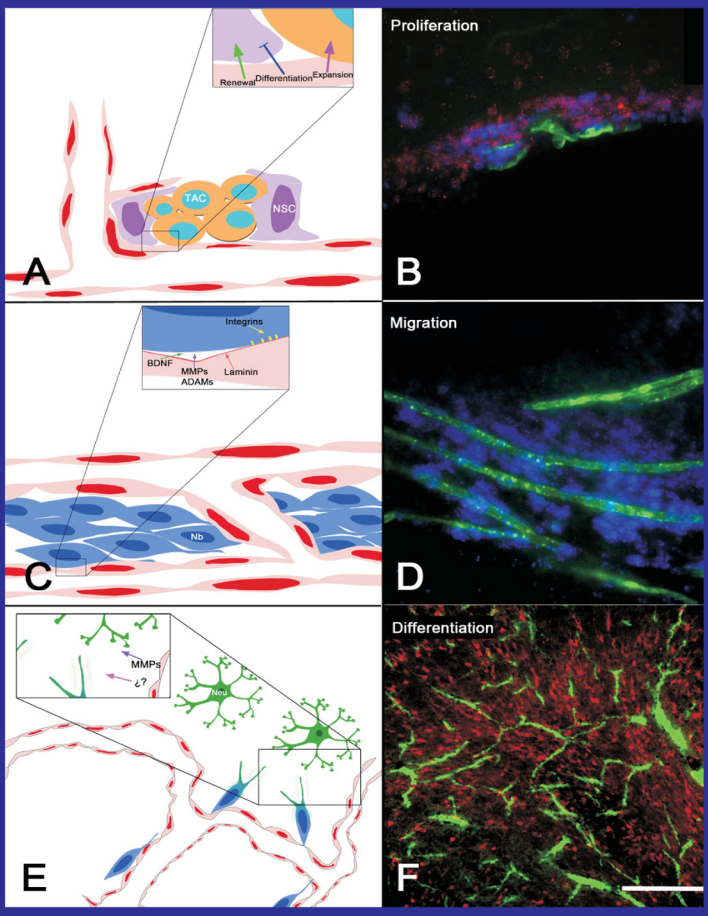
Endothelial Cell Plasticity in the Normal and Injured Central Nervous System



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Preface

It was once thought that blood vessels were passive elements that only provided metabolic and structural support for the central nervous system (CNS). However, the vasculature and the cells that comprise it are dynamic and may participate in many, if not all, aspects of CNS function. Endothelial cells, in particular, are highly heterogeneous and very plastic entities well suited and ideally placed to mediate the interactions between the vascular tree and the rest of the nervous system. There was a need to produce this volume to make the reader conscious of the fact that blood vessels have been long neglected with regard to their importance for assuring CNS function, development and maintenance. We think the chapters in this book are proficient in transmitting this point of view.

The title of this book emphasizes the very important fact that endothelial cells are capable of plastic changes. There are many examples of this throughout the book, some of which will be mentioned here. The basilar artery derives from migration of vein endothelial cells, showing that their phenotype is modifiable (Chapter 1). The microenvironment where endothelial cells reside in the forebrain determines the expression of homeobox transcription factors, which in turn determines their response to environmental signals (Chapter 1). The cells of the endothelium show extensive phenotypic heterogeneity and also functional diversity (Chapter 2). This variety favors the establishment and maintenance of compartmentalized microenvironments such as: neurogenic niches (SGZ and SVZ, Chapter 2), migratory routes (RMS, Chapter 2), sites that extensively contact blood-borne molecules (circumventricular organs, Chapter 2), regions where neurons differentiate (olfactory bulb) and locations that allow neuro-endocrine communication (median eminence, Chapter 4). Vascular cells, in particular the endothelium, are responsible for the dynamic and complex changes in blood flow and blood-brain barrier transport efficiency as a function of the local synaptic activity (neurovascular and neurobarrier coupling, Chapter 3). Plasticity of endothelial cells in the median eminence is ultimately in control of female reproduction (Chapter 4). Whether a cause or consequence of pathologies (drug-resistant epilepsy), the expression of transporter or carrier proteins is altered in endothelial

cells (Chapter 5). Tumor neovascularization is extremely plastic; the vessels can grow by intussusceptive or proliferating angiogenesis, differentiation of cancer stem cells and vasculogenic mimicry (Chapter 6). Plasticity or heterogeneity of endothelial cells causes the differences between the blood-brain barrier and blood-spinal cord barrier which render the spinal cord more susceptible to certain lesion types (Chapter 7). Changes in the phenotype of endothelial cells participate importantly in the damage and recovery from stroke (Chapter 8). Evidently, the plasticity of endothelial cells has a limit, revealed by the fact that they are as vulnerable as neurons to the diverse types of damage observed in Alzheimer´s disease (Chapter 9). Endothelial cells also crucially participate in neuro-immune communication in both health and disease. For example, leukocyte entry into the CNS during inflammation requires their interaction with endothelial cells via adhesion ligand molecules and chemokine receptors. Under non-inflammatory conditions the sites of leukocyte migration into the CNS are the choroid plexus and the meninges. The chemokines and adhesion molecules also underlie leukocyte-endothelial cell communication at these sites.

