Blood Vessels are in Control: Vasculature Regulates the Neural Niche in Cranial Sensory Ganglia

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SUMMARY

Cranial sensory ganglia are groups of neurons located in the head of chordates outside the central nervous system that allow individuals to sense and perceive information from the outer world. An important step in the proper formation and functioning of these cranial ganglia is the regulation of neural proliferation and differentiation. The control of stem cells behaviour is carried out by signals provided by their niche, which is formed by other cell types found around stem cells. Blood vessels have emerged as key components in the adult neural stem cell niche. However, the putative function of vasculature on neural behaviour has yet not been studied in the peripheral nervous system.

In the present work, I have described the anatomical relationship of cranial sensory ganglia with vasculature during their development, focusing in the statoacoustic ganglion and using zebrafish as a model system. Secondly, I have demonstrated that two independent signalling mechanisms exist from vasculature to the developing sensory neurons. Early in development, endothelial cells maintain neural cells' quiescence via Dll4/Notch1 signalling and cytoneme contacts. Later, blood flow onset is required for the differentiation of sensory neurons. Blood flow initiation also produces transcriptional changes in neural cells related to oxygen sensing and oxidative phosphorylation metabolism. In conclusion, my work sheds light into the role of vasculature in sensory neurogenesis that might be relevant to understand organ growth and their associated diseases.

RESUM

Els ganglis sensorials cranials són grups de neurones que es troben en el cap dels cordats, fora del sistema nerviós central, que permeten captar i percebre informació del món extern. Un pas important en la correcta formació i funcionament d'aquests ganglis cranials és la regulació de la proliferació i la diferenciació. El control del comportament de les cèl·lules mare és dut a terme per les senyals emeses en el seu nínxol, format pel altres tipus cel·lulars que resideixen al voltant de les cèl·lules mare. Els vasos sanguinis han esdevingut un component clau del nínxol de les cèl·lules mare neurals adultes. Tanmateix, la possible funció dels vasos en la regulació del comportament neural encara no s'ha estudiat en el sistema nerviós perifèric.

En aquest treball, he descrit la relació anatòmica del ganglis sensorials cranials amb la vasculatura del cap durant el seu desenvolupament, centrant-me en el gangli estaoacústic i utilitzant el peix zebra com a model animal. També he demostrat que, durant el desenvolupament, existeixen dos mecanismes de senyalització independents de la vasculatura a les neurones sensorials. En estadis inicials del desenvolupament, les cèl·lules endotelials mantenen a les cèl·lules neurals en quiescència mitjançant la senyalització Dll4/Notch1 i contactes per citonemes. En estadis més tardans del desenvolupament, l'inici del flux sanguini és necessari per la diferenciació de les neurones sensorials. L'inici del flux sanguini també produeix canvis transcripcionals en les cèl·lules neurals relacionades amb la detecció de la presència d'oxigen i la fosforilació oxidativa. En conclusió, la meva feina contribueix a la comprensió de la funció de la vasculatura en la neurogènesis sensorial que pot ser rellevant per entendre el creixement dels òrgans i les malalties que hi estan relacionades.

PREFACE

The term niche was first used in ecology by Grinnell in 1917 to describe the functional role and position of an organism in its environment. Much later the term niche was expanded to other contexts. For example, the adult neural stem cell (NSC) niche refers to the specialized brain microenvironment in which NSCs reside.

The nervous system is composed by a high number and diversity of cell types that are highly specialized and connected to conduct their function of sensing, integrating and responding to inner and outer stimuli.

Neurogenesis is the process by which neural stem cells produce all the cells of the nervous system. During neurogenesis, some neural cells must remain in quiescence, while others will proliferate, migrate, differentiate and integrate into a network through axogenesis. All these steps must be tightly regulated for their correct functioning at adult stages. This control is done, in part, by the surrounding cells that conform the niche.

Blood vessels have been proven to belong to the NSC niche. Their communication with neural cells to regulate neural development goes beyond the simple perfusion of oxygen and nutrients.

While the vast majority of these studies have been conducted in the adult central nervous system (CNS), the first articles on this interaction during embryonic stages have started to appear recently. Additionally, these studies have focused in the CNS, while very little is known about the NSCs niche in the peripheral nervous system.

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I. INTRODUCTION

One of the most fascinating questions in biology is to understand how, during development, a single cell is capable of generating diverse functional tissues and organs. For this to happen, cells with a broad potential known as stem or progenitor cells become committed to specific cell fates. Cell-cell communication between these new cells is crucial for generating structural diversity, regulating differentiation and growth, and allowing tissues and organs to organize in 3D complex structures.

The nervous system is one of the most complex tissues due to its high number and diversity of cell types, and also for the complexity of its cells connectivity, among them (neuronal synapses) and with other cell types (muscles, glia, blood vessels (BV) and sensory receptors).

In this thesis, I was interested in understanding the mechanisms that regulate the development of the cranial sensory nervous system. Cranial sensory ganglia are clusters of neurons that belong to the peripheral nervous system (PNS). These ganglia collect information from the outer world through sensory receptors and transmit this information to the central nervous system (CNS) for its processing. Specifically, I have studied the interaction between sensory neural and endothelial cells (ECs) for the development of the statoacoustic ganglion (SAG) of the inner ear and other sensory ganglia.

In order to better understand my results, I will first give a short overview on the development of the nervous and vascular system, two different but also surprisingly resembling organs; before fully explaining previous knowledge on the relationship between neurons and vessels, the neurovascular crosstalk. Finally, the role of filopodia as a signalling mechanism will also be introduced.

1. The stem cell concept and functions

Stem cells (SCs) are characterized by two intrinsic properties: self-renewal and potency. Self-renewal is the capacity to generate an identical daughter cell, while potency is the ability to generate a diverse array of differentiated cells (Spradling et al., 2001). Thanks to these properties SCs contribute to tissue development during embryogenesis, and their maintenance and regeneration after injury during adulthood. Depending on their source, thus, two major types of SCs can be found: embryonic SCs and adult SCs.

SCs can divide symmetrically producing two SCs, or asymmetrically, producing one SC and another cell that will start to differentiate. They give rise to progenitor or transit-amplifying cells (TACs), which are incapable of unlimited self-renewal but divide few times before differentiating. These cells are committed to a particular cell lineage (Seaberg and van der Kooy, 2003). Through this mechanism, amplification of mature cells is achieved while minimizing the chances of DNA alterations during replication in the long-living SCs (Reya et al., 2001).

In mammals, adult SCs where discovered in the 1960s in the bone marrow (Humphries et al., 1979; Jurásková and Tkadlecek, 1965; Till and McCulloch, 1961), but now they are known to be present in many other organs and scientists discovered epidermal, hair, melanocyte, muscle, tooth, gut, germline and neural SCs (NSCs).

