTION

Skin is the largest organ of the body and is responsible for several critical functions, such as control of body temperature and protection against external pathogens. Skin defects lead to the death of thousands of people per year and represent large costs for the health care system. In this regard, the development of scaffolds for dermal regeneration (SDR) has meant a significant breakthrough,

especially for patients presenting large burns (Machens et al., 2000). However, the clinical benefit of these scaffolds in chronic wounds has been disappointing due to lack of induction of dermal regeneration and re-epithelialization. We and others have shown that SDR-mediated skin repair is greatly improved if SDR are "bio-activated" by pre-implantation of cells (Markowicz et al., 2006; Falanga et al., 2007; Egaña et al., 2009).

A very promising cell source for skin repair is mesenchymal stem cells/multipotent stromal cells (MSC), which, in many applications, do not contribute to regeneration through direct differentiation into dermal, epithelial, or endothelial cells (tissue replacement), but rather act as trophic mediators, releasing chemotactic, immune modulatory, and pro-angiogenic factors (Caplan and Dennis, 2006; Fu and Li, 2009). MSC are easy to isolate from various tissue sources, including bone marrow and adipose tissue (da Silva Meirelles et al., 2006), can be robustly expanded *ex vivo* and present low immunogenicity, allowing both autologous and allogeneic transplants (Uccelli et al., 2008). Albeit an overall elusive function *in vivo* due to the absence of specific MSC surface markers, MSC seem to correlate with pericytes (Crisan et al., 2008). MSC from the bone marrow are the primary source for osteo-progenitor cells and play a critical role in supporting hematopoietic stem/progenitor cells (Sacchetti et al., 2007). It has also been shown that direct intradermal injection of human MSC promote wound healing in diabetic mice (Kim et al., 2012; Shin and Peterson, 2013).

MSC that are expanded for *in vitro* studies are commonly cultured in polystyrene plastic flasks or plates. The capability to attach to plastic is indeed a major defining characteristic of MSC (Dominici et al., 2006). However, parameters such as the high stiffness of plastic strongly affect MSC cell fate (McBeath et al., 2004; Engler et al., 2006), suggesting that characteristics attributed to MSC will vary according to the specific *in vitro* culture conditions. For example, when MSC cultured in three-dimensional (3D) scaffolds are implanted into nude mice, they generate more abundant and homogenous bone as compared to MSC cultured as monolayers (Braccini et al., 2005). MSC cultured in 3D scaffolds respond to hypoxia by expressing higher levels of the stem cell markers Oct-4 and Rex-1, and maintain a higher colony forming unit (CFU-F) potential as compared to cells in normoxia (Grayson et al., 2006). Hypoxia also enhances the motility of MSC, improving their therapeutic potential on blood flow restoration, as shown using a murine hind limb ischemia model (Rosová et al., 2008). Finally, we have recently shown that hypoxic pre-conditioning induces metabolic changes in MSC that promote their retention after intramuscular injection into immune deficient mice (Beegle et al., 2015).

This development suggests that combining scaffolds for dermal regeneration (SDR) with MSC for the treatment of chronic skin ulcers and wound repair would be an ideal strategy. We recently compared incorporation of adipose-tissue derived MSC into different scaffolds. In those studies, parameters such as seeding efficiency, distribution, attachment, survival, metabolic activity, and release of paracrine signals where measured, where the best results were obtained with Integra™ matrices (Wahl et al., 2015). Integra™ Matrix Wound Dressing is a bilayer scaffold composed of type I bovine collagen and chondroitin-6-sulfate with a thin silicon layer. We have also shown that Integra™ SDR seeded with a mesenchymal cell line show a strong increase of new blood vessel formation, following a skin wound excision model in nude mice (Egaña et al., 2009). These results strongly support the strategy of "bio-activating" Integra™ SDR by pre-seeding it with MSC.

Here we show that bone marrow-derived MSC implanted in three dimensional SDR promote key features for wound repair applications, such as sustained viability, migration of hematopoietic/endothelial progenitor cells (H/EPC) and response to hypoxia, inducing release of pro-angiogenic signals *in vitro*. Most importantly, our results also suggest that pre-incubation with hypoxia increases the angiogenic potential of MSC/SDR *in vivo*.

MATERIALS AND METHODS

Cell Isolation and Culture

MSC were obtained from either bone marrow samples of healthy human donors who gave informed consent to the research protocols (approved by the institutional review board of the Technical University of Dresden) or purchased from Allcells (Alameda, CA). Mononuclear cell (MNC) fraction of bone marrow aspirates were obtained by density gradient centrifugation [Percoll (1.073 g/l) for 30 min at 700 × g] and plated in plastic culture flasks with MSC culture media (see below). After 3 days, non-adherent cells were removed by 2–3 washing steps with PBS. MSC culture media used for viability assays, secretion of VEGF and SDF-1α and migration assays was low glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany). MSC culture media used for confocal imaging in SDR, analysis of distribution, hypoxia-induced angiogenic proteome array and *in vivo* studies was MEM-alpha (HyClone Thermo Scientific, Waltham MA) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville GA). In all cases, MSC from passages 2–6 where used for experimentation. All MSC are routinely characterized for immune phenotype ($CD14^-$, $CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, $CD105^+$, and $CD166^+$) and differentiation potential into both adipocytes and osteoblasts (not shown).

For isolation of $CD34^+$ cells, leukapheresis samples were obtained from healthy donors, whose stem cells were mobilized by administration of GM-CSF, following institutional approval. MNC were separated by density gradient [centrifugation over Percoll (1.073 g/l) 30 min at 700 × g]. $CD34^+$ cells were isolated using anti-CD34 coated paramagnetic microbeads according to manufacturer instructions (Miltenyi, Begisch-Gladbach, Germany).

Seeding MSC in SDR

For all experiments performed with MSC in SDR (Integra™ Matrix Wound Dressing; Plainsboro, NJ), the following protocol was used: Pieces of SDR matrices (10 mm diameter, approximate volume; 0.3 cm^3) were dried with sterile gauze, placed in 24-well plates and 2.5×10^5 MSC in 300 µl culture medium were dropped over the scaffold and quickly absorbed. After 30 min of

incubation in the cell culture hood, 1 ml of culture media was added to each well.

Imaging of SDR by Scanning Electron Microscopy

For scanning electron microscopy of SDR, scaffolds were washed with PBS and fixed with 2% glutaraldehyde for 1 h at room temperature and then overnight at 4°C. SDR were dehydrated in a series of acetone (30–100%) and critical-point-dried in a CO_2 system (Critical Point Dryer CPD 030, BAL-TEC GmbH, Witten, Germany). Samples were then sectioned and mounted on aluminum stubs and sputter-coated with gold (Sputter Coating Device SCD 050, BAL-TEC GmbH, Witten, Germany). Finally, samples were analyzed at 10 kV accelerating voltage in an environmental scanning electron microscope (XL 30; Philips, Eindhoven, The Netherlands).

Imaging of MSC in SDR by Laser Scanning Confocal Microscopy

For the observation of MSC in SDR, the following protocol was followed: 3 days after seeding MSC in SDR, scaffolds containing cells were rinsed with PBS, fixed for 30 min with paraformaldehyde (3.7% paraformaldehyde, 0.1% Triton in PBS), and blocked in 2% BSA in PBS prior to incubation in phalloidin-texas red to stain actin and To-Pro3 to stain DNA. After washing, scaffolds were imaged using a confocal microscope to assess the number of cells that were seeded, the distribution of cells and their morphology. Three SDR were examined, and each of which was seeded using MSC derived from three different donors. Since Integra™ Matrix was ∼1 mm in thickness, the matrices were sectioned, turned on their sides and z-section image series acquired (about 100 μm z-sect depth). Seven sections were imaged and analyzed so as to gather a representative sampling. Fluorescence imaging of cells upon and within the matrix were optimized so as to avoid spectral overlap since collagen was observed to auto-fluorescence in UV and FITC channels (emission ranging over 300–500 nm).

Cell Loading Capacity and Cell Retention In SDR

MSC cultured in SDR for up to 15 days were incubated for 3 h in fresh medium containing 5 ng/ml of MTT (3-(4,5-dimethyl-2-Thiazolyl)-2,5-Diphenyl-2H-Tetrazolium bromide) (Sigma-Aldrich, Turkirchen, Germany). Then, medium was removed and replaced by 300 μl dimethyl sulfoxide (DMSO). After 15 min incubation, DMSO was removed and absorbance was measured at 570 nm to quantify formation of formazan blue, which is proportional to the number of living cells. Scaffolds without cells were used as negative control.

VEGF and SDF-1α Release from MSC in SDR

Two days after seeding MSC in SDR, medium was replaced with DMEM + 2% FBS. Then, every 48 h medium was removed and replaced with fresh medium. VEGF and SDF-1α concentrations were measured by ELISA according to manufacturer instructions (Quantikine ELISA kits, R&D systems, Minneapolis, MN). Scaffolds without cells were used as negative controls.

Migration Assay

2×10^5 $CD34^+$ cells in 100 μl RPMI were added into inserts of 5 μm-pore transwell plates (Corning Inc., Corning, NY) with conditioned media of MSC-containing SDR or empty SDR (control) in the lower compartment. After incubation for 4 h, inserts were removed and cells that migrated into the lower compartment were counted using Trypan blue exclusion dye and a hemocytometer. The effect of blocking the CXCR4 receptor was evaluated by pre-incubation of the cells for 2 h in media containing 100 μM AMD3100 (Sigma).

Angiogenesis Proteome Array

MSC derived from three different donors were seeded in SDR as described above. After 24 h, medium was changed to MEM-alpha + 2% FBS and cells cultured for 48 h either under normal (21% O_2) or hypoxic (3% O_2) conditions. Next, media were collected and used in a Proteome Profiler Human Angiogenesis Array Kit, following manufacturer instructions (R&D Systems). Hypoxia was generated in an incubator at 37°C with 5% CO_2 humidified atmosphere and dedicated oxygen level (3% O2), as established by replacement with Nitrogen injections.

Endothelial Cell Migration in a Wound Excision Model *in vivo*

All animal procedures were performed strictly adhering to protocols approved by the Institutional Animal Care and Use Committee at UC Davis. Two excisional wounds were generated bilaterally on the flanks of immune deficient NOD/SCID IL2Rγ −/− (NSG) mice, as previously described (Egaña et al., 2009; Schenck et al., 2014). Briefly, animals were placed under anesthesia (1.5–3% isoflurane), fur was shaved and an area of skin was removed on both the left and/or right flank of the animal using a 10 mm biopsy punch. Then, a 15 mm piece of surgical mesh (TiMESH Titanized Polymers, Nuernberg, Germany) was placed under the wound edge, thus covering the wound bed. Next, an 8–10 mm piece of SDR was placed over the wound bed. Each condition, (SDR alone or SDR containing MSC pre-incubated for 2 days in either 21 or 3% O_2) was placed bilaterally into 3 animals for a total of 6 replicates. SDR were fixed at 6 points with skin glue (Histoacryl Topical Skin Adhesive, TissueSeal, Ann Arbor, MI), such that the wound edge overlapped 1–2 mm with the scaffold. Finally, wounds were covered with sterile gauze and adhesive tape and monitored daily.

After 14 days, animals were euthanized and SDR with surrounding tissue processed for histological analysis. Samples were transferred to a blocking solution (2% BSA in PBS) at room temperature for 2 h. Then, tissues were blotted dry with gauze and incubated in PBS containing 0.5% Triton X-100 and a 1:75 dilution of Biotinylated GSL I—isolectin B4 (Vector Laboratories, Burlingame, CA), was applied at room temperature for 1 h with gentle agitation on a platform shaker. GSL I—isolectin B4 has been shown to recognize terminal α-galactosyl residues found on the glycoprotein laminin secreted by endothelial cells while forming the basal lamina of blood

vessels (Benton et al., 2008). After washing 3 times for 5 min each with PBS containing 0.5% Triton X-100, Dylight 649-streptavidin secondary antibody (Vector Labs) at a 1:150 dilution was applied at room temperature for 1 h with shaking. After 3 additional washes with PBS + 0.5% Triton X-100 and a final wash with PBS, samples were mounted in VectaShield containing DAPI (Vector Labs). Imaging was done on an Olympus FluoView FV10i confocal microscope. Each sample was cut into up to four sections and each cut was placed on its cross-section for imaging. At least 35 images per condition were analyzed and averaged for the quantification of endothelial cell infiltration.

Statistical Analysis

All assays were repeated in at least three independent experiments. Results are expressed as averages with the standard error of the mean (SEM) as error bars, unless otherwise stated. One-Way ANOVA or Student's *t*-tests were used to compare samples, where $p < 0.05$ was considered significantly different.

RESULTS

To characterize MSC cultured in SDR, we first used scanning electron microscopy (SEM) to inspect the scaffold. We observed that the SDR has a randomly folded laminar structure, which generates large spaces of 50–200 μm in diameter (**Figure 1A**). To evaluate how MSC distribute within this scaffold, we seeded the MSC in SDR and cultured them for 3 days to allow maximum attachment and spreading within the scaffold. Then, samples were fixed and examined using confocal imaging. We counted cell nuclei within each z-section image and tabulated their distribution with respect to the scaffold fraction. Although cell distribution appeared relatively homogenous within the scaffold (**Figure 1B**), 85% of cells were found concentrated in the upper half of the scaffold (**Figures 1C,D**). Also using this z-stack analysis, we inferred an average seeding efficiency of 92%. MSC were observed to be well spread throughout the matrix as evidenced by actin staining (**Figures 1B,C**).