Chapter 1 shows that vascular development in the CNS is stereotyped but nonetheless requires active interaction between all cells comprising the neurovascular unit. Thus, the formation and preservation of the CNS and its vessels are mutually dependent to ensure their proper operation. Chapter 2 discusses the idea that endothelial cells contribute to the generation of new neurons from undifferentiated neural progenitors, which originate from multi-potent neural stem cells. Endothelial cells may participate in neural stem cell proliferation, neuroblast migration and differentiation of neurons during development and in the adult. Moreover, the phenotypic heterogeneity and functional diversity of endothelial cells, which arise with age, seem to be important for the spatial and temporal restriction of neurogenesis to specialized microenvironments known as neurogenic niches. Chapter 3 reveals the complexity of the diverse blood-neural barriers and their importance for the homeostasis of the nervous system. Even blood flow to the brain and blood-brain barrier permeability are dynamic and complex processes, since both change as a function of local neural activity. Chapter 4 explains that the vasculature of the median eminence is similar to a neurogenic niche. Additionally, endothelial cells importantly participate in female reproduction by stimulating the withdrawal of the tanycyte outgrowths that cover the nerve terminals of neurons that secrete gonadotropin-releasing hormone and by promoting axonal sprouting from these neurons in the direction of the pituitary portal blood vessels. Chapter 5 reveals that over-expression of drug transporter proteins in the endothelial cells which comprise the blood-brain barrier may be responsible for drug-resistant epilepsy. Chapter 6 discusses the diverse molecules that participate in tumor growth, primary and secondary tumor cell invasion

as well as tumor blood vessel angiogenesis. Chapter 7 expands upon the vessels of the spinal cord as well as vascular dysfunction, blood-spinal cord disruption, endothelial cell death and angiogenesis that follow spinal cord injury. Chapter 8 also touches upon angiogenesis and remodeling of the vasculature after stroke, detailing their molecular mediators. Finally, Chapter 9 highlights the importance of energy metabolism and blood vessel function in Alzheimer's disease.

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Contents

CHAPTER 1

Early Development of the Vascular System Supplying the Brain

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1.1 Introduction

Vascular development in the embryonic and fetal central nervous system (CNS) is a highly stereotypical process that begins at the cervical levels of the neural tube (NT) and progresses towards the caudal and cephalic poles of the embryo following the process of neurulation. Until a few years ago, the ontogeny of blood vessels within the nervous system was believed to be a "passive" process subordinated to the metabolic demands of the neural tissue (Park et al. 2003). An increasing body of evidence, however, shows that vascularization in the developing brain proceeds through a relatively autonomous process. The vascular network guarantees proper neuronal

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development by providing nutrients, migratory guidance and trophic modulatory factors that allow neural cells to proliferate and differentiate (Sun et al. 2010). Blood vessels also modulate synaptic plasticity (Hopper and Garthwaite 2006) and the interactions among neural, immune and endocrine cells during development (Matsumura and Kobayashi 2004). In addition, it is now widely recognized that the patterning of the brain's vascular and neural networks is coordinated by the same set of molecular signals (Zacchigna et al. 2008). Early neurovascular communication thus establishes a life-long relationship between these systems that guarantees the proper function of the CNS. On the other hand, deficient neurovascular communication leads to several pathological conditions (Girouard and Iadecola 2006).

The formation of blood vessels throughout the nervous system, as in the rest of the body, may occur *de novo* (i.e., vasculogenesis) or from pre-existing vessels (i.e., angiogenesis). In turn, angiogenesis encompasses two mechanisms (Risau 1997). The first one, known as non-sprouting angiogenesis, is characterized by blood vessel elongation and intussusception in the absence of cell proliferation (Burri et al. 2004). The second one, termed sprouting angiogenesis, leads to the growth of blood vessels following the proliferation of endothelial cell precursors (Heinke et al. 2012). A combination of these processes produces the intricate vascular patterns observed in the adult brain.

Vascular development in the vertebrate nervous system is a complex process. Throughout this chapter, information obtained from a variety of animal models is used to elaborate a general picture in an effort to provide the reader with a comprehensive view on how the vertebrate nervous system is vascularized.

1.2 Vascular Patterning in the Neural Tube

During embryonic development, the brain and spinal cord are formed by morphogenetic movements combined with the processes of differential growth and remodeling that shape the NT. Once formed, the NT enlarges at its rostral-most segments giving rise to the primary brain vesicles: the caudal-most rhombencephalon, the mesencephalon and cephalic-most prosencephalon. Each of these vesicles is transiently divided along its anterio-posterior axis by metameric units called neuromeres. These subdivisions represent morphogenetic fields and hence define cell migration and connectivity patterns, as well as cell fate within them (Puelles and Rubenstein 2003, Kiecker and Lumsden 2005). As development proceeds, differential proliferation in the rhombencephalon and prosencephalon leads to the formation of the mielencephalon/metencephalon and diencephalon/secondary prosencephalon, respectively, thus generating the so-called secondary brain vesicles (Puelles et al. 2013).