The ability of a cell to become a SC is determined by where it resides. Appropriate signalling must exist to regulate the maintenance of SCs and the production of differentiated cells. Schofield first introduced the concept of “stem cell niche” in 1978, described as the existence of an anatomical microenvironment that provide signals to maintain SCs undifferentiated, control survival, proliferation, fate specification and differentiation (Schofield, 1978). It also ensures a structural and trophic support, temporal and spatial information and physiological cues (Jones and Wagers, 2008). SCs microenvironment regulate proliferation and differentiation by paracrine and juxtracrine factors produced by the cells that make up the niche. As SCs leave the niche they no longer can sense these signals and thus commit differentiation (Moore and Lemischka, 2006).

NSCs give rise to the whole nervous system during development and can replace lost cells during adulthood in certain circumstances. Before going into the properties of the NSC niche, I will briefly introduce the nervous system and the main mechanisms for neuronal generation.

2. The nervous system of vertebrates

The vertebrate neural system is a complex highly organized structure responsible for sensing, integrating, processing and sending information from all parts of the body and the external environment. It is composed of multiple cell types: neurons, astrocytes, oligodendrocytes and microglia that communicate with each other to ensure the proper development and function of the system.

2.1 Neurogenesis during development

During the development of vertebrates, neurons are generated from three sources deriving from the ectoderm: the neuroepithelium of the neural tube, the precursor of the CNS; and the neural crest and ectodermal cranial placodes, which will generate, among others, the PNS (see Fig 1).

Neurogenesis is the process by which NSCs produce all the cells of the nervous system: neurons and macroglial cells (astrocytes and oligodendrocytes). During this process, some NSCs remain in quiescence, while others proliferate to give rise to progenitors that become specified, migrate, differentiate and integrate into a network through axogenesis. All these steps must be tightly controlled for their correct functioning, and thus, understanding how NSCs behaviour is regulated is of outstanding importance (Bjornsson et al., 2015).

2.1.1 Development and neurogenesis in the CNS

The development of the vertebrate CNS begins early during embryogenesis. The neural plate is specified by low levels of BMPs, FGFs and Wnts, and the expression of Sox family transcription factors (SoxB1 genes, Sox1, 2 and 3) establish the neural plate cells as neural precursors (Wilson and Edlund, 2001).

Then, the neural plate folds to form the neural tube (see Fig 1). The neural tube is formed by a single layer of neuroepithelial cells (Manabe et al., 2002; Zhadanov et al., 1999). Multipotent neuroepithelial cells are SCs that can undergo symmetric division, or proliferative division -giving rise to two new SCs- or asymmetric division (Malatesta et al., 2000). In asymmetric division one of the daughter cells will continue being attached to the ventricular surface and thus remains as a NSC, while the other cell detaches, migrates and differentiates (Gray et al., 1988; Hollyday, 2001; Huttner and Brand, 1997) (see Fig 2). Once the neural tube is constituted, the neurogenic program starts from multipotent neuroepithelial SCs that will become specialized into neurons, glial or ependymal cells. This process can be divided into three steps: first, cells will commit to a certain neural cell phenotype; secondly, they will become determined according to their position (anteroposterior and dorsoventral); and finally, the developmental decision to differentiate regulated by the time or birthday. Thus, combinatorial codes of position and

temporal identity regulate the neural progenitor population differences in the different regions of the neural tube that will eventually translate in the specific neuronal subtypes at precise positions and developmental times (Osterfield et al., 2003; Panchision and McKay, 2002; Temple, 2001).

Proneural genes of the homeodomain and basic helix-loop-helix (bHLH) family are the molecular codes that determine the where and when the different classes of neural cell are going to be generated. These transcription factors also promote the generation of different cell types, linking patterning with cell specification. Proneural bHLH genes, which include Mash1, Ngn1-3 and Math1, are necessary and sufficient to promote the generation of differentiated neurons. In order to activate neurogenesis, they first inhibit SoxB1 genes. They control the commitment of multipotent progenitors, the production of a particular neuronal subtype, division arrest, migration and terminal differentiation, through the activation of numerous downstream transcription factors. Homeodomain genes participate in the subtype specific differentiation neural programs. Neural differentiation bHLH genes, such as NeuroM (Neurod4) and NeuroD (Neurod1), are induced by proneural proteins and contribute to neuronal differentiation (Bertrand et al., 2002; Guillemot, 2007).

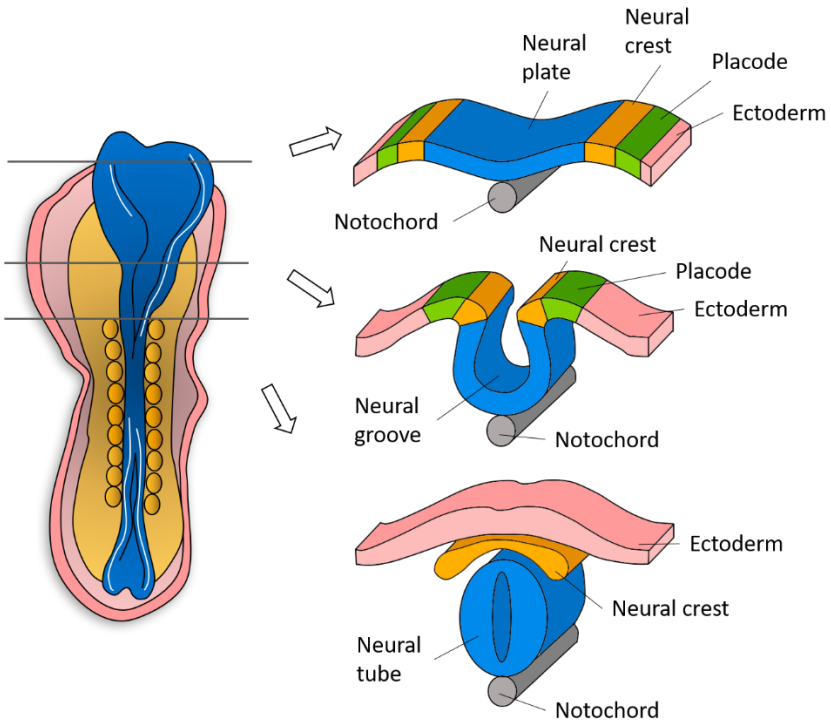

Figure 1. Formation of the neural tube, neural crest and placodes.

In the ectoderm the neural plate, neural crest and pre-placodal region are specified. Neural folds form along the embryo as parallel ridges, forming a neural groove in the centre. Neural crest cells begin to form at the crest of the neural folds. The neural folds meet at the midline and fuse forming the neural tube, and the neural crest cells disperse and leave the neural tube separated from the epidermis. Mouse embryo shown of 21 days after fertilization. Cross sections represent progressive closure of the neural tube.

Modified from Developmental Biology, 8th Edition.