Next we quantified cell load-capacity of SDR using a MTT assay. First, we demonstrate that there was no unspecific labeling with MTT substrate in empty scaffolds. In contrast, MSC-containing scaffolds show cells turning a dark blue color, which can be clearly visualized using microscopy (**Figure 2A**). Next, we seeded increasing doses of MSC and concluded that the SDR supports high cellularity, with the highest concentration tested equated to 3.6×10^6 cells per $1\,cm^2$ (**Figure 2B**). To evaluate whether MSCs were viable and if they proliferate within the SDR matrix, we cultured the cells for up to 15 days. As shown in **Figure 2C**, we found constant formazan blue formation, suggesting that MSC are viable and metabolically stable within the scaffold for at least 15 days, but show limited expansion.

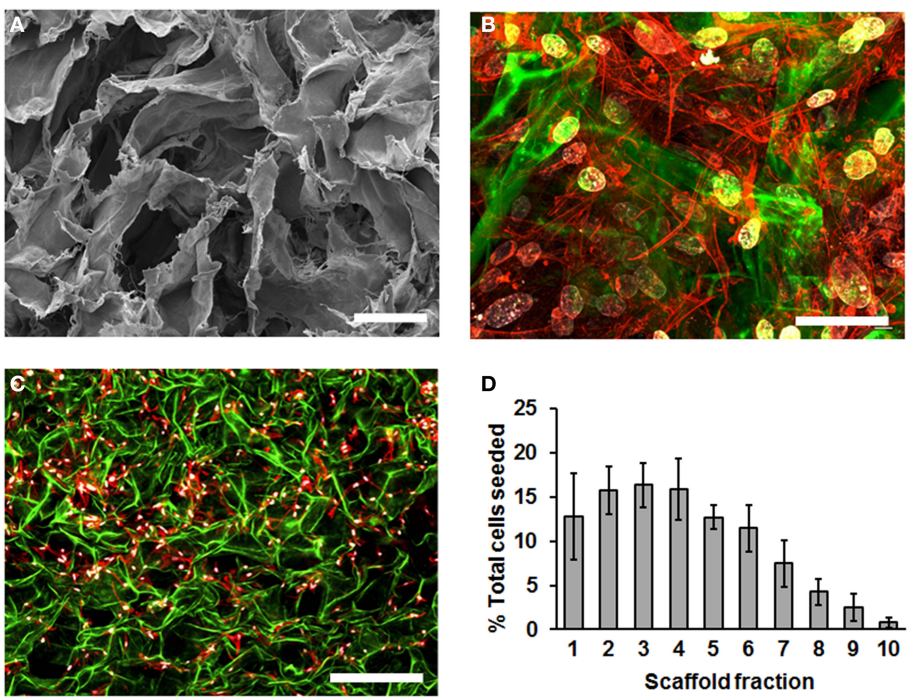

FIGURE 1 | SDR topology and distribution of MSC in them. (A) SDR as observed using scanning electron microscopy (SEM). SDR has a randomly folded laminar structure, which generates large spaces of 50–200 mm in diameter. **(B–D)** 2.5×10^5 MSC were seeded in SDR, cultured for 3 days to allow maximum attachment and spreading, and fixed for staining as follows: Nuclei were stained with DAPI (blue), actin cytoskeleton is labeled with TRITC-coupled phalloidin and auto-fluorescence of SDR is shown in green. Although cell distribution appears relatively homogenous within the scaffold surface, 85% of cells were found concentrated in the upper half of the scaffold **(C,D)**. To quantify cell distribution, cell nuclei were counted within each z-section image and their distribution tabulated with respect to the scaffold fraction. Also using this z-stack analysis, we inferred an average seeding efficiency of 92%. Scale bar represents 0.1 μm in **(A)**, 50 μm in **(B)**, and 500 μm in **(C)**.

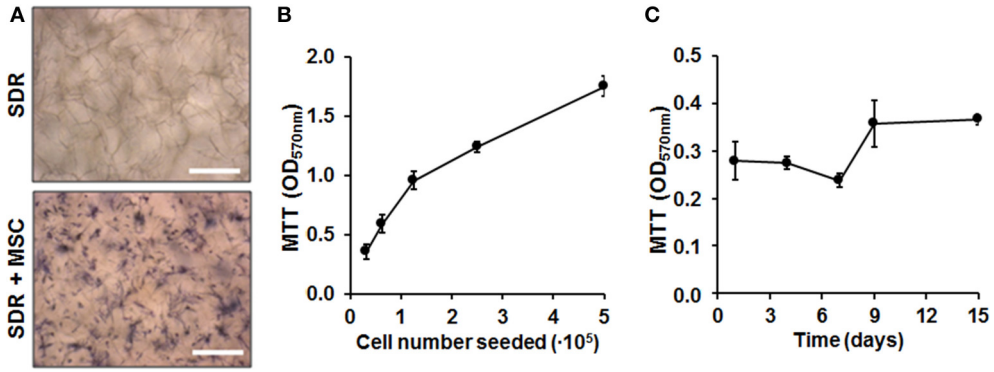

FIGURE 2 | Cell load capacity and cellular viability within SDR matrices. (A) MTT assay on empty- and MSC-containing SDR matrix demonstrates specific formazan formation by cells (dark blue). **(B)** MSC were seeded in 0.13 cm^2 pieces of SDR matrix and cultured for 24 h to allow maximum spreading and adhesion of cells. Then, scaffolds with cells were incubated with MTT substrate to determine relative cellularity based on formazan blue formation. **(C)** MSC in SDR matrices shows relatively constant MTT hydrolysis for up to 15 days. Scale bars represent 200 μm.

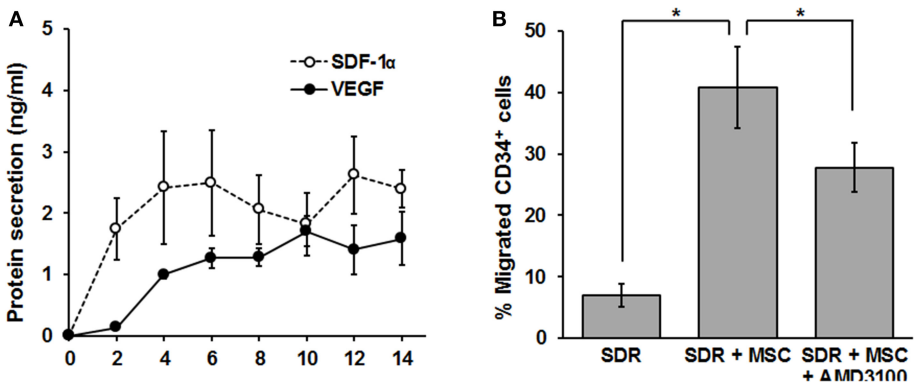

FIGURE 3 | MSC-containing SDR secrete VEGF, SDF-1α, and induce migration of $CD34^+$ H/EPC *in vitro*. (A) MSC-containing SDR were incubated with medium changes every second day. At every medium change, supernatants were collected, and VEGF and SDF-1α protein levels measured by ELISA. Average of MSC from 3 different donors are shown. **(B)** Supernatant of SDR (control) and SDR + MSC were collected after 48 h incubation and tested for $CD34^+$ H/EPC migration in a transwell assay, as described in the Materials and Methods Section. In addition, AMD3100 (100 ng/ml) was added to the SDR + MSC condition during the transwell assay. Average of $CD34^+$ of 3 different donors using MSCs from 2 different donors are shown. *$p < 0.05$.

In the following set of experiments, we determined whether the SDR could be "bio-activated" with MSC in terms of secreting functionally relevant levels of chemotactic and angiogenic signals. The development of new vasculature during the tissue repair process is driven by two major mechanisms: angiogenesis and vasculogenesis (Carmeliet, 2000). While the latter implies the recruitment of new endothelial progenitor cells (EPC), angiogenesis is commonly defined as the extension of pre-existing blood vessels and is therefore dependent on the proliferation and organization of endothelial cells (Carmeliet, 2000). Vascular remodeling (vasculogenesis) relies on the recruitment of $CD34^+$ EPC (Asahara et al., 1997). Since EPC mobilization and homing to the site of injury depends on the CXCR4/SDF-1α axis (Tachibana et al., 1998; Moore et al., 2001), we tested whether MSC-containing scaffolds could produce and release significant levels of SDF-1α. For this, MSC were seeded in SDR matrix, medium was changed every second day and SDF-1α protein levels were measured in the collected supernatant by ELISA. As shown in **Figure 3A**, SDF-1α levels are low for the first 2 days after seeding MSC in SDR. However, constant levels (ranging from 1.3 to 1.7 ng/ml) of SDF-1α were detected from days 4 to 14. Most importantly, MSC-containing scaffolds are capable of inducing migration of $CD34^+$ cells (comprising both hematopoietic- and endothelial- progenitor cells (H/EPC) in a transwell-migration assay, demonstrating that the chemotactic signals released are fully functional. Furthermore, the migration of $CD34^+$ cells was partially inhibited by the CXCR4 antagonist AMD3100, strongly suggesting that the migration of $CD34^+$ cells is at least partly due to SDF-1α released by MSC-containing SDR (**Figure 3B**).

As shown in **Figure 3A**, MSC-containing SDR also release constant levels of the angiogenic signal vascular endothelial growth factor (VEGF) (2.2 + 0.5 ng/ml). In addition, we tested whether MSC are able to respond to hypoxia when seeded in SDR. For this, seeded scaffolds were cultured for 48 h in the presence of either 21% (normoxia) or 3% O_2 (hypoxia). As shown

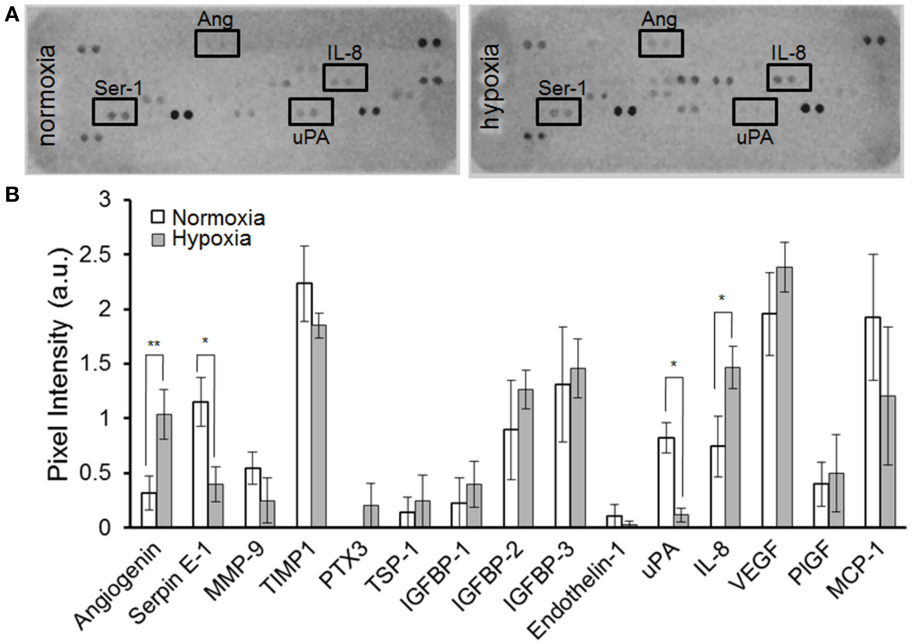

FIGURE 4 | MSC-containing SDR secrete angiogenic factors and respond to hypoxia. MSC-containing SDR were cultured under normoxic or hypoxic conditions for 2 days and supernatant collected for angiogenic factors as described in Section Materials and Methods. **(A)** Representative angiogenesis-proteome array. **(B)** Quantification of angiogenesis proteome array, using MSC from 3 different donors. $^{*}p < 0.05$; $^{**}p < 0.005$.

in **Figures 4A,B**, several angiogenic signals could be detected in supernatants of MSC-seeded SDR. Among them, angiogenin (Ang), and interleukin 8 (IL-8) were significantly up-regulated during hypoxia, while serpin-1 and urokinase-type plasminogen activator (uPA) protein levels were strongly inhibited by hypoxia.

Finally, we tested whether MSC-seeded SDR pre-incubated for 48 h in hypoxia would exert a functional effect *in vivo*. For this we generated bilateral excisional full skin defects in mice and implanted in each either SDR alone or MSC-containing SDR that were pre-incubated for 2 days in either normoxic or hypoxic conditions. **Figure 5** shows both representative images and the quantification of endothelial cells in the wound area that were secreting the specific glycoform of laminin found in the basal lamina of blood vessels, 14 days after surgery. Previous studies have shown that GSLI-isolectin B4 labels only the basal lamina of blood vessels secreted by endothelial cells within the dermal layers (Brabec et al., 1980; Benton et al., 2008). While no significant differences were found between SDR alone and MSC-containing SDR in normoxia, pre-incubation for 2 days in hypoxia had a highly significant effect promoting endothelial cell migration into wound edge, suggesting that hypoxic pre-conditioning is essential for promoting blood vessel formation in MSC-containing SDR.