Early vascular patterning of the NT resembles that of the neural tissue during neurulation (Copp et al. 2003); however, angiogenesis precedes neurogenesis (Gotz 2013). The formation of blood vessels is triggered by NT-derived signals that induce the elaboration of the perineural vascular plexus (PNVP) around it. In the brain *primordium*, the PNVP is formed through vasculogenesis and angiogenesis. Angioblasts originating from head paraxial mesoderm (i.e., vasculogenesis) and from the cephalic vascular plexus (i.e., angiogenesis) are incorporated within the PNVP vascular network (Walls et al. 2008). At the spinal cord, the PNVP is formed by angioblasts coming from the somites and the lateral mesoderm (i.e., vasculogenesis) (Klessinger and Christ 1996, Pardanaud et al. 1996, Pardanaud and Dieterlen-Lievre 1999, Ambler et al. 2001) and from the vertebral artery and dorsal longitudinal anastomotical vessel (i.e., angiogenesis) (Walls et al. 2008). Later, the PNVP originates the intraneural vascular plexus (INVP), which supplies the CNS with nutrients and oxygen (James and Mukouyama 2011) through a process of angiogenesis (Lee et al. 2009). The development of the PNVP and INVP follows a stereotypical pattern proceeding rostrally from the rhombencephalon (Walls et al. 2008), caudally from the cervical segments of the spinal cord and from ventral to dorsal along the NT (Kurz et al. 2004) (Fig. 1A).

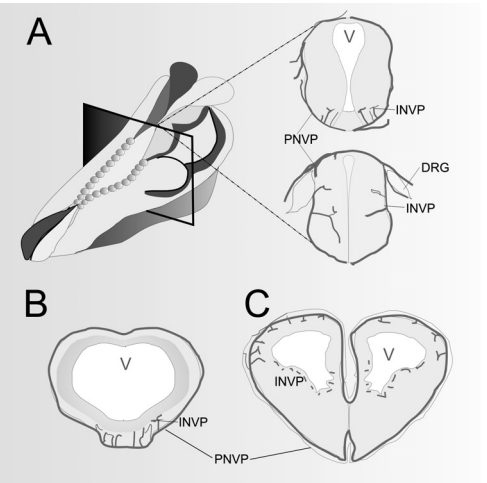

Figure 1. Early brain vascularization follows a stereotypical pattern. Neural tube closure instructs perineural vascular plexus (PNVP) formation, which starts at the rhombencephalic-cervical boundary (A, *left*). Intraneural vascular plexus (INVP) development is modulated by neuromere-specific local cues as demonstrated by its entry points into the central nervous system. PNVP-derived vessels sprout into the rhombencephalon (A, *top right*) and mesencephalon (B) ventrally while they enter the spinal cord laterally (A, *bottom right*). In the prosencephalon, pial and ventricular vessels develop independently (C). DRG= dorsal root ganglion, V= ventricle.

Color image of this figure appears in the color plate section at the end of the book.

1.2.1 Ontogeny of the Perineural Vascular Plexus

In mice, the cardiovascular system begins its formation by embryonic day (E) 7.3. At this age, the *endocardium primordia* appears in the thoracic segment of the embryo and a few hours later (E7.8), the aortic *primordia* can be clearly identified. A day later, the *endocardium* of the heart as well as the ventral and dorsal aortic segments are in place. A complete circulatory system is observed between E8–8.5 (Drake and Fleming 2000). Roughly by the same time, the cephalic vasculature begins the formation of the PNVP (Walls et al. 2008). At first, disconnected clusters of endothelial cells are identified throughout the cephalic mesenchyme and lateral mesoderm. Shortly afterwards, these cell clusters aggregate to form a rudimentary plexus at the level of the occipital somites. Then, capillaries grow towards the rostral and caudal aspects of the embryo. The latter reach the cervical somites a bit later in development. The NT is completely encased by the PNVP by E9.5 (Walls et al. 2008), an age that corresponds to incubation day (ID) four in chicken embryos (Kurz 2009).

The ontogeny of the PNVP is regulated by NT-derived signals, most important among them vascular endothelial growth factor (VEGF)-A. This factor induces differentiation and migration of presomitic mesoderm-derived endothelial progenitors after binding to the VEGF-2 receptor (VEGFR-2; Hogan et al. 2004). In addition, the PNVP in each side of the embryo develop independently from each other (Kurz and Christ 2002, Kurz 2009) due to the presence of NT-released chordin and noggin that work as endothelial cell repellent signals along the midline (Reese et al. 2004) (Fig. 2).

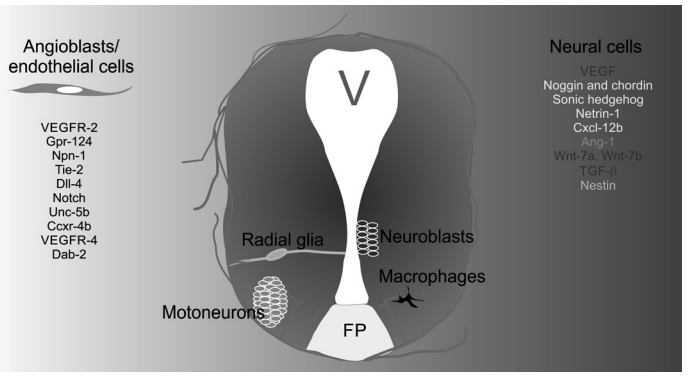

Figure 2. Neural tube-derived cues regulate perineural vascular plexus (PNVP) formation and vessel entry into the central nervous system (i.e., intraneural vascular plexus development). Some of the molecules expressed in angioblasts or endothelial cells are shown on the left, while neural tissue-derived signals are shown on the right (color code indicates the specific cell type that produces the protein). FP= floor plate, V= ventricle. For abbreviations of the molecules see text.

Color image of this figure appears in the color plate section at the end of the book.