As the neural tube matures, the brain transforms into a more complex tissue with numerous cell layers, and the multipotent neuroepithelial SCs become radial glial cells (RGC), which are NSCs. RGC exhibit residual properties of the neuroepithelial cells but also new astroglial properties, such as the expression of GFAP (glial fibrillary acidic protein) (Götz, 2003; Huttner and Brand, 1997; Kenneth Campbell and Magdalena Götz, 2002). As their predecessors, RGC maintain the apical-basal polarity and contact with both surfaces (Chenn et al., 1998; Wodarz and Huttner, 2003), but they are more fate-restricted (Malatesta et al., 2000; Williams and Price, 1995). Most of the neurons in the brain are derived from RGC (Anthony et al., 2004; Malatesta et al., 2003). RGC daughter cells will become intermediate progenitor cells (IPCs) that can divide symmetrically functioning as TACs (Lui et al., 2011; Noctor et al., 2004), and later undergo symmetric differentiating division generating terminally differentiated postmitotic cells. Post-mitotic neurons will use the pial connection of RGC to migrate, followed by glia-independent migration, to attain their final position (Miyata et al., 2001; Rakic, 1971) (see Fig 2).

The resulting neurons will be composed by a neural cell body or soma from which two different types of extensions grow. Fine and short branching extensions used to pick up electric impulses from other neurons are called dendrites. These structures allow the connection or synapse with other neurons. Some neurons have few, while other have extensive *dendritic arbors*. On the other hand, axons can be very long and allow the connection to far placed neurons or other cells and form the nerves. Nerve outgrowth is led by the tip of the axon, called growth cone, which moves and senses the environment to find their correct path thanks to the elongation and retraction of filopodia (Lamoureux et al., 1989, and further developed in **3.1. Similar morphological structures**).

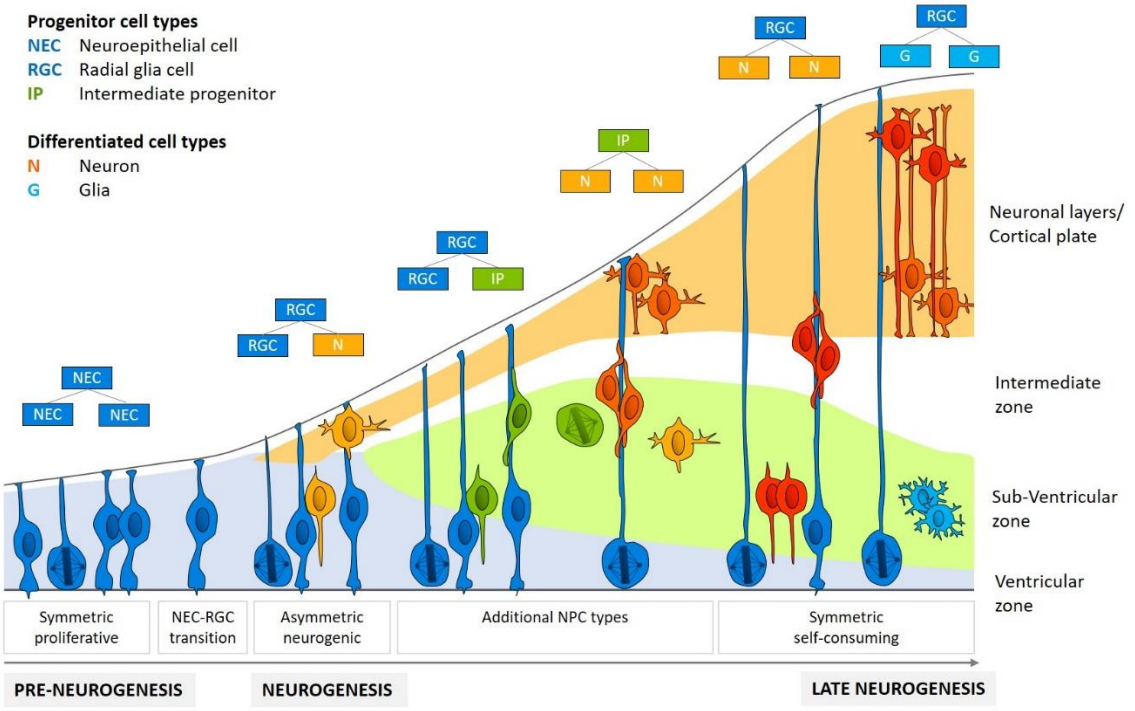

Figure 2. Schematic drawing of neurogenesis in the embryonic vertebrate brain.

The principal types of NPCs (NEC and RGC) are represented with their mode of division (symmetric and asymmetric), and their progeny indicated in different colours: intermediate progenitor cells, neurons and glia.

Modified from Paridaen and Huttner, 2014.

2.1.2 Development and neurogenesis in the cranial PNS

The PNS is composed of afferent sensory fibres from the sensory organs to the CNS (brain, brainstem and spinal cord), where interneurons will modulate the information. Thus, the PNS has the important role of mediating the relationship of the organism with its environment.

The cranial PNS is composed of cranial nerves and their associated cranial sensory ganglia, which relate to the paired sense organs. Cranial sensory ganglia are the condensation where somas of the sensory neurons of the cranial nerves reside.

Some cranial sensory ganglia have a dual embryonic origin, the proximal parts arise from neural crest cells (NCCs) and the distal portions from the ectodermal cranial placodes. Others have exclusively a placodal origin (Baker and Bronner-Fraser, 2001; Blentic et al., 2011; D'amico-Martel and Noden, 1983; Ladher et al., 2010; Schlosser, 2010).

Ectodermal Cranial Placodes

Ectodermal cranial placodes (or cranial placodes) are specified at the boundary between the neuroectoderm and ectoderm, they are located lateral to the NCCs and receive moderate levels of BMPs. Paired ectodermal thickenings are formed first in the pre-placodal region (PPR), a U-shaped domain around the anterior neural plate (Schlosser, 2010; Streit, 2004), defined by the expression of transcription factors Six1 and Eya1. The expression of this factors is maintained in some placodes (Kobayashi et al., 2000; Schlosser and Ahrens, 2004). Upon inductive signals, the PPR will segregate and form the placodes, which are specified for a unique sensory fate (Schlosser, 2005; Streit, 2004) (see Fig 3). The cranial placodes will contribute to non-neural cell types of the paired sensory organs related to hearing, balance, vision and olfaction. They also form the majority of the cranial sensory ganglia neurons, allowing the connection of sensory organs and the CNS. Some placodes are only neurogenic (the trigeminal and the epibranchial), while others will also give rise to a multitude of cell types, such as lens fibres, hair cells (HC) or epithelial cells.

Cranial placodes produce sensory neuroblasts that delaminate, migrate and coalesce to form ganglia (Begbie et al., 2002; Blentic et al., 2011; Graham et al., 2007; McCabe et al., 2009). The distinct cranial sensory ganglia send projections to the neural tube at

specific axial levels, coordinating the sensory input with the correct CNS region (Begbie and Graham, 2001; Osborne et al., 2005).