DISCUSSION

The reparative potential of skin is limited because wounds are typically replaced by scar formation, which restores tissue integrity, but not full functionality. This is critical in massive (e.g., burns) and specific (e.g., neck, hands, elbows) skin injuries where functional tissue is required. In this context, SDR have been used as templates to favor a skin regeneration process. However, an important limitation in efficacy of SDR to induce tissue regeneration is the lack of proper vascularization. This limitation could be circumvented by "bio-activating" the SDR with MSC, which are able to induce angiogenesis and recruit H/EPC via secretion of paracrine factors. Nevertheless, these properties of MSC have been studied *in vitro* under standard culture conditions, where cells are grown as monolayers adhered to plastic. In contrast, little is known about their performance in 3D structures. SDR provide a very different cell culture condition, generating several local microenvironments and gradients, which may influence the exocrine profile of MSC, as well as their proliferation and survival, among others.

Ultra-structural characterization of the SDR used in this study (Integra™ Matrix Wound Dressing) revealed 50–200 μm diameter pores within the scaffold. Of note, during the preparation process, the SDR was dried, possibly altering the fine structure morphology of the matrix. Commercially, this SDR is stabilized with glutaraldehyde and stored in sodium phosphate buffer. However, our images show a structure that allows a homogenous distribution of MSC, as observed by confocal microscopy. Of note, a key step in our protocol is the seeding process: if MSC are seeded into wet scaffolds, cells stay attached in higher densities at the surface of the SDR (not shown), presumably due to a high affinity of MSC to attach to collagen (Cool and Nurcombe, 2005). In contrast, slightly drying the SDR with subsequent addition of highly concentrated MSC

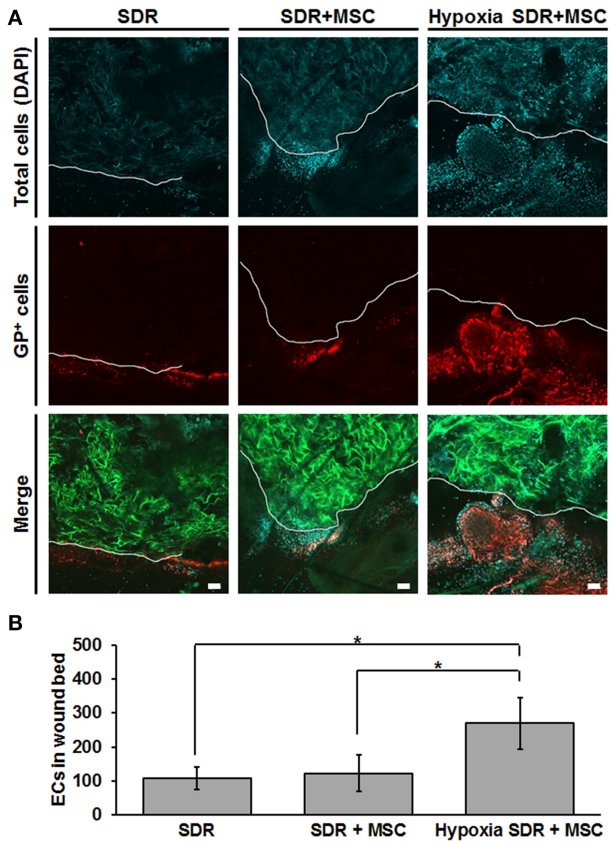

FIGURE 5 | Hypoxic pre-conditioning of MSC-containing SDR favors high cellular infiltration *in vivo*. MSC-containing SDR were cultured under normoxic or hypoxic conditions for 2 days and then implanted into immune deficient NSG mice, following a skin excision procedure. After 2 weeks, animals were euthanized and scaffold and surrounding tissue were analyzed using laser scanning confocal microscopy. **(A)** Green shows the SDR (due to auto-fluorescence of collagen), blue shows DAPI staining of cell nuclei, and red shows the staining of GSLI isolectin B4 which binds to the terminal α-D-galactosyl residues of the glycoprotein laminin (GP) expressed by endothelial cells within the basal lamina of neovascular structures. The white line indicates the border of the scaffold and surrounding tissue. **(B)** Quantification of endothelial cells (ECs), as determined by the average number of GP-positive cells within the first 200 μm of SDR near wound edge. Scale bar represents 50 μm. *$p < 0.05$.

(∼1000 cells/μl) promotes absorption of the cells into the SDR, favoring the penetration of cells into the scaffold. Addition of more culture media 20–30 min later ensures optimal MSC culture conditions. Our distribution analysis reveals that 85% of cells are concentrated in the upper half of the scaffold, suggesting that for clinical applications, the MSC-containing SDR should be placed with the upper side facing toward the wound, in order to favor the interaction of MSC with surrounding tissue. Alternatively, MSC could be seeded from both sides to further increase the total amount of cells incorporated. Direct interaction of MSC in the SDR was demonstrated as cells attached and spread, acquiring characteristic fibroblastic morphology. The adhesion of MSC is critical, since detachment of cells induces programmed cell death, a process known as anoikis (Frisch and Francis, 1994).

The notion that MSC attached to the scaffold remain viable is supported by detection of constant formazan formation. Our results also suggest that MSC may not extensively proliferate under these culture conditions. This is further supported by the steady levels of secreted SDF-1α and VEGF reached by 2–4 days after seeding. The proliferation of MSC could be limited due to a rather low volume of culture media added to each scaffold/well, limiting nutrient availability. Alternatively, integrin-mediated adhesion of MSC to the matrix may inhibit cell proliferation (Giancotti and Ruoslahti, 1999). The constant release of factors such as VEGF and SDF-1α could possibly have an important impact on the regeneration process. VEGF is a potent mitogen for endothelial cells and induces endothelial cell migration, sprouting, and survival (Ozawa et al., 2004). The CXCR4/SDF-1α axis is essential for stem cell homing and mobilization into damage tissues (Tachibana et al., 1998; Peled et al., 1999; Ceradini et al., 2004). Of note, SDF-1α has been shown to improve wound healing in diabetic mice (Badillo et al., 2007).

Here we show that MSC seeded in SDR respond to hypoxia, which alters their expression of angiogenic factors. We found that MSC in SDR under hypoxia increase Ang and IL-8 secretion. This effect has been reported in various cell types, including MSC cultured under standard conditions (Hung et al., 2007; Potier et al., 2007). Ang is an important RNAse that promotes neovascularization. When new vessels are required, Ang is incorporated into endothelial cells and transferred to their nuclei. There, it stimulates rRNA transcription, a rate-limiting step in ribosome biogenesis, protein translation, and cell growth (Wiedlocha, 1999; Tello-Montoliu et al., 2006). It has also been shown that angiogenin is necessary for angiogenesis induced by factors such as VEGF (Kishimoto et al., 2005). Expression of the chemokine IL-8 directly correlates with neovascularization (Yoneda et al., 1998) by stimulating both proliferation and migration of endothelial cells (Brat et al., 2005). We hypothesize that the observed reduction of PAI-1 and uPA under hypoxia are inter-related events, because interaction of PAI-1 with the uPA/uPA-receptor complex induces internalization of the ternary complex uPA-R/uPA/PAI-1, resulting in degradation of uPA and PAI-1, while uPA-R is recycled to the cell surface (Harbeck et al., 2004). According to our angiogenesis array, hypoxia only induced a modest increase in VEGF levels in MSC in SDR. However, using ELISA, we and others have observed that expression of VEGF is significantly increased in MSC under hypoxia (not shown). This is most likely due to limited sensitivity of the angiogenesis array, where VEGF detection approached saturation levels (maximal pixel intensity). The angiogenesis array is only a semi-quantification based on pixel intensity of the dots, rather than colorimetric optical density, with quantification based on a standard curve, as used in ELISAs.

The exocrine effects of MSC could have tremendous impacts on the development of new strategies to enhance vascularization and tissue regeneration in tissue engineering approaches. The use of MSC seeded scaffolds should be studied in massive or chronic wounds in *in vivo* settings. Indeed, our *in vivo* studies strongly support the notion that hypoxic pre-conditioning favors the infiltration of endothelial cells into the wound bed that are in

the early stages of neovascularization. The mechanism, however, remains unclear. One possibility is that hypoxic pre-conditioning enhances the survival of MSC after implantation, hence a greater number of endothelial cells is favored by increased presence of MSC (Beegle et al., 2015). Alternatively, hypoxia increases the secretion of angiogenic signals (as shown *in vitro*) that promote endothelial cell migration. These and other hypotheses need future experimental validation. In addition, future studies need to directly address the effect of hypoxic MSC-containing SDR in wound closure.

This work underlines the relevance of studying MSC not only under standard culture conditions, but also in environments that more closely mimic their potential clinical application. Our results suggest that seeding MSC into SDR potentially "bio-activates" the material to enhance the regenerative process. Finally, we show that hypoxic-preconditioning of MSC-containing SDR has a strong positive impact promoting endothelial cell infiltration toward the wound bed, possibly contributing the wound repair process.

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frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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Allergy Enhances Neurogenesis and Modulates Microglial Activation in the Hippocampus

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Allergies and their characteristic T_H2-polarized inflammatory reactions affect a substantial part of the population. Since there is increasing evidence that the immune system modulates plasticity and function of the central nervous system (CNS), we investigated the effects of allergic lung inflammation on the hippocampus—a region of cellular plasticity in the adult brain. The focus of the present study was on microglia, the resident immune cells of the CNS, and on hippocampal neurogenesis, i.e., the generation of new neurons. C57BL/6 mice were sensitized with a clinically relevant allergen derived from timothy grass pollen (Phl p 5). As expected, allergic sensitization induced high serum levels of allergen-specific immunoglobulins (IgG1 and IgE) and of T_H2 cytokines (IL-5 and IL-13). Surprisingly, fewer Iba1$^+$ microglia were found in the granular layer (GL) and subgranular zone (SGZ) of the hippocampal dentate gyrus and also the number of Iba1$^+$MHCII$^+$ cells was lower, indicating a reduced microglial surveillance and activation in the hippocampus of allergic mice. Neurogenesis was analyzed by labeling of proliferating cells with bromodeoxyuridine (BrdU) and determining their fate 4 weeks later, and by quantitative analysis of young immature neurons, i.e., cells expressing doublecortin (DCX). The number of DCX$^+$ cells was clearly increased in the allergy animals. Moreover, there were more BrdU$^+$ cells present in the hippocampus of allergic mice, and these newly born cells had differentiated into neurons as indicated by a higher number of BrdU$^+$NeuN$^+$ cells. In summary, allergy led to a reduced microglia presence and activity and to an elevated level of neurogenesis in the hippocampus. This effect was apparently specific to the hippocampus, as we did not observe these alterations in the subventricular zone (SVZ)/olfactory bulb (OB) system, also a region of high cellular plasticity and adult neurogenesis.

Keywords: allergy, neurogenesis, microglia, systemic inflammation, hippocampus, T_H2 polarization

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INTRODUCTION

In comparison to the broad interest focusing on the influence of T_H1 inflammatory parameters on the central nervous system (CNS; e.g., Cunningham et al., 2009; Henry et al., 2009; Jurgens and Johnson, 2012; Jurgens et al., 2012; Kahn et al., 2012; Kranjac et al., 2012; Valero et al., 2014), only a small number of studies deals with the effects of T_H2 immunity on the brain (e.g., Tonelli et al., 2009;

Sarlus et al., 2012, 2013). This is in striking contrast to the fact that chronic T_H2-polarized immune reactions, which are a main characteristic of allergies, affect a substantial and increasing part of the population world-wide (Bieber, 2011; Fiocchi, 2011; Pawankar, 2011). The WAO estimates that 400 million people in the world suffer from allergic rhinitis and 300 million from asthma (Brozek et al., 2010; Pawankar, 2011).

Allergies are misguided responses of the immune system in which normally non-pathogenic stimuli, such as tree and grass pollen, dust mites, or animal dander, lead to immune responses characterized by the synthesis of allergen-specific IgE antibodies, the activation of T_H2 immune cells and the production of the key T_H2 cytokines IL-4, IL-5, and IL-13 (for a review, see Bloemen et al., 2007; Galli et al., 2008). At later stages of a persisting allergic immune response, also other T_H subsets, e.g., T_H1 and T_H17, may be activated leading to an increased production of pro-inflammatory cytokines, such as IFNγ and TNFα, or the T_H17 cytokine IL-17 (reviewed in Holgate and Polosa, 2008).

There is increasing evidence that allergic reactions might influence immune status and functions of the CNS. In a rodent model of allergic rhinitis, reduced social interaction and anxiety-like behavior were observed, accompanied by the induction of a T_H2-biased cytokine mRNA profile (IL-4, IL-5, IL-13) in the olfactory bulb (OB) and the prefrontal cortex (Tonelli et al., 2009). In another model of airway-induced allergy, the allergic reaction was associated with increased levels of the immunoglobulins IgG and IgE in CNS tissue, and with enhanced tau phosphorylation (Sarlus et al., 2012), a risk factor for the development of Alzheimer's disease (AD). Chronic airway-induced allergy in mice modifies gene expression in the brain toward insulin resistance and inflammatory responses (Sarlus et al., 2013). In mice, in which a food allergy was induced shortly after weaning, reduced social behavior, increased self-grooming, reduced alternation in the T maze as well as decreased dopamine levels in the prefrontal cortex were observed (de Theije et al., 2014). Another study in juvenile mice, which were exposed to a long-term OVA-based asthma regime, showed impaired learning and memory in the Morris water maze, disturbed long-term potentiation in the hippocampal CA1 region and reduced cell proliferation in the hippocampal neurogenic niche (Guo et al., 2013).