1.2.2 Ontogeny of the Intraneural Vascular Plexus

In mouse embryos between E9.5 and E10, the PNVP forms branches that penetrate the developing NT from the *pia mater* deep into the ventricles (Kurz et al. 1996, Fantin et al. 2013). In vertebrates, this process starts at the rhombencephalic floor plate (Kurz et al. 1996) and then moves towards the cephalic and caudal poles of the embryo (Rovainen and Kakarala 1989, Kurz 2009, Marin-Padilla 2012) (Fig. 1A, *top right*). Blood vessels entering the spinal cord branch from primitive arteries (Kurz et al. 1996). These blood vessels reach the transition zone located between the ependymal and mantle layers, where they give rise to arch-like ventral sprouts (Kurz and Christ 2002) that later join lateral venous sprouts originating from the PNVP (Kurz et al. 2004). INVP angiogenesis at the forelimb bud level in mouse embryos follows a slightly different pattern to that seen in the chick embryo at cervical levels. It begins in the lateral border of the NT at E9.8 and then grows following a medial-ventral gradient until reaching the arch-like vessels in the floor plate at E10.2 (Nagase et al. 2005) (Fig. 1A, *bottom right*).

Since the INVP is formed by angiogenesis, it involves endothelial cell proliferation, migration and maturation. All these processes are known to be modulated by NT-derived growth factors (Risau 1997). Accordingly, in the chick spinal cord, the initial angiogenic sprouts are induced by the VEGF-VEGFR-2 pathway (James et al. 2009). In mice, this same process seems to be regulated by angiopoietin (Ang)-1, sonic hedgehog (Nagase et al. 2005) and epidermal growth factor (Sato et al. 1995). In addition, the orphan G protein-coupled receptor-124 (Anderson et al. 2011), transforming growth factor (TGF)-β (Mu et al. 2008) and wingless-related integration site (Wnt)-7a or 7b (Stenman et al. 2008) are required for the occurrence of normal angiogenic sprouting from the PNVP along the neural axis (Fig. 2).

Lastly, the final architectural features of the CNS vasculature require tightly controlled processes that involve a myriad of factors. For example, blood vessel fine tuning within the hindbrain requires the presence of neuropilin (Npn)-1 (Gerhardt et al. 2004), delta-like (Dll)-4 ligand (Suchting et al. 2007) and netrin-1 (Lu et al. 2004) to either facilitate or inhibit angiogenic sprout formation and branching (Fig. 2).

1.3 Vascularization in the Developing Brain

As previously mentioned, the process of vascularization throughout the vertebrate embryonic-fetal CNS generally follows specific gradients that are determined by multiple cues, such as NT closure (Copp et al. 2003, Kurz et al. 2004) and segmentation (Vasudevan et al. 2008), VEGF expression levels (Breier et al. 1992, Kawasaki et al. 1999) and local instructive signals released by discrete cellular domains (Hashimoto et al. 2001, Sure et al.

2001). Hence, this section of the chapter will review literature that clearly illustrate these general principles.

1.3.1 Development of the Hindbrain Vascular Network

During early vertebrate development, the full extent of the hindbrain is divided into eight units called rhombomeres (Kiecker and Lumsden 2005). In the zebrafish, the first vascular buds emerge from the primordial hindbrain veins. These buds ramify through the rostral aspect of the hindbrain most frequently at the levels of rhombomeres two, three or five, less often at rhombomeres four and six and never at rhombomere one, seven or eight (Fujita et al. 2011). Blood vessels first invade the center of each rhombomere to then grow dorsally until reaching the halfway point of the dorso-ventral axis. At that point, they turn laterally and ventrally forming a loop that allows blood vessels of both sides to form anastomoses with each other, with posterior communicating vessels at cephalic segments and with the basilar artery at the caudal segments (Ulrich et al. 2011). Thus, it is clear that vascularization along the zebrafish hindbrain does not occur simultaneously. Even though hindbrain vascularization exhibits a metameric pattern, rhombencephalic vessel sprouts do not occur at inter-rhombomere boundaries (Ulrich et al. 2011). Finally, it is worth emphasizing that blood vessel patterning shows spatial congruence with axonal tracts and neuronal nuclei that develop before them. For instance, the basilar artery runs along the medial longitudinal fascicle. Interestingly, some of its branches establish close associations with the branchiomotor and reticulospinal neurons (Ulrich et al. 2011), but avoid oligodendrocytes and neurons of the abducens motor nucleus in rhombomeres five and six (Fujita et al. 2011).

The pattern of hindbrain vascularization is controlled by several factors. In the zebrafish, NT-derived VEGF is necessary for formation of the primordial veins, the basilar artery (Covassin et al. 2006) and the INVP (Fujita et al. 2011, Ulrich et al. 2011). In addition, the chemokine C-X-C motif ligand-12b is required to generate hindbrain vascular buds and to orient basilar artery sprouts to the hindbrain midline, following the activation of phosphatidylinositide 3-kinase in endothelial cells after binding its receptor cxcr-4a (Fujita et al. 2011). The venous markers VEGFR-4 and disabeled (dab)-2 are present in the PNVP during early development. In contrast, dab-2 expression is reduced during the formation of the basilar artery. The basilar artery seems to originate primarily, if not entirely, from migration of primordial vein-derived endothelial cells (Fujita et al. 2011, Ulrich et al. 2011); suggesting that the endothelial cell phenotype is rather plastic during development, with some venous markers being expressed in cells that will later form part of the arterial walls (Fujita et al. 2011).