From anterior to posterior, the olfactory placode produces migrating cells that enter the forebrain to contribute to the neuroendocrine compartments (Tarozzo et al., 1995), olfactory sensory cells and non-neuronal sustentacular cells of the olfactory epithelium. It will transduce the odour and pheromone signals to the CNS (Croucher and Tickle, 1989). The lens placode forms the lens and is the only placode that does not form neurons. The trigeminal placode forms the trigeminal ganglion, which functions as a relay station for temperature, pain and touch of facial skin, jaws and teeth (Baker et al., 1999; Begbie et al., 2002). The otic placode (OP) is one of the best known and widely studied

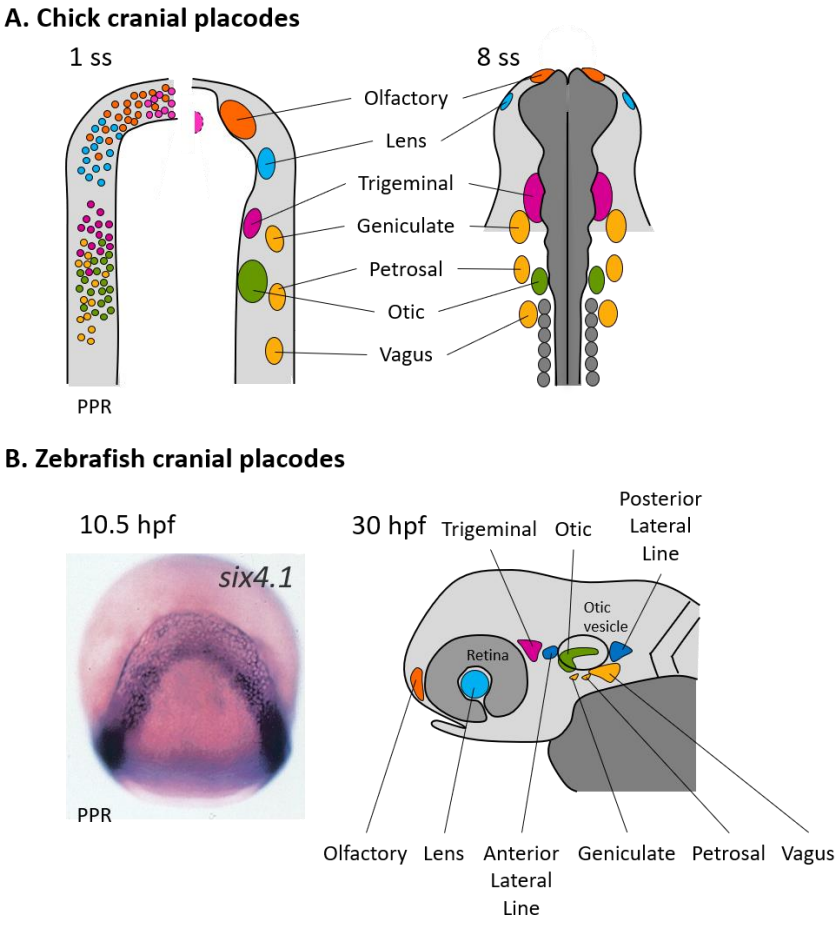

Figure 3. Cranial placodes form sensory neurons.

A. Fate map of the cranial placodes in the developing chick embryo at the neural plate (left) and 8 somite (right) stages. **B.** PRR in a zebrafish embryo at 10.5 hpf (left), and their derivative cranial placodes in the zebrafish embryo at 30 hpf (right). PRR, Pre-placodal Region.

Modified from Developmental Biology, 8th Edition and Kobayashi et al., 2000.

and will be developed further in the next section. It is located adjacent to r5 and r6 and generates all the structures of the inner ear and neurons of the VIIIth ganglion (SAG) (for review, see Brown et al., 2003; Maier et al., 2014; Torres and Giráldez, 1998; Whitfield, 2015). In fish and aquatic amphibians the OP is surrounded by the lateral line placodes, which develop the lateral line organs in the head and trunk with mechano- and electro-receptors and the neurons that innervate them (Northcutt et al., 1994, 1995; Schlosser, 2002; Schlosser and Northcutt, 2000). Finally, lateral to the OP, there are the three epibranchial placodes -geniculate, petrosal and nodose- that contribute to the fascial, glossopharyngeal and vagus ganglia, respectively. They innervate taste buds, visceral organs and the heart (D'amico-Martel and Noden, 1983; Schlosser and Northcutt, 2000) (see Fig 3).

2.1.2.1 The Inner Ear and Statoacoustic Ganglion

The vertebrate inner ear is a sensory organ with a complex morphology and high sensitivity. It is responsible for the sound and equilibrium detection.

In mammals, the inner ear is composed of multiple compartments also known as the labyrinth: the spiral cochlea is the auditory organ; the utricle and saccule sense linear acceleration in the horizontal and vertical axis respectively, and also the gravity pull; and three semicircular canals allow the sensing of head rotation (Cantos et al., 2000; Fekete and Wu, 2002). The epithelial layer that specializes in sensory functions is located in specific regions and is called sensory patches. They contain mechano-sensory receptors – HC –, as well as supporting and secretory cells, associated with sensory neurons. Different sensory patches are placed at each inner ear structure according to their function: cristae are found in the semicircular canals, maculae in the saccule and utricle, and the organ of Corti (mammals) or basilar papilla (birds) in the spiral cochlea. In zebrafish, sacculus is the primary auditory organ as no cochlea is present, while the maculae and cristae are very similar to those of vertebrates, including humans (Jiang and Tierney, 1996; Whitfield et al., 1996) (see Fig 4).

HCs have a kinocilia and a bundle of stereocilia that sense mechanic perturbations, causing the depolarization of HCs. This information will be collected by bipolar sensory neurons of the VIII ganglion, which will transduce the information to the corresponding nuclei in the hindbrain (Kelley, 2006; Rubel and Fritzsch, 2002). The inner ear is suspended in endolymph, essential for HCs functioning (Li et al., 2013c).

The VIIIth ganglion is also known as SAG, auditory vestibular ganglion and vestibular and spiral ganglia, depending on the species. In zebrafish this ganglion is named as SAG. While the organization of the vestibular region is very similar amongst vertebrates, the auditory part is almost absent in aquatic vertebrates.

Zebrafish has become an important model for the study of the inner ear and SAG development.

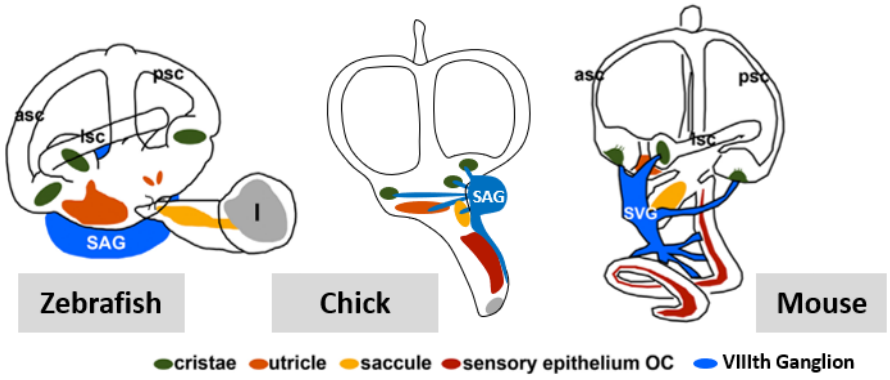

Figure 4. The adult inner ear in vertebrates.