There are only few findings suggesting that allergic reactions, like allergic rhinitis and asthma, affect cognitive functions in humans. Individuals suffering from seasonal allergic rhinitis, for example, perform worse in cognitive tests (Hartgerink-Lutgens et al., 2009). Moreover, there is a positive correlation between allergic rhinitis and mood disorders, such as anxiety and depression (reviewed in Sansone and Sansone, 2011). Similarly, children with asthma have higher rates of depression, behavioral disorders, and learning disabilities (Blackman and Gurka, 2007; Blackman and Conaway, 2012). There is also a correlation between allergies and epilepsy in children (Silverberg et al., 2014). While elderly asthma patients can profit from anti-asthmatic treatment, at least temporarily, with improved cognitive functions (Bozek et al., 2010), patients suffering from seasonal allergic rhinitis have a slower processing speed during attention tasks—also during symptom-free periods (Trikojat et al., 2015).

While there are indications that chronic systemic inflammation might contribute to neurodegenerative diseases (reviewed in Perry, 2010; Czirr and Wyss-Coray, 2012; Cunningham, 2013), the data about a possible influence of allergy on neurodegeneration is still conflicting. A longitudinal study in a population-based twin sample showed a positive association between a history of atopy and dementia (Eriksson et al., 2008). However, another study reported recently that AD patients who also suffered from allergies had an improved biomarker profile, closer resembling that of healthy subjects (i.e., higher $A\beta_{42}$ levels in the cerebrospinal fluid), and had a better cognitive performance, which might indicate a beneficial effect of allergy on AD (Sarlus et al., 2015).

There is accumulating evidence that the immune system, e.g., via cytokines and chemokines, strongly modulates CNS functions like learning and memory, and also adult neurogenesis, the generation of new neurons in the adult CNS (reviewed in Yirmiya and Goshen, 2011). Thus, the aim of the present study was to investigate if an allergic reaction influences the hippocampus, specifically the dentate gyrus, which contains one of two classical neurogenic niches in the adult CNS and which is known for its central role in cognitive functions (reviewed in Marín-Burgin and Schinder, 2012; Bond et al., 2015). In comparison, we also analyzed the subventricular zone (SVZ), from which neuronal progenitors migrate via the rostral migratory stream to the OB to integrate into the neuronal networks, the second classical neurogenic niche (reviewed in Bond et al., 2015).

Based on these data, we hypothesized that a systemic allergic reaction affects neurogenesis and microglia in the hippocampus. Further, we expected that the effect of a T_H2-polarized allergic response on microglial activation might differ from the well described reaction to a systemic LPS challenge.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (aged 10–12 weeks) were purchased from Charles River Germany and afterwards kept under standard animal housing conditions with free access to food and water at the animal facility at the University of Salzburg, Austria. All experimental procedures were approved by the Austrian Ministry of Science and carried out in compliance with International Ethical guidelines.

Allergy Induction

Recombinant Phl p 5.0101 (Phl p 5) was purchased from Biomay AG. The animals were divided into two groups: controls ($n = 9$) and allergy model ($n = 10$). The control group received all treatments using only the vehicle solution

(phosphate-buffered saline, PBS). Animals of the allergy group were immunized intraperitoneally (i.p.) with 1 μg Phl p 5 adjuvanted with $Al(OH)_3$ (Alu-Gel-S from Serva) in PBS (50% v/v, total volume: 200 μl) at weeks 1, 2, and 7. In week 11, starting 4 days before the perfusion (day 75), this group was challenged three times with a daily dose of 5 μg Phl p 5 in 40 μl PBS intranasally (i.n.; on days 71, 74 and 75). During this procedure, all mice (also the controls) were briefly anesthetized with isoflurane.

Analysis of Blood Parameters

Blood samples were taken at the end of the experiment (day 75), and incubated for 1 h at 37°C. After centrifugation (10 min), the sera were collected and stored at −80°C until measurements. Serum levels of Phl p 5-specific IgG1 and IgG2c were determined by a luminescence-based ELISA, and biologically functional IgE was measured *in vitro* by a rat basophil leukemia (RBL) cell assay. Additionally, cytokines, chemokines and the growth factor VEGFα were measured with a Luminex Multiplex Assay (Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel, Merck) according to the manufacturer's instructions.

Luminescence-Based ELISA Assay to Analyze Serological IgG Levels

Levels of Phl p 5-specific IgG1 and IgG2c were determined using a luminescence-based ELISA assay as previously described (Weinberger et al., 2013). In short, 96-well plates for immunoassays (Greiner) were coated for 24 h at 4°C with recombinant Phl p 5 (per well 50 μl of 1 μg/ml Phl p 5 in PBS). Afterwards, plates were washed with 0.1% Tween-20 in PBS (v/v) and incubated with blocking buffer (0.1% (v/v) Tween 20 and 2% (w/v) skim milk in PBS, pH 7.5) for 1 h at RT, before washing the plates again. Then, the plates were incubated with serum diluted (1:10,000) in blocking buffer for 1 h at RT, washed again, before the horse radish peroxidase (HRP)-conjugated antibodies for the detection of IgG1 (Zymed) or IgG2c (Zymed; diluted 1:1000 in blocking buffer) were added to the wells for 1 h at RT. After that, the luminometric assay (BM chemiluminescence substrate, Roche) was developed by adding the substrate (luminol diluted 1:2 in H_2O) to each well. After 3 min incubation, chemiluminescence (photon counts/s) was determined using an Infinite M200 Pro Plate Reader (Tecan).

RBL Cell Assay to Measure Biologically Functional IgE

The serum level of IgE was measured using a RBL cell assay as previously described (Weinberger et al., 2013). Briefly, RBL-2H3 cells (ATCC CRL-2256) were seeded in 96-well culture plates (Greiner) at a density of 6×10^5 cells/ml and grown over night in 100 μl culture medium per well at standard culture conditions (37°C, 95% relative humidity, 5% CO_2). The culture medium was RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, and 100 μM 2-mercaptoethanol. Next day, cells were incubated for 2 h with different serum dilutions (1:50, 1:100, and 1:200). Untreated wells were used to assess background and maximum release values. To remove unbound antibodies, plates were washed twice with 200 μl Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM D-glucose, 12 mM $NaHCO_3$, 10 mM HEPES, and 0.1% (w/v) bovine serum albumin (BSA); pH 7.2). Then the cells were incubated for 30 min in 100 μl of 0.1 μg/ml recombinant Phl p 5 diluted in Tyrode's buffer to induce crosslinking of FcεR-bound IgE and degranulation of RBL cells. To determine maximum release, cell membranes were disrupted by adding 10 μl of a 10% (v/v) Triton X-100 solution. After that, 50 μl of the cell culture supernatants were transferred into fresh 96-well plates (Greiner), where they were incubated for 1 h with 50 μl assay solution at a final concentration of 80 μM 4-methylumbelliferyl N-acetyl-b-D-glucosaminide (4-MUG, Sigma) in 0.1 M citrate buffer (pH 4.5). To stop the reaction, 100 μl glycine buffer (0.2 M glycine and 0.2 M NaCl, pH 10.7) were added and fluorescence (in relative fluorescence units) was measured in a fluorescence microplate reader (Infinite M200 Pro, Tecan). Background values were subtracted from all measured values, and the results were presented as percentage of the maximum release value.

Detection of Proliferating Cells to Determine Cell Fate

For the detection of proliferating cells, a solution of 10 mg/ml bromodeoxyuridin (BrdU; Sigma-Aldrich) in 0.9% NaCl (w/v), in a dosage of 50 mg/kg body weight, was once injected i.p. in week 7 on day 47 (4 weeks before the end of the experiment).

Bronchoalveolar Lavage (BAL) and Tissue Processing

In week 11, mice were deeply anaesthetized by i.p. injection of a mixture of ketamine (273 mg/kg body weight), xylazine (71 mg/kg body weight) and acepromazine (4 mg/kg body weight) in a physiological NaCl solution. During deep anesthesia (which was carefully evaluated), tracheotomy and a bronchoalveolar lavage (BAL) were performed. In short, the lungs were washed twice with 1 ml of ice cold PBS and the fluid was collected back into the syringe. This BAL fluid was stored on ice until flow cytometric analysis (FACS Canto II, BD Bioscience).

After BAL, mice were transcardially perfused, first with a 0.9% NaCl (w/v) solution, and then with phosphate-buffered 4% paraformaldehyde (pH 7.4). Afterwards, brains were removed, postfixed overnight in 4% paraformaldehyde, cryoprotected in phosphate-buffered 30% sucrose (w/v), and sectioned on dry ice with a sliding microtome. The sections (thickness: 40 μm) were stored at −20°C in a cryoprotection solution (made of equal parts glycerin, 0.2 M phosphate buffer, ethylene glycol and H_2O).

Analysis of the BAL Fluid

BAL samples were centrifuged (7 min, 250 × g, 4°C) and 450 μl of supernatants were taken and mixed with 50 μl of 10% BSA and 1% NaN_3 in H_2O and frozen at −80°C. Cytokine levels in

the BAL fluid were analyzed using a Luminex Multiplex Assay (MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel, Merck) according to the manufacturer's instructions.

For analysis of different immune cell populations in the BAL fluid, cell pellets were re-suspended in 100 µl of the remaining supernatant and transferred into a 96-well V-bottom plate (Greiner), and then centrifuged (5 min, 250 × g, 4°C). After that, cell pellets were re-suspended in 30 µl antibody staining mix: CD45-PerCP/Cy5.5 (1:400, 30-F11, Biolegend), CD4-BV421 (1:200, GK1.5, Biolegend), CD19-PE-Cy7 (1:100, 6D5, Biolegend), Gr-1-APC (1:200, RB6-8C5, eBioscience), Siglec-F-PE (1:200, E50-2440, BD Biosciences), CD8-FITC (1:100, 53-6.7, eBioscience) and incubated for 10 min on ice. Afterwards, the cells were washed with 100 µl FACS buffer (0.5% BSA and 2 mM EDTA in PBS), and then incubated for 5 min at RT in 100 µl Red Blood Cell (RBC) Lysis Buffer (eBioscience) to remove residual RBCs. After another washing step (using FACS buffer), cell pellets were re-suspended in 120 µl FACS buffer and transferred into FACS tubes. Cells were analyzed on a FACS Canto II flow cytometer (BD Bioscience) and data were recorded for 30 s at a rate of 120 µl/min to calculate the absolute cell numbers per BAL. For flow cytometric analysis, at first total leukocytes were gated based on their expression of CD45. The FSC/SSC plots were used to exclude cell debris, and then in FSC-W/FSC-A plots the single cells were gated. Neutrophils were identified based on their high Gr1 expression. The other cells were gated according to their expression of CD4, CD8, and SiglecF. SiglecF is highly expressed on eosinophils and monocytes. Monocytes were then separated from eosinophils by their autofluorescence in the BV-510 channel. In general, the observed CD19 staining was weak; therefore, $CD19^+$ cells were identified after exclusion of the previously gated cell types.

Immunohistochemistry

Immunohistological stainings were performed as previously described (Kandasamy et al., 2014), using the following antibodies and dilutions. Primary antibodies: rat anti-BrdU (1:500, BU1/75, AbD Serotec), rabbit anti-CD68 (1:500, ab125212, Abcam), rabbit anti-doublecortin (1:250, 4604, Cell Signaling), guinea pig anti-GFAP (1:500, GP52, Progen), rabbit anti-Iba1 (1:300, 019-19741, Wako), goat anti-Iba1 (1:250, ab107159, Abcam), anti-mouse MHCII (I-A/I-E; 1:100, 14-5321-82, eBioscience), mouse anti-NeuN (1:500, A60, Merck Millipore), mouse anti-PCNA (1:500, sc-56, Santa Cruz). Secondary antibodies: donkey anti-rat Alexa 488, donkey anti-goat, -mouse Alexa 568, donkey anti-rabbit, -guinea pig Alexa 647 (all 1:1000, Invitrogen, Life technologies), donkey anti-rat Cy5, donkey anti-mouse biotinylated (1:1000, Jackson Immuno Research), goat anti-rabbit biotinylated, rabbit anti-rat biotinylated (all 1:500, Vector Labs). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride at a concentration of 0.5 µg/µl (DAPI; Sigma-Aldrich).

Image documentation and analysis were done using a Zeiss Axioplan light microscope or a confocal scanning laser microscope (Zeiss LSM 700) with LSM Software (ZEN 2012) for fluorescent stainings.

Quantitative Analysis of Immunohistological Stainings

Image acquisition and quantification were done blinded (i.e., without knowing group or mouse number). For quantitative analysis, a representative tenth of one brain hemisphere was analyzed by collecting every 10th section, with an interval of 400 µm between sections. This 10th of a hemisphere was used for immunohistochemistry with a chromogenic dye. The total number of stained cells within the regions of interest was counted using a Zeiss Axioplan light microscope. In the dorsal hippocampal dentate gyrus, the total numbers of $PCNA^+$, $BrdU^+$, $Iba1^+$ and DCX^+ cells were counted. In addition, the total number of $BrdU^+$ cells in the SVZ was determined.