In mice, the pattern of parenchymal vascularization of the hindbrain follows the same basic sequence described before. At E9.5, pial vessels sprout radially into the ventricular zone and then turn laterally just below the ependymal layer to form the subventricular vascular plexus by E10.25–E12.5 (Gerhardt et al. 2004) (Fig. 1A, *top right*). The initial growth of these blood vessels is guided by radial glia and promoted by VEGF released from cells in the subventricular zone (Fantin et al. 2013). Subventricular lateral branching involves Npn-1 (Fantin et al. 2013) and the late formation of anastomosis is regulated by yolk sac-derived macrophages that enter the brain parenchyma independently from blood vessels (Fantin et al. 2010). Additionally, hindbrain vessel branching is negatively regulated by Dll-4 located in endothelial cells (Suchting et al. 2007) (Fig. 2).

1.3.2 Development of the Midbrain Vascular Network

During embryonic development, the mesencephalon does not subdivide as the other regions of the brain. Its alar plate gives rise to the *tectum*, whereas its basal plate differentiates into the *tegmentum*. In chick embryos, midbrain vascularization proceeds sequentially following a ventral to dorsal gradient starting at the *tegmentum*. At the initial stages, only a handful of vessels derived from the PNVP enter the midbrain orthogonally through the mantle layer (Roncali and Ambrosi 1982) (Fig. 1B). A day later, new vessels invade the ventrolateral *tectum* from the pial surface. At ID6, scant radial vessels sprout into the dorsomedial *tectum* (Roncali et al. 1985a). During the following days (ID 7–14), vessel density augments since radial vessels increase in number and length, branch and anastomose with one another to form the midbrain subventricular plexus. Later in development (ID 14–21), all vessels become surrounded by astrocytic endfeet, promoting endothelial cell maturation and the formation of the blood-brain barrier (BBB) (Bertossi et al. 1993). It has been suggested that vascular and neuronal development are somehow linked since the first vascular sprouts enter the ventrolateral *tectum* at a time when peak proliferative activity in the neuroepithelium is taking place. Also, branching activity of blood vessels entering the posterior *tectum* coincides with intense neuronal differentiation (Roncali and Ambrosi 1982, Roncali et al. 1985a).

In *Xenopus laevis* embryos, the process of hindbrain vascularization (Rovainen and Kakarala 1989) is fully comparable to that described for the chick. However, neither the removal of tectal visual afferents in this model (Rovainen and Kakarala 1989) nor chronic hypoxia in the chick embryo (Roncali et al. 1985b) significantly disturbs the progression of hindbrain angiogenesis, challenging the notion that angiogenesis and neurogenesis are interdependent.

In the zebrafish midbrain, oxygenated blood arrives through the basal communicating artery and deoxygenated blood departs through the choroidal vascular plexus (Isogai et al. 2001). Non-sprouting and sprouting angiogenesis contribute to create a highly intricate and anastomosed midbrain vascular network (Chen et al. 2012). Vessel regression and remodeling is commonly observed in the vascular bed that derives from the choroidal vascular plexus (Chen et al. 2012). Vascular pruning, promoted by diminished mechanical (i.e., hemodynamic) forces, improves the efficiency of the blood flow throughout the vascular network. Reduced blood flow activates the rat sarcoma (Ras)-related C3 botulinum toxin substrate (Rac)-1, resulting in endothelial cell migration from pre-existing vessels into adjacent vessels (Chen et al. 2012).

In the rat midbrain, as in other parts of the CNS, VEGF promotes angiogenesis by increasing the proliferation of endothelial cells. Interestingly, VEGF also stimulates astrocyte proliferation and improves neuronal survival, particularly of dopaminergic neurons, in mesencephalic explants (Silverman et al. 1999).

1.3.3 Development of the Forebrain Vascular Network

Early in vertebrate development, the forebrain is divided into two vesicles: the diencephalon and the secondary prosencephalon. According to recent cell fate mapping and gene expression studies, the diencephalon gives rise to the pretectum and thalamic nuclei, whereas the secondary prosencephalon forms the hypothalamus, retina, telencephalon and olfactory bulbs (Puelles et al. 2013). In this context it is worth noting that, with the exception of the telencephalic structures and the retina, we lack information on the vascular development of many structures derived from the prosencephalon. Hence, this part of the chapter will comment mostly on aspects of the development of the telencephalic and retinal vascular beds.

In mouse embryos, the initiation of telencephalic vascularization occurs by E10 to E11. During this time frame, the PNVP generates sprouts that invade the dorsolateral aspect of the telencephalic vesicle and ventral ventricular vessels appear (Vasudevan et al. 2008). In contrast to what happens in caudal vesicles, telencephalic structures are vascularized following a precise ventro-dorsal ventricular gradient that is independent from pial-derived vessels (Fig. 1C). Furthermore, as is the case of neuronal populations, endothelial cell fate and migration in telencephalic structures is regulated by region-exclusive homeobox proteins. Endothelial cells in the ventral telencephalon express the thyroid transcription factor-1 and distal-less like-1 and -2 proteins. In contrast, endothelial cells migrating through the dorsal telencephalon produce the paired box (Pax)-6 protein. These region-exclusive homeobox transcription factors differentially

modify the expression of molecules critical for endothelial development, such as VEGF and brain derived neurotrophic factor, and guide endothelial cell migration through cell autonomous region-specific signals (Vasudevan et al. 2008).