Schematic drawings of adult inner ears in zebrafish, chick and mouse, their sensory patches and VIIIth ganglion (SAG).

Modified from Maier et al., 2014; Neves et al., 2013.

Inner ear development

Almost all the different cell types that compose the inner ear derive from the OP: epithelial cells, supporting cells, HCs, secretory cells and sensory neurons. Sensory neurons of some cranial ganglia derive both from NCCs and the cranial placodes (D'amico-Martel and Noden, 1983), but the SAG is somewhat unique as neurons arise exclusively from the OP (Breuskin et al., 2010; van Campenhout, 1935; D'amico-Martel and Noden, 1983), and NCCs only participate in glia formation (D'amico-Martel and Noden, 1983; Harrison, 1924).

Otic placode development

After the PPR is determined, a common otic/epibranchial precursor domain (OEPD) is formed, followed by the induction of the OP. Later, the OP segregates from the lateral

line and epibranchial placodes (McCarroll et al., 2012; Steventon et al., 2012). After induction, the OP forms the otic vesicle (OV) via invagination in amniotes (Sai et al., 2014) and hollowing in fish (Hoijman et al., 2015) (see Fig 5). The NCCs also participate in the ear development (Dutton et al., 2009; Freyer et al., 2011; Sandell et al., 2014; Takano-Maruyama et al., 2012; Watari et al., 2001), forming the SAG glia (D'amico-Martel and Noden, 1983; Hemond and Morest, 1991) and helping in the SAG projection to the CNS (Sandell et al., 2014). The PNS glia consists of Schwann cells, supporting neuronal axons and neurites, and satellite cells, which surround and myelinate ganglia neurons.

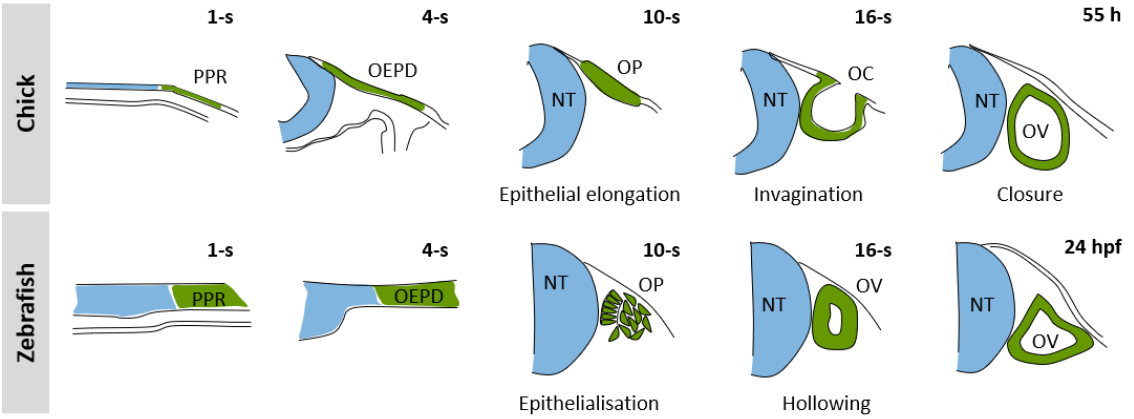

Figure 5. Formation of the otic vesicle in chick and zebrafish.

The PRR is first specified to later split into larger placodal domains such as the OEPD. This structure later segregates to form the OP, which appears as a thickening in the chick. The OP invaginates by apical constriction forming an OC, which eventually forms the OV. In zebrafish cells of the OEPD are not epithelialized. By 10 s, placodal precursors coalesce into an unorganised mass of cells next to the posterior hindbrain. The OP appears by progressive epithelialisation from medial to lateral positions. Subsequently, the OP undergoes a process of hollowing, in which establishment of apicobasal polarity leads to the separation of the apical membranes and emergence of intercellular spaces that will be fluid-filled and expanded to generate the lumen.

PRR, pre-placodal region; OEPD, otic/epibranchial precursor domain; NT, neural tube; OP, otic placode; OC, otic cup; OV, otic vesicle; s, somites; hpf, hours post fertilization.

Modified from Alsina and Whitfield, 2017.

Neurogenesis of the statoacoustic ganglion

Early in development the OP becomes subdivided in an anterior neurogenic domain and a posterior non-neurogenic domain. The neurogenic domain gives rise to the neurons of the SAG and partially overlaps with the sensory domain that will form sensory HCs in chick and mouse, at later stages (Raft et al., 2007; Satoh and Fekete, 2005); while in fish, sensory HCs arise both at the anterior and posterior poles of the OV, concomitantly with neuronal precursor cells (Haddon and Lewis, 1996; Radosevic et al., 2014). The non-neurogenic domain gives rise to support, secretory and epithelial cells. In zebrafish, Foxi1 and Dlx3b/4b specify the neuronal and sensory competence respectively (Hans et al., 2013). Tbx1 and her9, on the other hand, restrict the neurogenic domain (Radosevic et al., 2011; Raft et al., 2004), in zebrafish and mice. Expression of Tbx1 is regulated by external signals such as RA and Hedgehog (Hh) expressed in the surrounding mesenchyme, in zebrafish and chick (Bok et al., 2011; Hammond et al., 2010; Radosevic et al., 2011) (see Fig 6 A). In chick, members of the Notch signalling pathway also participate in the neurogenic/non-neurogenic fate determination (Abelló et al., 2007) (see BOX 1). Finally, BMPs also participate in the determination of the non-neurogenic domain (Abelló et al., 2007).

Neurogenesis in the inner ear does not differ extensively from neurogenesis in the CNS. Specification of neural precursors from Sox2/3 neuroepithelial cells occur under the regulation of Fgf and RA gradients in mice and zebrafish (Maier and Whitfield, 2014; Radosevic et al., 2011; Vemaraju et al., 2012). Specification of neuronal precursors occurs in the anterior OV floor, by the activation of *Neurog1* expression and Notch-mediated lateral inhibition (Adam et al., 1998; Andermann et al., 2002; Haddon et al., 1998; Ma et al., 1998). High levels of Neurog1/Delta1 induce the expression of other bHLH genes such as *NeuroD1* (Adam et al., 1998; Bell et al., 2008; Kim et al., 2001; Liu et al., 2000). Neuroblasts expressing *NeuroD* soon delaminate to continue their development outside of the inner ear, they migrate a short distance and enter a transit-amplification phase that allows the precursor population to expand (Kim et al., 2001; Raft et al., 2007). These precursor cells eventually differentiate; they exit cell cycle, lose expression of *NeuroD* and express differentiation markers such as Islet1, POU4f1 and the survival factor IGF1 (Camarero et al., 2001; Huang et al., 2001; Radde-Gallwitz et al., 2004), to compose the SAG (see Fig 6 B and C). In zebrafish, it is thought that Fgf5-dependent feedback inhibition from the differentiated neurons regulate both specification and maturation of neuroblasts, regulating the final number of neurons composing the