To investigate the cell fate of $BrdU^+$ cells in the dorsal dentate gyrus, a BrdU/NeuN/GFAP fluorescence staining was analyzed. For each animal, z-stacks of the dorsal dentate gyrus were made in a 10th brain hemisphere on a Zeiss LSM 700 laser scanning microscope. In these image stacks all $BrdU^+$ cells were counted, and at the same time, it was also determined if these cells were co-labeled for NeuN or GFAP. Thus, the percentage of $BrdU^+NeuN^+$ or $BrdU^+GFAP^+$ cells was determined per animal. Similarly, $BrdU^+$ cells in the granular cell layer of the OB were analyzed in a region of 400 × 400 × 40 µm.

To analyze the activation state of $Iba1^+$ cells in the dorsal dentate gyrus, the numbers of $Iba1^+MHCII^+$ and $Iba1^+CD68^+$ cells were counted in z-stacks (generated using a with Zeiss LSM 700 equipped with the Zeiss ZEN 2012 software) of four visual fields (400 × 400 × 40 µm) per animal. For the OB, one visual field of the granular cell layer was analyzed.

To estimate the analyzed volume for each region, the corresponding tissue area in the middle of the stack was measured and then multiplied by 40 µm. Then the cell densities were determined by dividing the total number of counted cells by this volume and presented as cells/mm^3.

Statistics

Data are shown as Mean + standard deviation (SD). Statistical significance was determined in Prism 5 (Graphpad Software Inc) using independent samples *t*-tests (the corresponding *p*-values are represented as: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). To adjust for multiple comparisons between the two groups, we computed for each large family of comparisons (cytokines in serum, cytokines in BAL fluid, and cell types in BAL fluid) a Holm-Šídák correction for multiple *t*-tests (at a global alpha level of 0.05) and reported the multiplicity adjusted *p*-values (in brackets) in addition to the unadjusted *p*-values. Statistical outliers were identified using the Grubb's test ($p < 0.05$) in the QuickCals GraphPad Software[1].

[1]http://graphpad.com/quickcalcs/grubbs1.

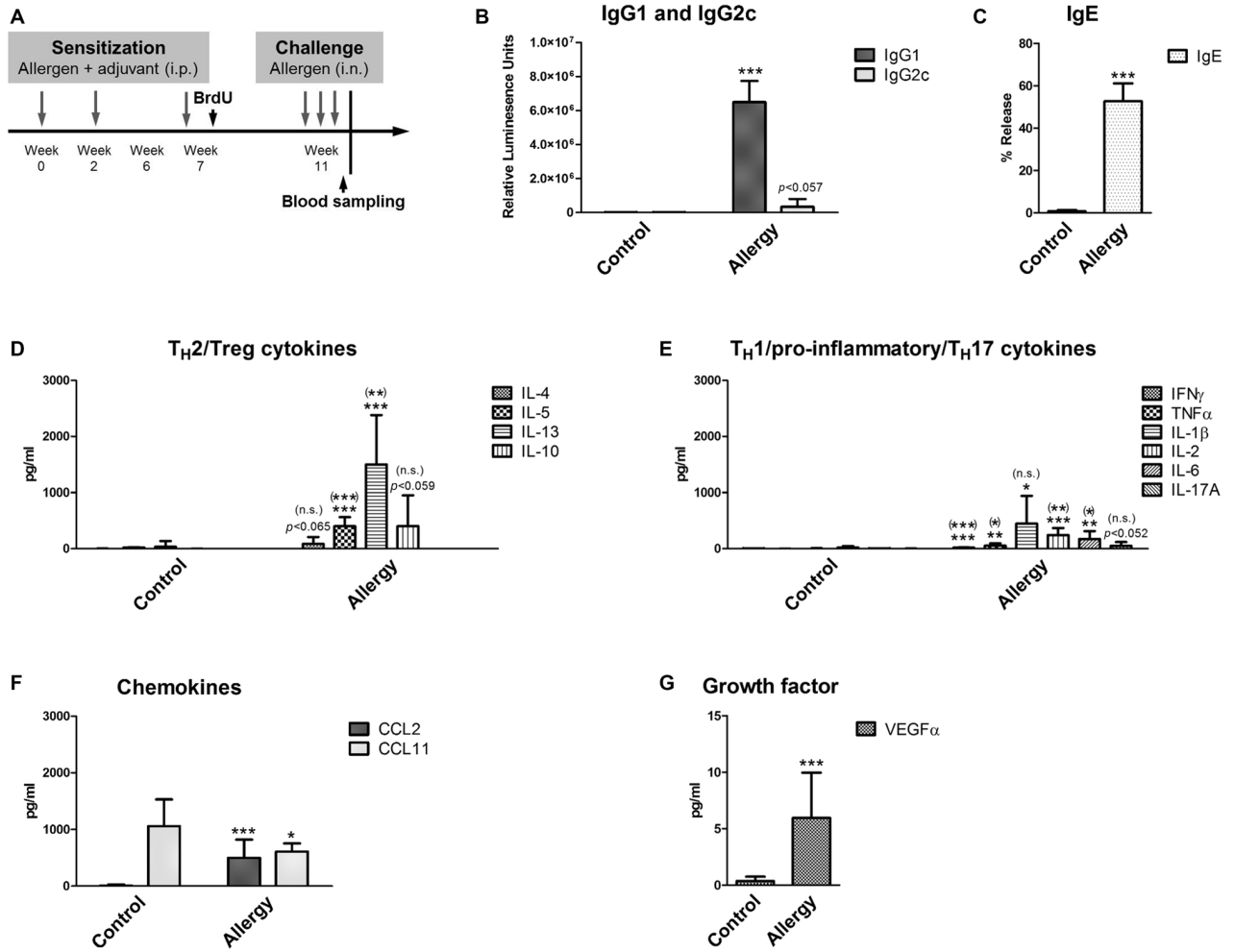

FIGURE 1 | Experimental design and serological analysis. (A) Setup of the experiment. **(B–D)** Serological changes in Phl p 5-sensitized and rechallenged mice confirmed a T_H2-polarized immune response. At the end of the experiment, sera were analyzed for **(B)** Phl p 5-specific IgG1 and IgG2c, and **(C)** biologically functional IgE. **(D)** Serum levels of T_H2 cytokines (IL-4, IL-5 and IL-13) and the T_H2/Treg cytokine IL-10. **(E)** Serum levels of T_H1/pro-inflammatory cytokines (IFNγ, TNFα, IL-1β, IL-2, IL-6) and the T_H17 cytokine IL-17A. **(F)** Levels of the chemokines CCL2 and CCL11 and of **(G)** the growth factor VEGFα in the serum. Data are shown as Mean + SD. (control: $n = 9$, allergy: $n = 10$). Statistical significance was determined using independent samples t-tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.s. - not significant), multiplicity adjusted p-values (Holm-Šídák correction for multiple t-tests) are reported in brackets ((*)$p < 0.05$, (**)$p < 0.01$, (***)$p < 0.001$; (n.s.) - not significant).

RESULTS

Allergic Mice Have a T_H2-polarized Immune Reaction

The allergen used in the present study, Phl p 5, is derived from timothy grass pollen and frequently responsible for allergy symptoms in human patients (Matthiesen and Løwenstein, 1991; Sekerkova et al., 2012). After sensitization, lung inflammation was induced in C57BL/6 mice via intranasal application of the allergen (experimental set-up see **Figure 1A**). To exclude that any of the observed effects were caused by experimental procedures (e.g., handling of the animals or anesthesia), the controls underwent all experimental steps at the same time as the allergy group and received the vehicle solution (PBS) during sensitization and challenge.

First, the allergic status of the sensitized mice was confirmed by measuring blood and lung parameters. For this, the levels of allergen-specific immunoglobulins were determined. In mice, the T_H2 cytokine IL-4 is necessary for the immunoglobulin class switch to IgG1 and IgE, whereas the T_H1 cytokine IFNγ would cause a class switch to IgG2c. Thus, the immunoglobulin measurements (**Figures 1B,C**) showed that mice sensitized to the allergen had a T_H2-polarized immune response, since we observed high IgG1 (**Figure 1B**) and IgE (**Figure 1C**) levels, whereas IgG2c was not significantly elevated in comparison to controls (**Figure 1B**).

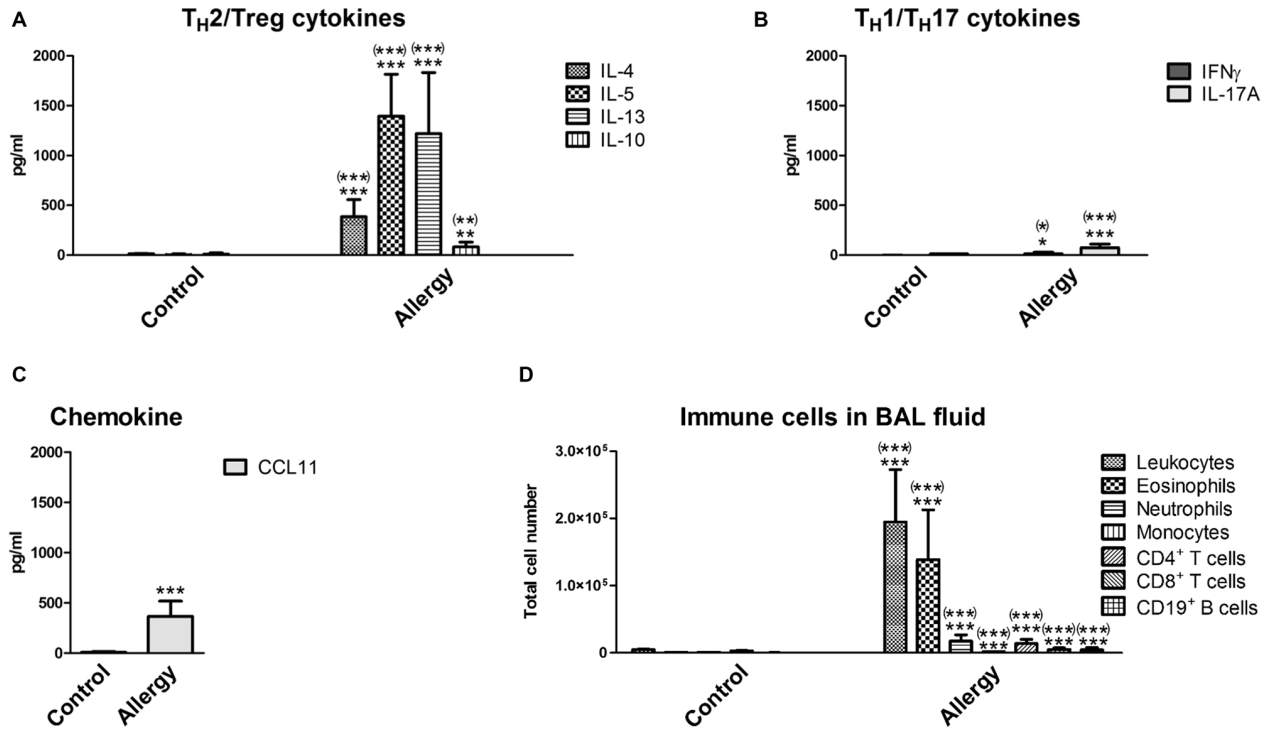

FIGURE 2 | Cytokines and immune cells in bronchoalveolar lavage (BAL) fluid. Analysis of immune parameters in the BAL fluid confirmed a T_H2-polarized immune reaction in the lungs of allergic mice. **(A)** BAL fluid levels of T_H2 cytokines (IL-4, IL-5, and IL-13) and the T_H2/Treg cytokine IL-10. **(B)** BAL fluid levels of the T_H1 cytokine IFNγ, the T_H17 cytokine IL-17A, and **(C)** the chemokine CCL11. **(D)** Immune cells which infiltrated into the lungs were analyzed in the BAL fluid using flow cytometry. Leukocytes, eosinophils, neutrophils, monocytes, T cells ($CD4^+$ or $CD8^+$) and $CD19^+$ B cells were detected. Data are shown as Mean + SD. (control: $n = 9$, allergy: $n = 10$). Statistical significance was determined using independent samples t-tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.s. - not significant), multiplicity adjusted p-values (Holm-Šídák correction for multiple t-tests) are reported in brackets ((*)$p < 0.05$, (**)$p < 0.01$, (***)$p < 0.001$; n.s. - not significant).

As expected, high serum levels of T_H2 cytokines IL-5 and IL-13 were observed in the allergy model (**Figure 1D**). The T_H2 cytokine IL-4 was also modestly, but not significantly, elevated in the allergy model in comparison to controls. The cytokine IL-10 (**Figure 1D**), which can be derived from T_H2 but also from regulatory Tr1 cells, was not significantly increased in allergic mice. Additionally, we found a modest increase of the pro-inflammatory cytokines IFNγ, TNFα, IL-1β, IL-2 and IL-6 in the sera of allergic mice (**Figure 1E**). Also, the serum levels of the T_H17 cytokine IL-17A were slightly, but not significantly, elevated in the allergy model (**Figure 1E**).

The chemokine CCL2, which is an effective attractant for monocytes, was significantly increased in the allergy model, whereas surprisingly CCL11, a signaling molecule attracting eosinophils was significantly decreased in the serum of allergic mice (**Figure 1F**). The growth factor VEGFα was also increased in the allergy model (**Figure 1G**).