Glial cells contribute a great deal to forebrain vascularization. Radial glia prevent regression of the vasculature irrigating the cerebral cortex by inhibiting Wnt signaling and the expression of matrix metalloproteinases (Ma et al. 2013). On the other hand, astrocytes and their precursors expand their processes onto vessel walls, covering and making widespread contacts with the vasculature. Thus, glia lend structural support in addition to guiding growing vessels and migrating neuroblasts. Additionally, glial cells participate in BBB development, since only vessels associated with glia express BBB markers (Virgintino et al. 1998).

Interestingly, neurogenesis follows a progression pattern similar to that seen for the vasculature during telencephalic development. However, vascularization takes place first (Vasudevan et al. 2008), suggesting that endothelial cells importantly participate in corticogenesis (Li et al. 2013). In support, VEGF gene deletion in endothelial cells alters neuronal proliferation and migration, as well as axonal tract formation in the developing cortex (Li et al. 2013).

Finally, a dynamic analysis of hypophyseal vascularization was recently reported in zebrafish embryos (Gutnick 2011). Hypophyseal vascular formation begins 24 hours after the formation of the hypothalamic-neurohypophyseal tract. Arteries irrigating the neurohypophysis form within the hypophysis itself, lengthen forward and attach to the palatocerebral arteries. Afterwards, the main lateral vein sinuses lengthen anteriorly, develop medial offshoots and come round the posterior neurohypophysis. The hypophyseal veins connect bilaterally to each other and to the hypophyseal arteries to form a loop. Interestingly, neural-derived oxytocin and its receptor, located in endothelial cells, control the development of the neurohypophyseal vessels (Gutnick 2011).

1.3.4 Development of the Retinal Vascular Network

As mentioned earlier, the secondary prosencephalon provides the anatomical *substratum* necessary for the retina to be formed. In a three-week old human embryo, bilateral symmetrical evaginations called optic cups emerge from the anterolateral surface of the prosencephalon; the retina proper arises from the ventral region of these structures. As the retina *primordium* evaginates by the fourth week of human gestation, the central hyaloid artery arises from the choroidal veins that surround the optic cup (Anand-Apte and Hollyfield 2011). However, this blood vessel is a transient structure. In all species so far studied, it disappears as ontogeny proceeds

(Fruttiger 2002, Saint-Geniez and D'Amore 2004). The process of hyaloid artery regression involves Wnt-7b (released by macrophages) binding to the frizzled (Fzd)-4 receptor. This process also requires the participation of the low-density lipoprotein receptor related protein (Lrp)-5 (Kato et al. 2002, Lobov et al. 2005), Norrin (a Fzd-4 receptor ligand) (Richter et al. 1998), Ang-2 acting on tyrosine kinases with immunoglobulin-like and epithelial growth factor-like domains (Tie)-2 (Hackett et al. 2002) and fibroblast growth factor receptors (Rousseau et al. 2003).

As soon as the hyaloid artery degenerates, a vascular plexus forms at the distal-most segment of the optic nerve. In the mouse, this vascular network is called the primary vessel plexus (PVP) and it spreads over the nerve fiber layer during the first ten days after birth (Dorrell et al. 2002). Newly formed vessels grow towards the outer side of the retina and, between days seven and nine, constitute a secondary vascular plexus at the outer edge of the inner nuclear layer (INL). During the third postnatal week, an intermediate vascular plexus is formed between the superficial and deep plexuses (Dorrell et al. 2002). The formation of the PVP is likely promoted by retinal astrocytes that experience hypoxia and release VEGF (Pierce et al. 1996, West et al. 2005) by the day of birth. Retinal astrocytes originate from Pax-6 positive optic nerve astroblasts (Mi et al. 1999, Chu et al. 2001), are present in the optic nerve by E15 (Dorrell et al. 2002) and enter the retina days later (Ling and Stone 1988, Dorrell et al. 2002, Fruttiger 2002). In addition to releasing VEGF, these cells also express platelet derived growth factor receptor (PDGFR)-α (Mudhar et al. 1993) and vimentin (Chu et al. 2001). Once the PVP is formed, the central retinal artery and vein develop first followed by their branches. This vascular developmental pattern seems to respond to the graded availability of VEGF across the retina (Ruhrberg et al. 2002, Stalmans et al. 2002, Gerhardt et al. 2003). When retinal oxygen concentrations are adequate, blood vessel branching, astrocyte proliferation and VEGF release stop (West et al. 2005).