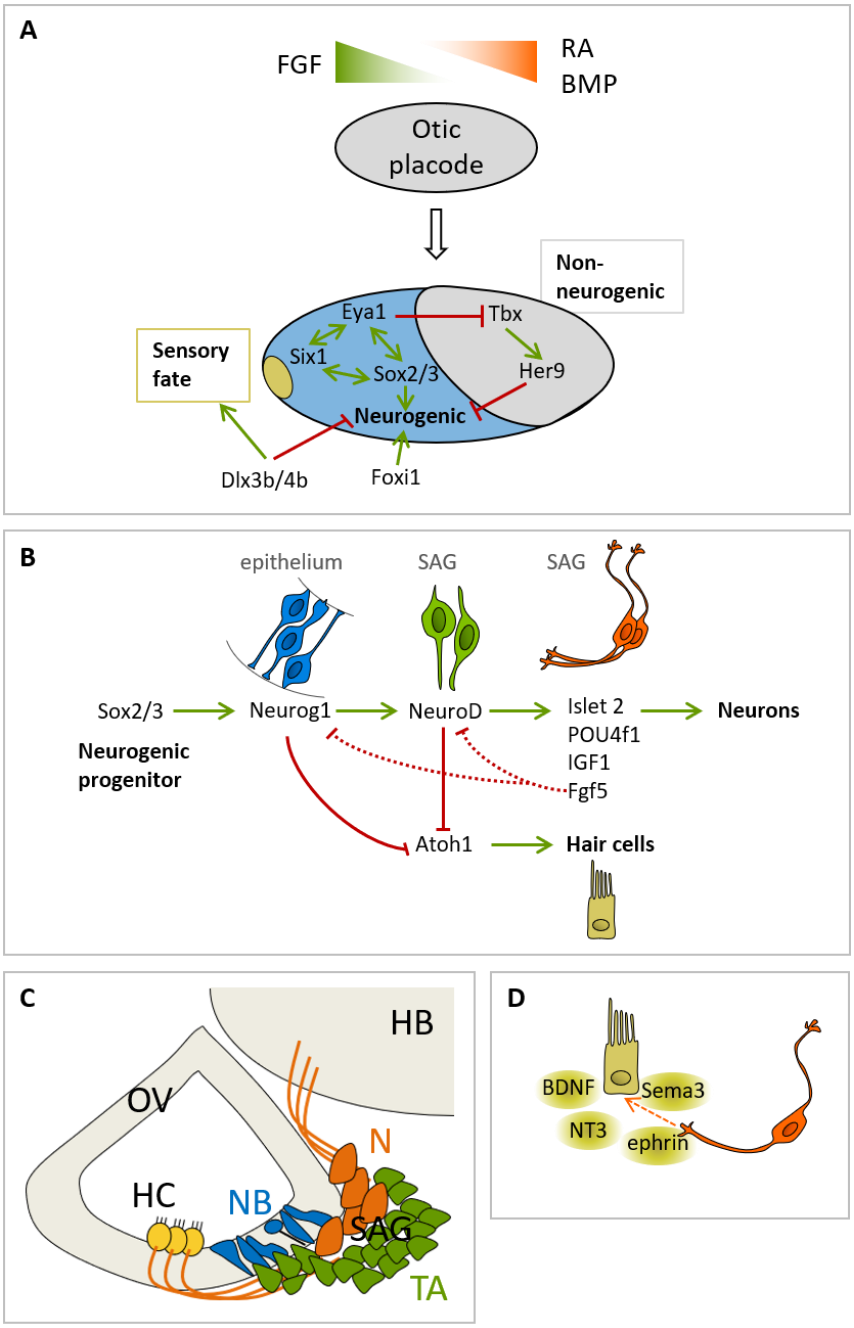

Figure 6. Signals involved in the SAG neurogenesis.

A. Establishment of the neurogenic (blue) and non-neurogenic (grey) regions in the otic placode and the signals and genetic networks involved in it. **B.** Neurogenic progenitors give rise to neurons and sensory cells and the genetic network responsible of it. **C.** Schematic representation of the different stages of the SAG development. Otic neurogenesis: neuroblasts (NB, blue) are specified in the otic epithelium, delaminate and accumulate beneath the otic vesicle and enter a transit amplifying (TA, green) phase.

(legend continued on next page)

These cells finally differentiate into sensory neurons (orange), which innervate hair cells (light green). **D.** Signals secreted by HCs to attract SAG axons, in mouse and chick.

RA, retinoic acid; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; OV, otic vesicle, HB, hindbrain; HC, hair cell, NB, neuroblast; TA, transit-amplifying, N, neuron.

Modified from Maier et al., 2014; Vemaraju et al., 2012.

SAG (Vemaraju et al., 2012) (see Fig 6 B). Finally, other factors such as birthdate or position will determine neuronal innervation of different HCs (Bell et al., 2008; Sapede and Pujades, 2010; Zecca et al., 2015). In mouse, neuronal subtype identity of cochlear versus vestibular fate is acquired by Gata3 expression being restricted to auditory neurons (Jones and Warchol, 2009; Karis et al., 2001; Lawoko-Kerali et al., 2004; Lu et al., 2011).

SAG neurons are bipolar, they extend one axon to the CNS and another one to innervate the HC of the different sensory patches of the inner ear. It is not clear what determines their innervation pattern, but it is known that sensory HCs secrete chemoatractants to guide sensory axons. Some of the signals have identified in mice and chick. Examples are neurotrophins (BDNF and NT3) and their receptors TrkB and C (Fariñas et al., 2001), Semaphorin 3A (sema3A) and neuropilin (Nrp)/plexin receptors (Gu et al., 2003; Katayama et al., 2013), and ephrin/Ephs (Allen-Sharpley et al., 2013; Bianchi and Gray, 2002; Kim et al., 2016; Zhou et al., 2011) (see Fig 6 D).

SAG degeneration and regeneration

Sensory HCs are lost through aging, noise and chemical exposure, for instance by aminoglycoside antibiotics. In mammals, damaged HCs cannot be replaced and, upon their loss, neurons innervating them also enter cell death (Schuknecht and Gacek, 1993; Webster and Webster, 1981). Contrary, fish, amphibians, lizards and birds are capable of HC regeneration thanks to supporting cells transdifferentiation (Avallone et al., 2003; Liang et al., 2012; Roberson et al., 2004; Stone et al., 1999; Taylor and Forge, 2005).

In species where HC regeneration takes place, re-innervation also occurs (Hennig and Cotanche, 1998; Zakir and Dickman, 2006). However, re-enervation does not always correlate with functional recovery (Lawner et al., 1997; Strominger et al., 1995). Efforts

have also been focused on trying to replace lost auditory neurons (Chen et al., 2012b; Coleman et al., 2007; Corrales et al., 2006; Hu et al., 2005; Okano et al., 2005).