In the lungs of the allergic mice similar changes were observed. In the BAL fluid, the levels of the typical T_H2 cytokines (IL-4, IL-5 and IL-13) and of IL-10 were increased (**Figure 2A**). The T_H1 cytokine IFNγ and the T_H17 cytokine IL-17A were also slightly increased (**Figure 2B**). In contrast to the serum, the chemokine CCL11 was markedly elevated in the BAL fluid, which was expected since CCL11 is important for the recruitment of eosinophils (**Figure 2C**). Indeed, the number of infiltrating leukocytes and eosinophils was very high in the BAL fluid of allergic mice (**Figure 2D**). Also neutrophils, T cells ($CD4^+$ and $CD8^+$) and $CD19^+$ B cells invaded the lungs of the allergy model (**Figure 2D**). Unexpectedly, we found a small, but significant, reduction in the number of monocytes in the BAL fluid of allergic mice (**Figure 2D**).

Allergy Modulates Microglia in the Hippocampal Neurogenic Niche

The neurogenic niche of the hippocampus is located in the subgranular zone (SGZ) of the dentate gyrus. The neurons which are generated from the neural stem cells in the SGZ then integrate into the granular layer (GL) of the dentate gyrus (reviewed in Bond et al., 2015). Microglia, the tissue macrophages of the CNS, play an important part in regulating the neurogenic niche (reviewed in Kokaia et al., 2012; Sierra et al., 2014). Since these cells are especially reactive to immune signals from the periphery (Hoogland et al., 2015), we checked whether they are influenced by allergy using the marker Iba1 which

labels microglia and macrophages (**Figure 3A**). Surprisingly, a significant reduction in the number of Iba1$^+$ cells was observed in allergic mice in the GL and SGZ of the dorsal hippocampal dentate gyrus (control: 2456 ± 295 cells per hemisphere, allergy: 1924 ± 275 cells per hemisphere; $p < 0.0013$; **Figure 3B**).

To check if also other commonly used markers for microglial activation were altered in allergic mice, we quantified MHCII, which is important for antigen presentation, and CD68, which is associated with lysosomes. In both, controls and the allergy group, these markers were mainly found in intracellular compartments, presumably lysosomes or endosomes (**Figure 3C**). While significantly fewer Iba1$^+$ cells also expressed MHCII in allergic mice (control: 7172 ± 913 cells/mm^3, allergy: 5555 ± 766 cells/mm^3; $p < 0.0001$; **Figure 3D**), there was no change in the numbers of Iba1$^+$CD68$^+$ cells (control: 10103 ± 609 cells/mm^3, allergy: 10300 ± 1002 cells/mm^3; $p < 0.6345$; **Figure 3E**).

Allergy Activates Microglia in the Granular Cell Layer of the OB

The OB is the brain region into which SVZ-derived newly generated neurons integrate (reviewed in Bond et al., 2015). Using the same markers as in the hippocampus, we analyzed microglial activation in the OB (**Figure 4A**) to find out if the changes we observed were specific for the hippocampus.

In contrast to the hippocampal neurogenic niche, allergy elevated the number of Iba1$^+$ microglia in the granular cell layer of the OB (control: 9890 ± 546 cells/mm^3, allergy: 11271 ± 985 cells/mm^3; $p < 0.0027$) (**Figure 4B**). In parallel, also the numbers of Iba1$^+$MHCII$^+$ cells (control: 8433 ± 919 cells/mm^3, allergy: 9829 ± 915 cells/mm^3; $p < 0.0060$) (**Figure 4C**) and of Iba1$^+$CD68$^+$ cells (control: 9370 ± 638 cells/mm^3, allergy 10676 ± 1123 cells/mm^3; $p < 0.0098$) (**Figure 4D**) increased. This indicates that allergy leads to more activated microglia in the OB.

Allergy Increases the Number of Immature DCX$^+$ Neurons in the Hippocampus

To investigate hippocampal neurogenesis in Phl p 5-sensitized mice after re-exposure to the allergen, we first quantified total cell proliferation in the GL and SGZ of the dorsal dentate gyrus. The marker PCNA labeled cells which were proliferating shortly before the animals were sacrificed, i.e., during allergen challenge (**Figure 5A**). This analysis showed that the number of PCNA$^+$ proliferating cells in the dentate gyrus was not changed in the allergy model (control: 1579 ± 223 cells per hemisphere; allergy: 1606 ± 179 cells per hemisphere; $p < 0.7847$; **Figure 5B**).

Next, we evaluated if there were changes in immature DCX-expressing neurons in the hippocampal neurogenic niche (**Figure 5C**). Indeed, allergic mice had an increased number of DCX$^+$ cells in the GL and SGZ of the dorsal dentate gyrus (control: 2659 ± 337 cells per hemisphere, allergy: 3395 ± 591 cells per hemisphere; $p < 0.0064$; **Figure 5D**).

Taken together these results suggest that even though the proliferation rate at the end of the experiment was not changed, either more immature neurons were generated already earlier after the sensitization phase or the differentiation of DCX$^+$ cells was delayed.

Allergy Increases Production of Mature Neurons (BrdU$^+$NeuN$^+$) in the Hippocampus

For cell fate analysis, mice received a single injection of BrdU after the last sensitization step and 4 weeks before the end of the experiment. BrdU, a thymidine analog, incorporates into the DNA of proliferating cells. Thus, a BrdU$^+$ nucleus indicates a cell that had been dividing at the time of injection, i.e., after the sensitization was completed, and survived until the end of the experiment (**Figure 6A**). A quantification of the total number of BrdU$^+$ cells in the GL and SGZ of the dorsal dentate gyrus showed that the allergic mice had a significantly higher number of BrdU$^+$ cells than the control group (control: 206 ± 40 cells per hemisphere, allergy: 339 ± 107 cells per hemisphere; $p < 0.0046$; **Figure 6B**).

Since the increase in BrdU$^+$ cells in the hippocampal GL and SGZ of allergic mice could not be explained by microglial cells, as their numbers actually decreased, we further analyzed the cell fate of these BrdU$^+$ cells and investigated if they became NeuN$^+$ mature neurons or GFAP$^+$ astrocytes or radial glia (**Figure 6C**). In allergic mice, the number of BrdU$^+$NeuN$^+$ mature neurons increased significantly (control: 1264 ± 271 cells/mm^3, allergy: 1766 ± 382 cells/mm^3; $p < 0.0065$; **Figure 6D**). There was also a slight, but not significant increase in the number of BrdU$^+$GFAP$^+$ cells (control: 446 ± 154; 566 ± 211; $p < 0.2027$; **Figure 6D**). However, the percentages of BrdU$^+$ cells which were either positive for NeuN or GFAP were unchanged (**Figure 6E**).

These results indicate that the observed increase in BrdU$^+$ cells is due to an increased net production of mature NeuN$^+$ neurons in the hippocampal neurogenic niche and not caused by a change in the differentiation fate of the cells.

Allergy Neither Affects the Numbers of BrdU$^+$ Cells in the SVZ nor the Cell Fate of BrdU$^+$ Cells in the OB

To assess if the pro-neurogenic effect of allergy was specific for the hippocampus, or if it was also affecting the other classical neurogenic niche, we analyzed BrdU$^+$ cells in the SVZ (**Figures 7A,B**) and OB (**Figures 7C–E**). In the SVZ, in both groups, hardly any BrdU$^+$ cells were left (**Figure 7A**), and there was no significant difference between the groups (control: 76 ± 33 cells per hemisphere, allergy: 93 ± 37 cells per hemisphere; $p < 0.3188$; **Figure 7B**). Next, we did an analysis of cell fate of BrdU$^+$ cells in the OB (**Figures 7C–E**). There was no significant difference in the densities of BrdU$^+$ cells between the groups (control: 9114 ± 1425 cells/mm^3, allergy: 9396 ± 2105 cells/mm^3; $p < 0.7534$) and also the density of BrdU$^+$NeuN$^+$ (control: 8059 ± 1149 cells/mm^3, allergy: 8216 ± 1819 cells/mm^3;

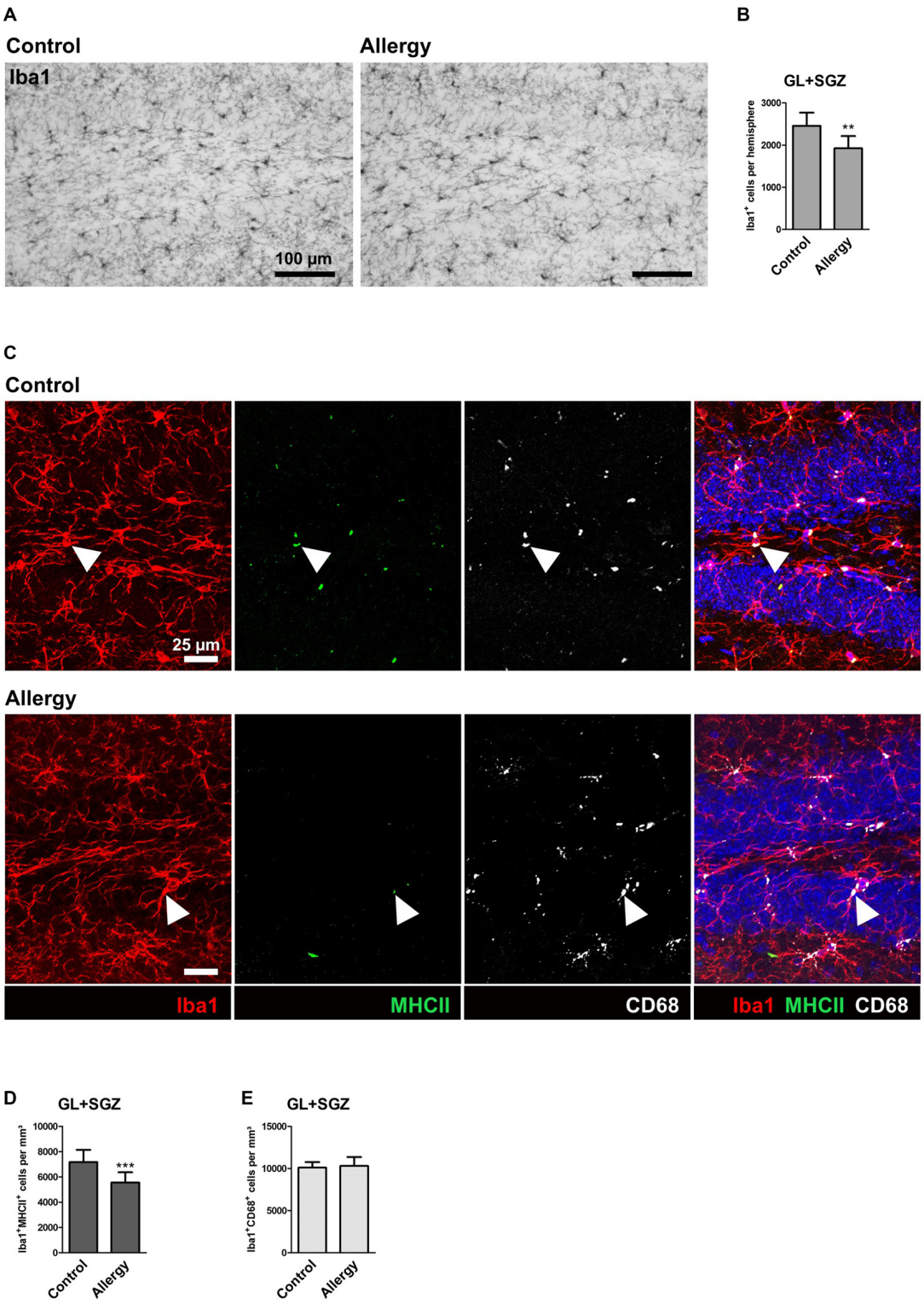

FIGURE 3 | Reduced numbers of Iba1$^+$ microglia and of microglia expressing MHCII in the hippocampal neurogenic niche of allergic mice. (A) Iba1$^+$ microglia in the hippocampal dentate gyrus. **(B)** The number of Iba1$^+$ cells in the granular layer (GL) and subgranular zone (SGZ) of the dentate gyrus is lower in allergic mice than in controls **(C)** Triple labeling of Iba1 (red), MHCII (green) and CD68 (white). Cell nuclei are stained with DAPI (blue). Triple-positive cells are indicated by arrow heads. **(D)** The number of Iba1$^+$MHCII$^+$ cells was decreased in the neurogenic niche of allergic mice, whereas **(E)** Iba1$^+$CD68$^+$ cells were not affected. Values are depicted as Mean + SD. (control: $n = 9$, allergy: $n = 10$). Statistical significance was evaluated using independent samples t-tests and is indicated in comparison to the control group ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Scale bars: **(A)** 100 µm, **(C)** 25 µm.