Diverse genes and their protein products participate in modeling the retinal angio-architecture. For instance, the tailless homologue promotes astrocyte proliferation (Miyawaki et al. 2004). Apelin, Dll-4, platelet derived growth factor (PDGF)-B, uncoordinated (Unc)-5b and VEGFR-2 support angiogenesis during the formation of the primary inner vascular plexus (Saint-Geniez et al. 2002, Gerhardt et al. 2003, Claxton and Fruttiger 2004, Lu et al. 2004, Suchting et al. 2007). Other ligand-receptor families that also participate in retinal angiogenesis are Ephrin-Eph, Semaphorin-Plexin, Slit-Roundabout and Netrin-Unc (Eichmann 2005). Müller glial cells appear by E18-P12 in the rat (Rapaport et al. 2004). These cells produce Norrin which, together with tetraspanin-12, interacts with Fzd-4 and Lrp-5 to induce angiogenesis by activating the β-catenin and sex-determining region Y-related high mobility group box-17 signaling pathway

in retinal endothelial cells (Junge et al. 2009, Ye et al. 2009). Additionally, mutual signaling among endothelial cells, smooth muscle cells and pericytes promotes retinal vascular maturation. Indeed, smooth muscle cells and pericytes secrete Ang-1 that acts on endothelial Tie-2 receptors; while endothelial cells release PDGF that acts on PDGFR-β in pericytes or smooth muscle cells (Suri et al. 1996, Hellstrom et al. 1999). In addition, VEGF released by INL glial cells promotes angiogenesis in the outer vascular plexus (Stone et al. 1995, although see Fruttiger 2007). Similar to the primary plexus, the center matures first and then the periphery. Veins of the primary plexus grow angiogenic sprouts that enter the retina following Müller cell extensions, perpendicularly to the primary plexus. They make a 90° turn upon reaching the inner and outer boundaries of the INL; therefore, two vessel systems are laid down parallel to the primary plexus (Fruttiger 2007). Ang-2 produced by horizontal INL cells might stimulate angiogenesis by inhibiting Ang-1-Tie-2 signaling (Hackett et al. 2000, Hackett et al. 2002). Norrin and Fzd-4 also participate in outer plexus vascular development (Xu et al. 2004b, Luhmann et al. 2005).

1.4 Brain Vascular Malformations

The term brain vascular malformation (BVM) groups a series of disorders that affect the anatomical and cytological arrangement of the blood vessels that nourish the brain. Arterio-venous (AVMs; tangles of abnormal and poorly formed blood vessels) and cavernous (CaV; clusters of abnormal dilated vessels also called cavernoma) malformations are the two most prevalent types of BVMs. Although BVMs were first thought to arise during embryonic development, it is now known that they can appear at any age throughout the organism's life (Minakawa et al. 1989, Clatterbuck et al. 2000). BVMs arise at sites where capillaries would normally be located. In an AVM, arteries and veins seem to coalesce forming a meshwork of direct, high-flow shunts. In a CaV, the vessels are thin-walled, dilated, low-flow and may contain thrombi. Capillary dysplasia might be the first event that leads to BVMs (Braverman et al. 1990, Rigamonti et al. 1991). A single lesion probably results in the production of multiple malformations (Sato et al. 2004, Shenkar et al. 2008).

Mutations in a single copy of the endoglin or activin-like kinase receptor (ALK)-1 genes result in loss of protein function and subsequent hereditary hemorrhagic telangiectasia (HHT) type 1 or 2, respectively (Goumans et al. 2009). Similarly, mutations in the SMAD (small, mothers against decapentaplegic)-4 gene are also associated with HHT (Goumans et al. 2009). On the other hand, mutations in Kirsten-Ras-revertant interaction trapped (KRIT)-1, osmosensing scaffold for mitogen activated protein kinase kinase kinase-3 (OSM) and programmed cell death (PDCD)-10 genes are the most

likely factors that cause CaVs (Uhlik et al. 2003, Glading et al. 2007, Ma et al. 2007, Crose et al. 2009).

The prevailing hypothesis regarding development of BVMs is that they arise as a result of two hits. The first hit corresponds to a hereditary mutation in a copy of the above genes, while the second hit can be a somatic mutation in the second copy or a localized environmental alteration. This hypothesis might explain why BVMs are confined to a small area of the vasculature. Evidence supporting the idea comes from CaV patient brain tissue showing germline and somatic biallelic mutations in a subpopulation of endothelial cells within the lesion site (Gault et al. 2005, Akers et al. 2009, Gault et al. 2009, Pagenstecher et al. 2009). Further evidence comes from AVMs in which hemodynamic stress enhances local inflammation and/or the expression of angiogenic factors (Hashimoto et al. 2006, Goettsch et al. 2008). In support of this possibility, previous studies have shown that venous hypertension increases the expression of hypoxia-inducible factor-1 and VEGF (Zhu et al. 2006). Also, patients suffering BVMs have augmented serum VEGF levels and up-regulated expression of VEGF and Ang-2 in the lesion (Jung et al. 2003, Hashimoto et al. 2005). Local increases in the expression of these angiogenic factors induce endothelial cell proliferation (Hashimoto et al. 2001, Sure et al. 2001); thus increasing the occurrence of vascular dysplasia, which is aggravated by increments of local blood-flow in endoglin and ALK-1 mutant mice (Xu et al. 2004a, Hao et al. 2008).