Further research needed

Thus, otic neurogenesis is achieved by a coordinated process of specification, delamination, proliferative expansion and terminal differentiation. Some of the signals regulating the sequential steps of SAG development have been identified, being those mainly produced by the inner ear or SAG itself (Vemaraju et al., 2012).

However, it is not known whether other cell types apart from neurons regulate cranial sensory neurons behaviour. It has not been studied before if cranial sensory NSCs develop in a niche, which are the cells that constitute it nor the signals that regulate their behaviour for the proper development of neurogenesis in the cranial sensory system.

2.2 Neurogenesis during adulthood

During late development, RGCs are the primary precursors of neurons and glia (Anthony et al., 2004; Malatesta et al., 2003; Miyata et al., 2001; Noctor et al., 2004). Postnatally, RGCs differentiate into astrocytes (Alves et al., 2002; Schmechel and Rakic, 1979; Voigt, 1989), but some of them are retained as NSCs in the adult brain. Thus, neurogenesis persists during adult stages (Merkle et al., 2004). In these cells, the embryonic pathways must be conserved, despite additional complements of regulatory mechanisms may also be active (Chichung Lie et al., 2004; Machold et al., 2003; Molofsky et al., 2003).

For many years it was thought that the brain, with its extraordinary structure, connectivity and cell type diversity did not have SCs, and that neurogenesis could only occur during development. This dogma was challenged in the 60s by Joseph Altman, who suggested that new neurons could be added in the cortex, hippocampus and olfactory bulb (OB) (Altman, 1962; Altman and Das, 1965). Direct demonstration of adult neurogenesis and functional integration of new neurons came in the 80s in songbirds (Burd and Nottebohm, 1985; Paton and Nottebohm, 1984). The presence of NSCs in the mammalian brain was demonstrated *in vitro* in the 90s (Gage et al., 1995; Lois and Alvarez-Buylla, 1993; Reynolds and Weiss, 1992; Richards et al., 1992). Since then, neurogenesis and the existence of NSCs in adult mammalian brain, including humans (Eriksson et al., 1998), has been widely confirmed. Still, the possibility that other neurogenic regions exist and the search for new sites of adult neurogenesis continues.

In the brain, adult NSCs only reside in a specialized brain microenvironment called NSC niche. A continuous communication between SCs and their niche is required for maintaining stemness, and to control their proliferation and differentiation. In the adult brain there are two regions containing NSCs that will generate neurons and macroglia. These two neurogenic regions are the Ventricular-Subventricular Zone (V-SVZ) and the Subgranular Zone (SGZ). The V-SVZ is the largest neurogenic region in the adult brain, found in the walls of the two lateral ventricles and 5-6 cells thick. The V-SVZ produces neurons that migrate long distances through the rostral migratory stream (RMS) to the OB (Luskin, 1993; Menn et al., 2006) and functionally integrate into the existing circuitry (Belluzzi et al., 2003; Carleton et al., 2003). These new born neurons contribute to fine odour detection and odour-reward discrimination (Grelat et al., 2018; Li et al., 2018; Lledo and Saghatelyan, 2005; Shingo et al., 2003). V-SVZ neurogenesis increases upon injury and migration is directed toward the injury site (Arvidsson et al., 2002). The second neurogenic region is the SGZ, which is placed between the hilus and the dentate gyrus.

SGZ progenitors migrate short distances to the granule cell layer and produces excitatory neurons (Seri et al., 2004). They are involved in learning, memory and pattern separation (Clelland et al., 2009; Ming and Song, 2011; Sahay et al., 2011; Tronel et al., 2012). Exercise and enriching environments increase hippocampal neurogenesis (Dranovsky et al., 2011; Praag et al., 1999), while stress and social isolation reduce it (Dranovsky et al., 2011; Goshen et al., 2008; McEwen, 1999). New neurons generation allow, therefore, adaptative responses to environmental factors. Still, in adult mammals, NSCs are mainly quiescent and only become activated under specific situations such as learning, exercise or injury (Gould and Tanapat, 1997; Gould et al., 1999; Kempermann et al., 1997; van Praag et al., 2005; Rochefort et al., 2002).

These two niches differ structurally but share some key features. In both cases NSCs contact different regions of their niche, allowing the regulation of their behaviour, and the interaction with BV. The V-SVZ niche s composed by different neural cell types: NSCs (type B1 cells); IPCs or TACs (IPC/TAC, type C cells); and neuroblasts (type A cells) (see Fig 7 A and B). B1 cells can be divided into quiescent and activated NSCs (Morizur et al., 2018). These last ones produce IPC/TACs or C cells. C cells have a round morphology, few processes and divide 3 to 4 times before generating migrating interneurons or A cells. A cells will also divide and eventually group to enter the RMS to reach the OB (Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Luskin, 1993; Ponti et al., 2013). It seems B1 cells can also produce oligodendrocytes *in vitro*, but it is not yet clear if they are multipotent *in vivo* and can generate both neurons and glia (Casper & McCarthy, 2006; Fogarty, 2005; Nait-Oumesmar et al., 1999; Menn et al., 2006) (see Fig 7 A and B).

The SGZ niche has at least two populations of astrocytes: radial astrocytes, which extend a process into the granule layer, are nestin^{+} and proliferate; and horizontal astrocytes, which extend a basal process under the granule cell layer and are nestin^{-}. These two types are also called B cells, which will give rise to intermediate precursors (type D cells). Type D cells will progressively generate more differentiated progeny (type D1, D2 and D3), which will eventually differentiate into granule neurons (type G) (Kronenberg et al., 2003; Seri et al., 2004) (see Fig 7 A and C).