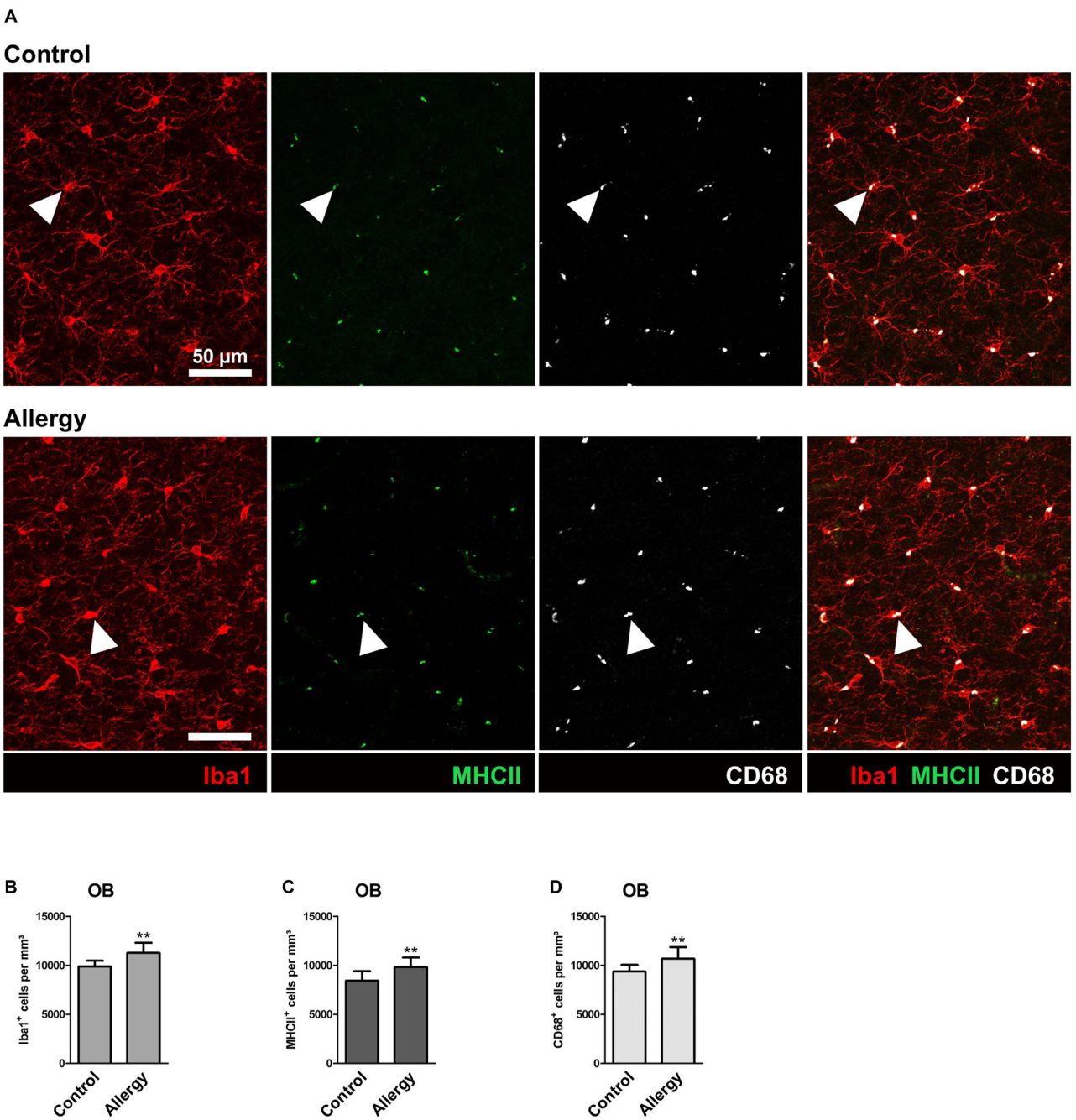

FIGURE 4 | Number of Iba1$^+$MHCII$^+$ and Iba1$^+$CD68$^+$ microglia is increased in the olfactory bulb (OB) of allergic mice. (A) Triple labeling of Iba1 (red), MHCII (green) and CD68 (white). Triple-positive cells are indicated by arrow heads. **(B,C)** Increased numbers of **(B)** Iba1$^+$, **(C)** Iba1$^+$MHCII$^+$ and **(D)** Iba1$^+$CD68$^+$ cells in the granular cell layer of the OB. Values are depicted as Mean + SD. (control: $n = 9$, allergy: $n = 10$). Statistical significance was evaluated using independent samples *t*-tests and is indicated in comparison to the control group ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Scale bars: 50 µm.

$p < 0.8363$) or BrdU$^+$GFAP$^+$ cells (control: 889 ± 355 cells/mm^3, allergy: 990 ± 451 cells/mm^3; $p < 0.6178$) did not change significantly (**Figure 7D**). In both cases, around 90% of these BrdU$^+$ cells in the OB were also positive for NeuN (control: 88.6 ± 2.9%, allergy: 87.5 ± 3.6%; $p < 0.4834$; **Figure 7E**).

DISCUSSION

In the present study, the effects of a T_H2-polarized systemic inflammation on the neurogenic niche in the hippocampus were analyzed in a model of grass pollen allergy. Surprisingly, allergy seems to have a positive impact on the production of new

neurons and leads to a down-regulation of microglial activation in this region.

The analysis of immunological parameters in sera and lungs confirmed that in the allergy model a T_H2-polarized allergic reaction was induced. As expected, the allergy model showed the typical immunoglobulin pattern for an allergic immune response (high levels of allergen-specific IgE and IgG1, low levels of IgG2c). In addition to a robust increase of T_H2 cytokines in serum and BAL fluid, also a modest, but significant, induction of pro-inflammatory cytokines was observed. Moreover, CCL2 an important chemoattractant for monocytes was increased in the serum. These immune parameters are in line with what has been described for patients suffering from allergies affecting the airways (Kuna et al., 1996; Holgate et al., 1997).

Microglia are the tissue macrophages of the CNS and are responsible for CNS immune surveillance. They react to pathogenic events and are "activated" in a multi-step process (Kettenmann et al., 2011), which leads to an upregulation of specific proteins, e.g., MHCII for antigen-presentation or CD68 which is associated with lysosomes and endosomes (Boche et al., 2013). Microglia are also part of the hippocampal neurogenic niche, and have regulatory functions there (Gemma and Bachstetter, 2013; Sierra et al., 2014). The allergic immune response in our model affected these immune cells in the hippocampal neurogenic niche in an unexpected way: allergy led to a "deactivation" of microglia in this region, since both, their numbers and their MHCII expression were reduced.

Actually, there is one study showing that—in the absence of inflammatory stimuli—the rate of neurogenesis and microglial numbers, specifically in the dentate gyrus, are inversely correlated (Gebara et al., 2013). This fits to our results, since we observed an increase of neurogenesis accompanied by a reduction in microglial numbers ($Iba1^+$ cells). The further reduction of microglia that are expressing MHCII (from 58.8 ± 5.1% in controls to 44.7 ± 4.6% in allergic mice, $p < 0.00001$; data not shown) might suggest that the affected microglia were either less phagocytic or less inclined to present the phagocytosed antigens as MHCII is involved in this process. A reduced phagocytic activity could be explained by a lack of cellular debris due to an increased survival of newly generated immature DCX^+ neurons, since superfluous progenitors are normally phagocytosed by microglia (Sierra et al., 2010). However, the number of microglia expressing CD68, a widely used marker for microglial activation, which is located in phagosomes and lysosomes, was

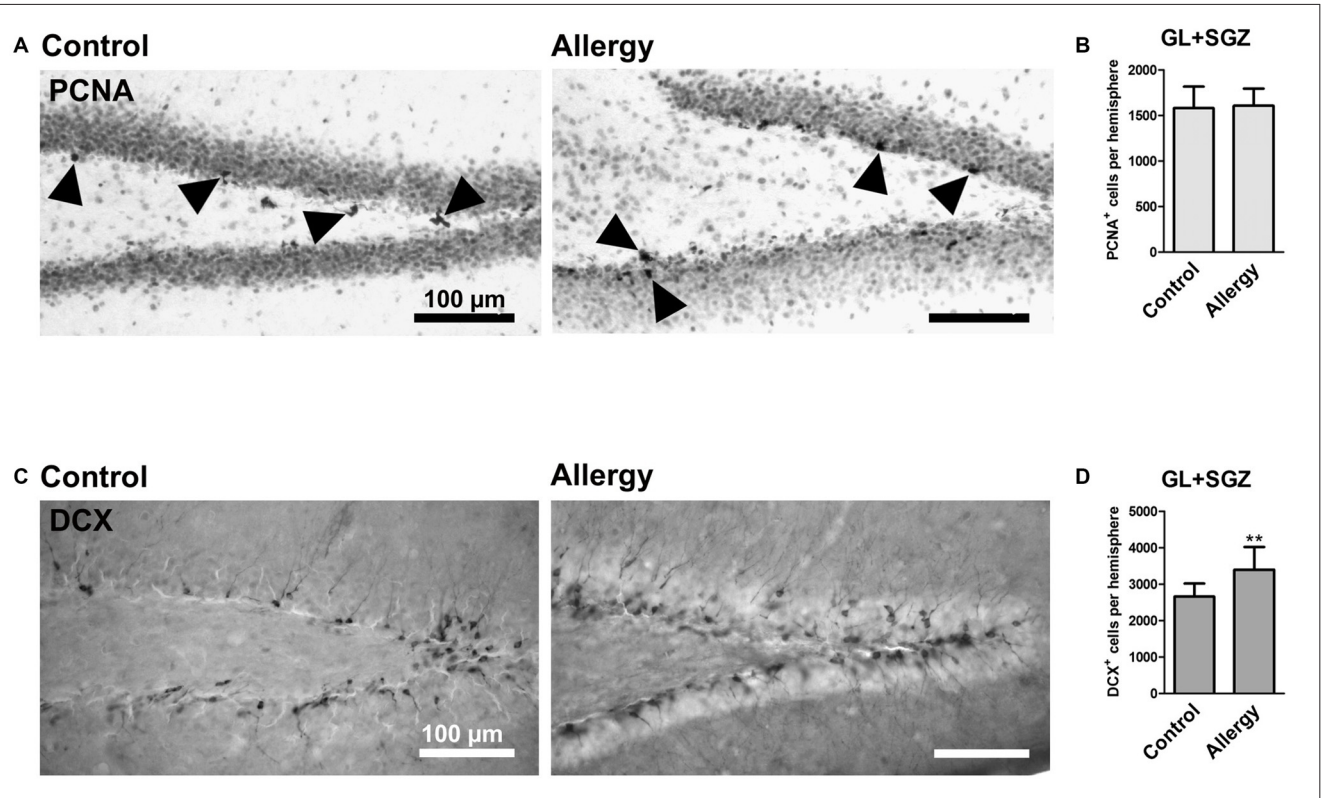

FIGURE 5 | More DCX^+ immature neurons, but unchanged cell proliferation, in the hippocampal dentate gyrus. (A) Proliferating cells stained with PCNA (arrowheads) in controls and the allergy group. **(B)** Allergic mice and controls had the same number of $PCNA^+$ cells in GL and SGZ of the dentate gyrus. **(C)** DCX^+ immature neurons in controls and allergy group. **(D)** Allergic mice had significantly more DCX^+ cells in GL and SGZ. Values are depicted as Mean + SD (control: $n = 9$, allergy: $n = 10$). Statistical significance was evaluated using independent samples t-tests and is indicated in comparison to the control group ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Scale bars: 100 µm.