Endoglin, ALK-1 and SMAD-4 constitute part of the TGF-β signaling cascade (ten Dijke et al. 2008). All these proteins are expressed in endothelial cells during development and their expression is reduced in adulthood, unless needed for vascular repair (Ma et al. 2000, Jonker and Arthur 2002, Seki et al. 2003, van Laake et al. 2006, ten Dijke et al. 2008). Both ALK-1 and SMAD-4 are essential for vascular recruitment of smooth muscle cells (Lan et al. 2007, Park et al. 2008). Thus, these data suggest that the principal problem in AVMs and HHTs is a defective function of the endothelial TGF-β pathway which results in secondary deregulation of smooth muscle development. This is supported by the finding that bone morphogenetic protein-9 or 10, also members of the TGF-β family, are the physiological ALK-1 ligands (David et al. 2007, Scharpfenecker et al. 2007). Interestingly, mice with reduced expression of ALK-1 or endoglin have dilated, thin-walled arteries with reduced smooth muscle cells and lower expression of ephrin B2, an arterial marker (Urness et al. 2000, Sorensen et al. 2003). Thus, defects in differentiation between arteries and veins (or their specification) may also participate in the pathogenesis of AVMs. In addition, monocytes might be also involved in BVMs since they express endoglin. Furthermore, even though endoglin or ALK-1 knockout mice show no major defects of vasculogenesis, they lack secondary capillary beds and pericyte vascular

coverage. These mice die during gestation likely from severe impairments in vascular development (Li et al. 1999, Urness et al. 2000). Alterations in a single copy of endoglin or ALK-1 in mice result in spontaneous vascular dysplasia, telangiectasia and random hemorrhages. Small vessel malformations are observed in these mice together with disappearance of vascular smooth muscle cells (Bourdeau et al. 1999, Satomi et al. 2003, Srinivasan et al. 2003, Torsney et al. 2003, Park et al. 2008). Also, endothelial nitric oxide synthase is dysfunctional in mice with an altered copy of endoglin, resulting in production of superoxide and impaired vascular function (Toporsian et al. 2005).

KRIT-1, OSM and PDCD-10 are expressed by endothelial cells, glia and neurons (Petit et al. 2006, Plummer et al. 2006). Endothelial, but not glial or neuronal, expression of KRIT-1 and OSM is needed for normal cerebral vascular formation and function (Hogan et al. 2008, Boulday et al. 2009, Whitehead et al. 2009). All these proteins constitute a single complex that interacts with cytoskeletal, signaling, cell junction and extracellular matrix proteins necessary to maintain vascular structural stability, such as: Ras-related protein-1 (Bos 2005, Carmona et al. 2009), heart of glass-1 (Whitehead et al. 2004, Beraud-Dufour et al. 2007, Glading et al. 2007, Kleaveland et al. 2009), β1-integrin and integrin cytoplasmic domain-associated protein-1α (Zhang et al. 2008, Faurobert and Albiges-Rizo 2010, Hilder et al. 2007). KRIT-1 also promotes β1-integrin action, such as endothelial adhesion to the extracellular matrix (Rupp and Little 2001), lumen formation (Iruela-Arispe and Davis 2009) and recruitment of pericytes and smooth muscle cells to vessels (Astrof and Hynes 2009).

Finally, endothelial cells with altered OSM function have cytoskeletal defects, diminished junctions between them and impaired barrier function (Hogan et al. 2008, Whitehead et al. 2009). Accordingly, OSM knockout mice show lesions that resemble those observed in cerebral CaV patients (Plummer et al. 2006). OSM is named due to its ability to restore cell shape and volume after hyperosmotic shock. It acts as a scaffold protein for Rac-1 and upstream mitogen activated protein kinases (Uhlik et al. 2003). Thus, OSM regulates cytoskeletal dynamics and cell shape via mitogen activated protein kinase signaling. Cytoskeletal dynamics are also important for maintaining cell polarity; KRIT-1, OSM and PDCD-10 maintain cell polarity by modulating Ras homolog family member A (RhoA) activity on the cytoskeleton. Indeed, OSM increases RhoA degradation by binding the SMAD specific E3 ubiquitin protein ligase-1 (Crose et al. 2009). KRIT-1 and PDCD-10 also inhibit RhoA activity. Although the mechanism of KRIT-1 action is unknown, PDCD-10 binds to protein kinases of the germinal center kinase sub-family to stimulate Golgi apparatus assembly and activate moesin (Preisinger et al. 2004, Fidalgo et al. 2010, Zheng et al. 2010).

1.5 Conclusions

1. The early establishment of blood vessels in the CNS follows a highly stereotyped pattern that is conserved throughout vertebrate evolution, suggesting a genetic underpinning. Nonetheless, CNS vascularization is an active process requiring communication between all cells of the neurovascular unit. This is particularly true for intraneural sprouting angiogenesis which follows a very specific ventro-dorsal pattern highly regulated by local signals throughout the entire antero-posterior axis.
2. Multi-directional communication among resident neuronal and glial cell populations with the newly formed endothelial cells is very important for brain function. Endothelial cells are perfectly located between the blood and the neural tissue, giving them the capacity to regulate molecular trafficking and consequently neural function. Similarly, neurons and glia can help in determining the phenotype of endothelial cells endowing them with a high specificity and heterogeneity. Although the metabolic demands of the neural tissue do not participate during early vascularization, the establishment and maintenance of the CNS and its vasculature are interdependent to guarantee their normal function.
3. The close interdependence between neural and endothelial cells is demonstrated by their response to the same signals. For example, VEGF and its receptors participate in nearly all aspects of CNS vascular development. This trophic factor also plays a prominent role in astrocyte proliferation, neuronal survival and neural cell differentiation.
4. Endothelial cells are likely to exert functions besides those commonly attributed to them (e.g., nutrient delivery) during CNS development. Exploring this possibility awaits the development of experimental designs capable of providing an adequate milieu for neural cells in the absence of blood vessels. Once these conditions are established, the re-introduction of controlled numbers of endothelial cells will undoubtedly provide scenarios in which to test their role on various aspects of neuronal and glial biology during brain development.

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