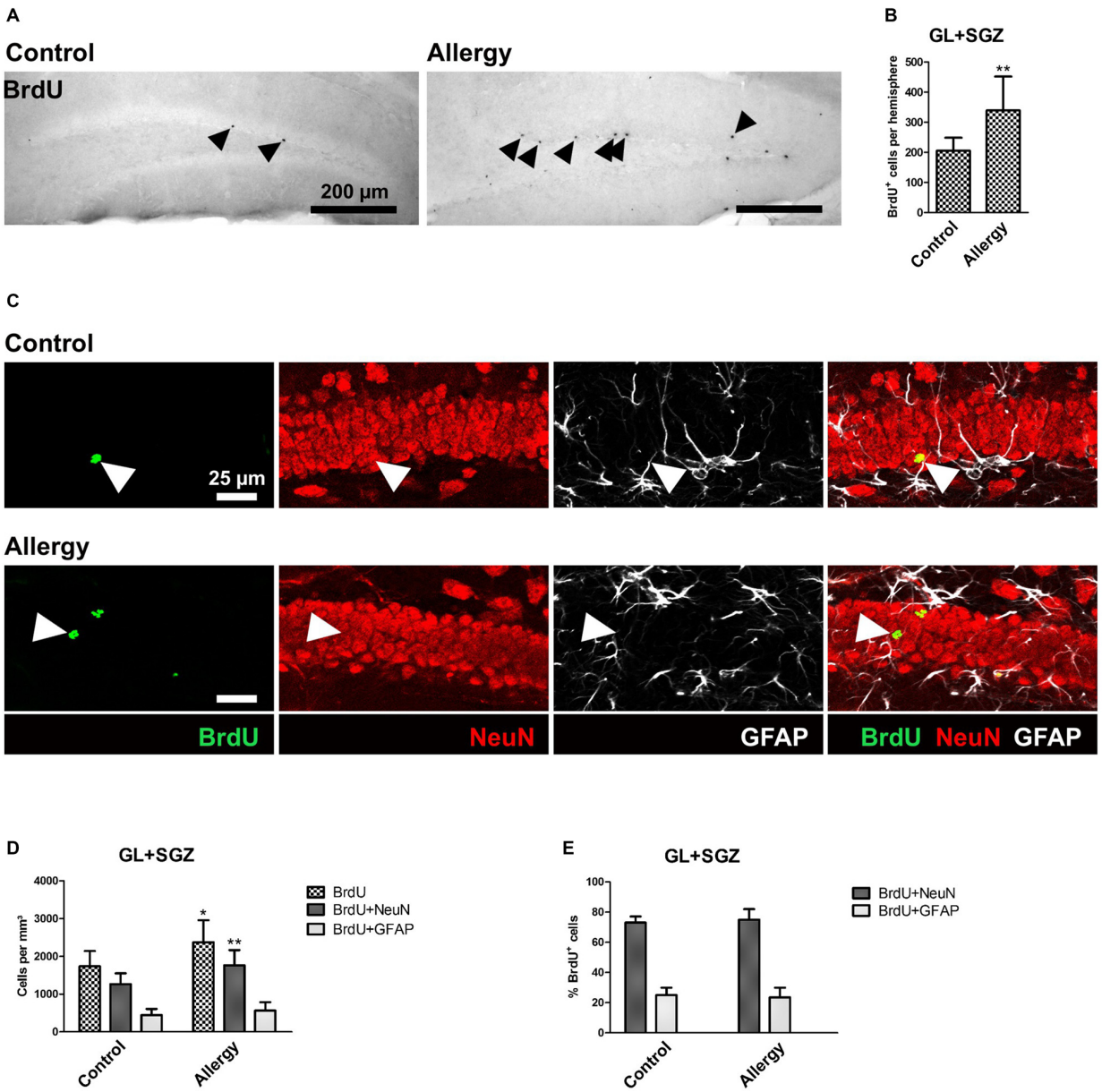

FIGURE 6 | More BrdU$^+$ and BrdU$^+$NeuN$^+$ cells in the hippocampal dentate gyrus. (A) BrdU$^+$ cells in the dorsal dentate gyrus. BrdU was injected once 4 weeks before the end of the experiment. **(B)** Increased number of BrdU$^+$ cells in the hippocampal neurogenic niche in allergic mice. **(C)** Triple labeling of BrdU (green), NeuN$^+$ mature neurons (red) and GFAP$^+$ astrocytes (white). BrdU$^+$NeuN$^+$ mature neurons are indicated by arrowheads. **(D)** In allergic mice not only the number of BrdU$^+$ cells, but also of BrdU$^+$NeuN$^+$ mature neurons increased in comparison to controls. **(E)** The percentage of BrdU$^+$ cells which became mature NeuN$^+$ neurons or GFAP$^+$ radial glia (or astrocytes) remained unchanged. Values are depicted as Mean + SD. (control: $n = 9$, allergy: $n = 10$). Statistical significance was evaluated using independent samples t-tests and is indicated in comparison to the control group ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Scale bars: **(A)** 200 µm, **(C)** 25 µm.

unaffected, suggesting that allergy affects predominantly antigen presentation.

At first glance, our findings are in contrast to numerous studies which found that microglia are activated by systemic inflammation (reviewed in Hoogland et al., 2015). However, those studies exclusively used stimuli inducing T_H1-polarized immune responses, i.e., LPS, bacteria, or viruses (reviewed in Hoogland et al., 2015), which could explain the different outcome. Moreover, in our allergy model, the deactivation of microglia seemed to be specific for the hippocampal dentate gyrus, since we actually observed an activation of microglia accompanied by upregulation of MHCII and CD68 in the OB (**Figure 4**). The OB is not only the region into which SVZ-derived newly generated neurons are integrated, but also seems to contain an especially reactive sub-population of microglia (Lalancette-Hebert et al., 2009), which could explain why

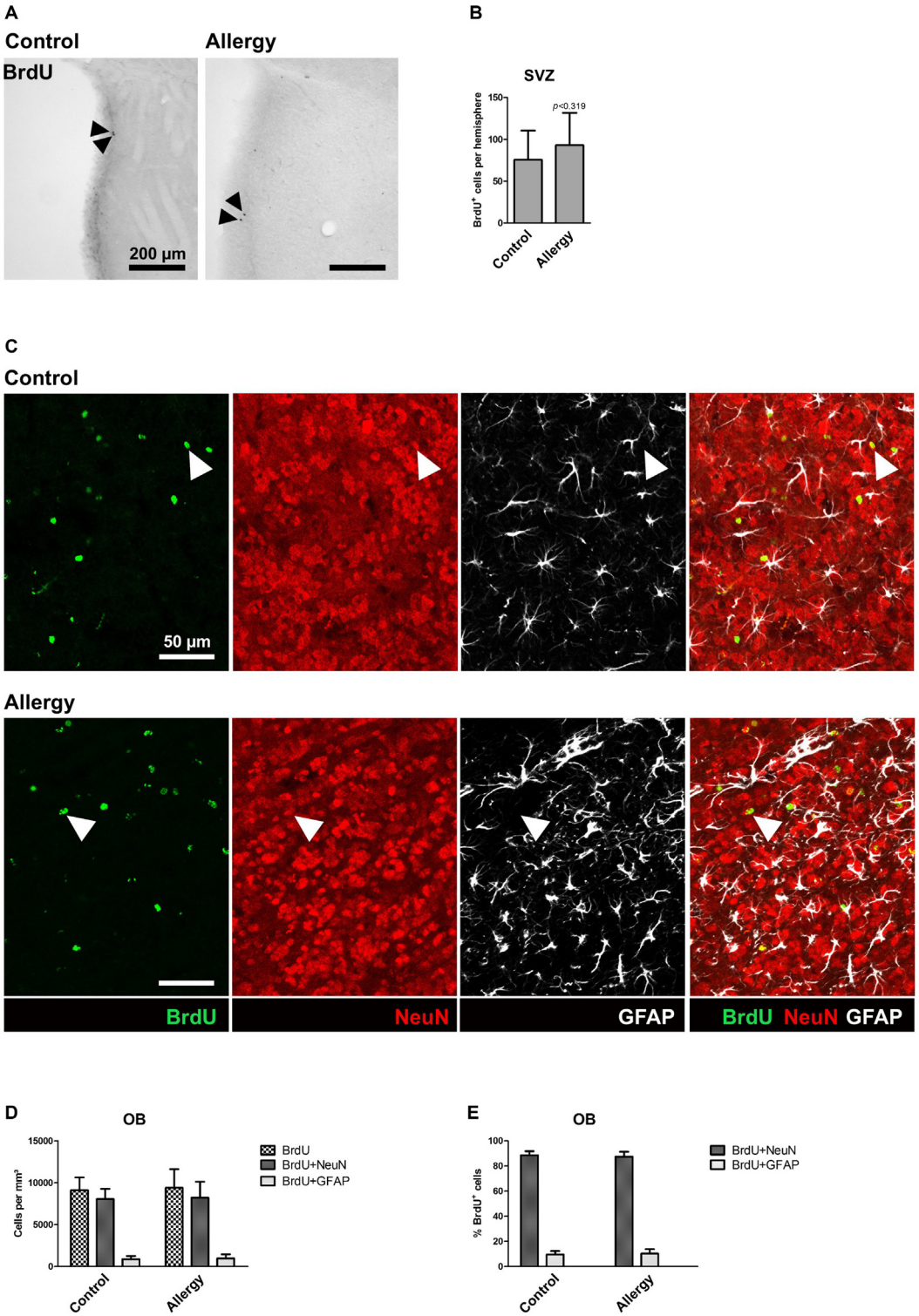

FIGURE 7 | Unchanged number of BrdU$^+$ cells in the subventricular zone (SVZ) and no alterations in cell fate of BrdU$^+$ cells in the OB. (A) BrdU labeling in the SVZ. Arrowheads indicate BrdU$^+$ cells. **(B)** There is no significant difference in the number of BrdU$^+$ cells in the SVZ between controls and allergic mice. **(C)** BrdU labeling in the OB. The images show a triple labeling of BrdU (green) NeuN (red) and GFAP (white). Arrowheads indicate BrdU$^+$NeuN$^+$ cells. **(D,E)** Neither the density **(D)** nor the percentage **(E)** of BrdU$^+$NeuN$^+$ or BrdU$^+$GFAP$^+$ cells in the OB differs significantly between controls and allergic mice. Values are depicted as Mean + SD (control: $n = 9$, allergy: $n = 10$). Statistical significance was evaluated using independent samples t-tests and is indicated in comparison to the control group ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Scale bars: **(A)** 200 µm, **(C)** 50 µm.

allergy has an opposite effect on microglia in this region. Alternatively, the intranasal delivery of the allergen might have a much more pronounced effect on the OB system compared to the hippocampus. For this reason, it might be interesting to investigate whether this difference between hippocampal and OB microglia is also present in allergy models affecting other parts of the organism, e.g., the skin.

Why are microglia deactivated in the hippocampus of allergic mice? It is tempting to assume that this might be a regulatory mechanism protecting the hippocampus, which is central for many important processes, from the immune response in the periphery. An alternative hypothesis would be that this down-regulation is directly caused by the elevated levels of T_H2 cytokines in the blood. It is even more challenging to speculate about the functional consequences of this observed down-regulation of microglial activation below the normal "surveying state" in the young hippocampus. If immune surveillance in the hippocampus is down-regulated for extended periods, this may have detrimental consequences. However, with the current experimental set-up we do not know if this microglial deactivation is transient or persists for longer periods. Of course, the allergy-induced changes in hippocampal microglia were rather subtle, which could be due to the fact that the starting point for the down-regulation was a young healthy condition. Therefore, it would be highly interesting to investigate what allergy does to microglia in the aged CNS, which might already be somewhat primed for a pro-inflammatory activation (Norden and Godbout, 2013).

Concomitantly with microglial deactivation, hippocampal neurogenesis was increased, i.e., we observed higher numbers of DCX^+ immature neurons and $BrdU^+NeuN^+$ mature neurons. Since BrdU was injected after the sensitization period (4 weeks before the end of the experiment) and the allergen challenge started only 4 days before the animals were sacrificed, it seems that already the sensitization has an impact on hippocampal neurogenesis. With the current experimental setup it is only possible to analyze the cumulative effect of both phases, but it would be interesting to study the effect of sensitization alone, and further time points in which also the challenge period is extended. Additionally, it would be worth investigating whether alternative sensitization and challenge routes (e.g., in a model for food allergy) have a similar impact on microglia and neurogenesis in the hippocampus.

So far, we can only hypothesize that the observed increase in hippocampal neurogenesis may also have functional consequences on long-term potentiation or learning and memory. For this, further studies including electrophysiological analysis and behavioral tests are needed.

We did not observe any changes in proliferation at the end of the challenge period. This is in contrast to a study which showed that in immature mice, chronic asthma leads to a reduced proliferation in the hippocampal neurogenic niche (Guo et al., 2013). An explanation for these different results could be that the latter study particularly investigated animals of a very young and thus, probably more vulnerable age, starting the OVA sensitization in 3-weeks-old mice and then extended the OVA challenge over a period of 9 weeks (Guo et al., 2013), whereas the present allergy model started in young adult mice (2-months-old) and the actual challenge period only lasted for 4 days. Moreover, commercially available OVA often contains substantial levels of LPS, which lead to additional and potentially confounding immune responses, whereas the Phl p 5 used in the present study was essentially LPS-free. However, similar to the study of Guo et al. (2013), also in our model an increase of serum levels of VEGFα was observed. Within the neurogenic niche, VEGFα supports neurogenesis (Schänzer et al., 2004; reviewed in Kokaia et al., 2012), and it also has been shown that peripheral VEGF is necessary for exercise-induced neurogenesis (Fabel et al., 2003). Taken together, these results suggest that a peripheral increase of VEGF might affect neurogenesis, but this is probably context-dependent.

High serum levels of CCL11 (eotaxin-1) were recently described to inhibit hippocampal neurogenesis (Villeda et al., 2011). While in the BAL fluid, as expected, elevated levels of CCL11 were only found in the allergy group, in serum both, control mice as well as allergic mice, displayed high levels of CCL11. In fact, serum levels of CCL11 were even slightly decreased in allergic mice. This is in line with data showing that eotaxin is elevated in nasal secretion of allergic patients during the pollen season, whereas no difference was observed in eotaxin serum levels—neither in or out of pollen season, nor between healthy and allergic donors (Pullerits et al., 2000).

Depending on the type of immune response, systemic inflammation might elicit diverse effects on the CNS. Our data suggests that a T_H2-polarized allergic immune response might promote neurogenesis and down-regulate microglia in the hippocampus. However, at the moment it is not clear if this also has a beneficial effect on CNS functions and what happens if this immune response persists for a longer time. Clearly, more experiments investigating the impact of different types of systemic inflammation on the CNS are needed to further the understanding about the interplay between peripheral immune activation and CNS functions.

AUTHOR CONTRIBUTIONS

BK: designed the study, analyzed and interpreted data, did histology and microscopy, and drafted the manuscript. HM: participated in the histology. SS participated in the analysis of the immunological parameters in sera and lung fluids. JT: participated in the design of the study and discussion of the results. RW: designed the study, analyzed and interpreted data, carried out the animal treatments and analysis of the blood and lung parameters. LA: participated in the study design and coordination, and the discussion of the results. All authors contributed to revising the manuscript and approved the final version.

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