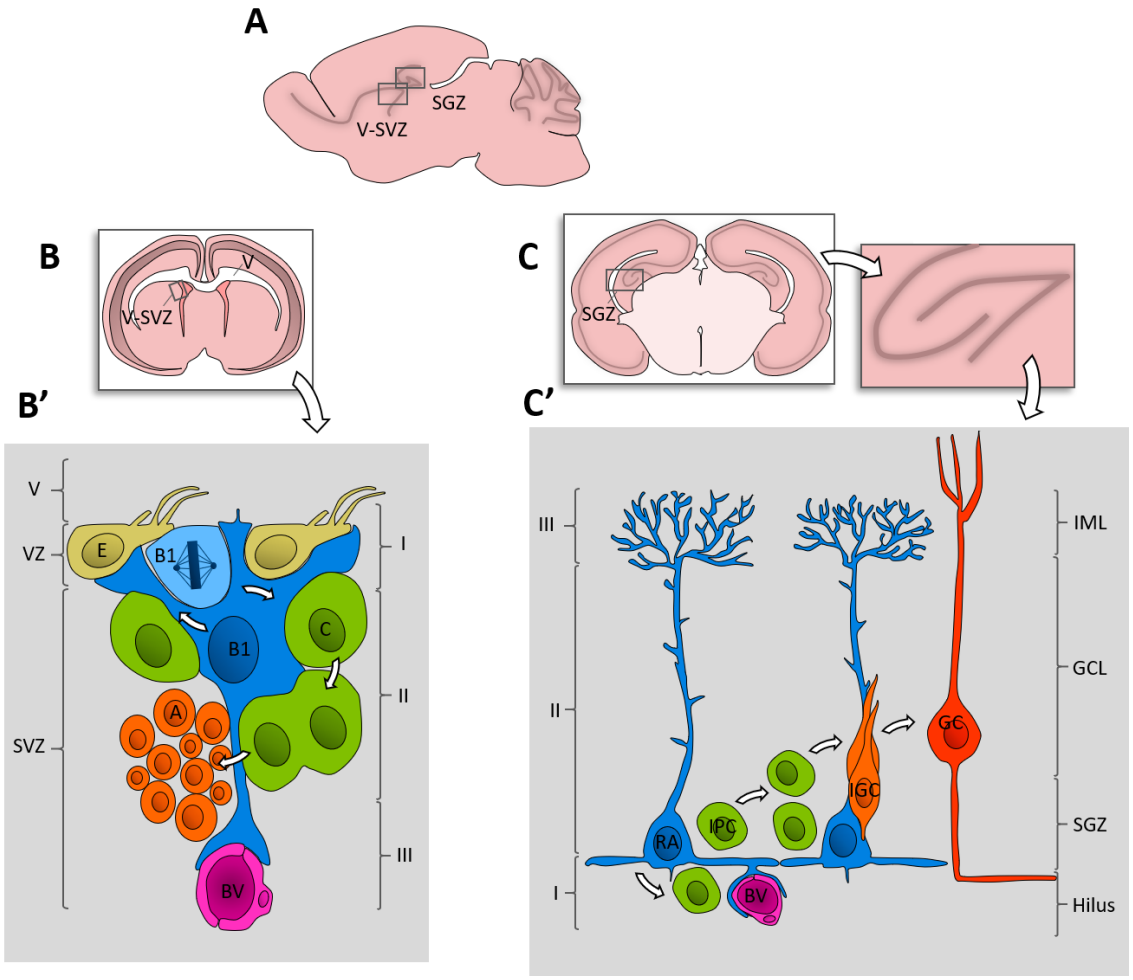

Figure 7. Adult Neural Stem Cell Niches: the V-SVZ and the SGZ

A. Drawing of a lateral section of the adult mouse brain. The V-SVZ and the SGZ are indicated. **B.** Drawing of a transverse section of the adult mouse brain showing the location of the V-SVZ. **B'.** The cellular composition and the domains of the V-SVZ. B1 cells (blue) are surrounded by E cells forming a pinwheel structure in the ventricular surface. B1 cells give rise to C cells (green), the transit-amplifying cells that will give rise to the A cells (red). B1 cells can be divided in three domains. Domain I (proximal) contains the primary cilium and contacts the CSF and the E cells (light green). Domain II (intermediate) is in close proximity with C and A cells. Domain III (distal) comprises a specialized end-foot process that contacts BV. **C.** Drawing of a transverse section of the adult mouse brain showing the location of the SGZ.

(legand continued on next page)

C'. The cellular composition and the domains of the SGZ. RA (blue) give rise to IPCs (green), which differentiate into GCs. RA can be subdivided in three domains. Domain I (proximal) faces the hilus, harbours a primary cilium and contacts BV and other RA. Domain II (intermediate) contains the cell body and interacts with IPCs and GCs. Domain III (distal) contacts other glial cells, axons and synaptic terminals in the IML.

V-SVZ, ventricular-subventricular zone; SGZ, sub-granular zone; E, ependymal cell; CSF, cerebrospinal fluid; BV, blood vessels; RA, radial astrocytes; IPC, intermediate progenitor cell; IGC, immature granule cell, GC, granule cell; IML, inner molecular layer; GCL, granule cell layer.

Modified from Fuentealba et al., 2012.

1.2.1 Domains of the NSC niche

B1 cells of the V-SVZ, or NSC, have a very peculiar morphology that allows them to interact with multiple environments. B1 cells have an apical-basal polarity and are organized in clusters. A subpopulation of type B cells have an apical process called primary cilium. The proximal domain of B1 cells is formed by this primary cilium, which penetrates the cerebro-spinal fluid (CSF) that fills the ventricles to sense changes in signals in this humoral compartment (Doetsch et al., 1999). Signals in the CSF can promote NSC proliferation (Bauer and Patterson, 2006; Lehtinen et al., 2011). The proximal part of B-cells is also surrounded by ependymal cells, which form a pinwheel around the apical processes and secrete signals that regulate NSC stemness and proliferation (Holmberg et al., 2005; Lim et al., 2000; Mirzadeh et al., 2008; Peretto et al., 2004; Ramírez-Castillejo et al., 2006) (see Fig 7 B').

The intermediate domain of B1 cells is in contact with IPCs and neuroblasts, allowing the NSCs to receive feedback signalling from them, to regulate their proliferation and stemness maintenance (Alfonso et al., 2012; Fernando et al., 2011; Kirby et al., 2015; Liu et al., 2005) (see Fig 7 B').

Finally, the distal domain is formed by a specialized end-food process that allows B1 cells to interact with BV (Kacem et al., 1998) (see Fig 7 B' and 8). BV perfuse all the body with oxygen, nutrients and signals. However, it is now clear that vasculature in the NSC niche goes beyond the support function. Many studies have reached the conclusion that local microvasculature bed is needed to direct NSC quiescence, proliferation, self-renewal and differentiation, as well as for providing a cellular scaffolding for neuroblasts migration. BV have been demonstrated to be such an important component for NSC

regulation that they can be considered a niche itself, named as “vascular niche”, and it will be developed in a separated section (see **3.5. Vessels instruct on Neurons behaviour and functions**). Finally, fractons -a new structure of the extracellular matrix (ECM)- have been found to be an extension of BV ECM that contact all the cells in the V-SVZ niche and bind neurogenic growth factors (Douet et al., 2013; Kerever et al., 2007; Mercier and Douet, 2014) (see Fig 7).

The integration of all these multiple signals, coming both from neural and nonneural cell types, will regulate NSCs renewal and differentiation. There is an increasing interest on understanding how the NSC microenvironment or niche both helps maintain the NSC pool and facilitates neuroblast production.

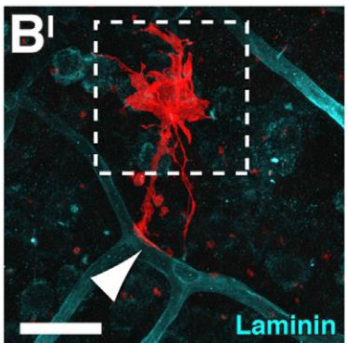

Figure 8. NSC contacting a blood vessel through an end-food process.

Confocal image of a whole mount showing projections of B cells (mP2-mCherry^{+} cells, red) contacting blood vessels (immunostained with laminin, cyan).

Modified from Codega et al., 2014.

3. The vascular system

Besides the neural cells, the other major cell type present in the nervous system/brain is the vascular network.

3.1 Vasculature functions and morphology

The vascular system is composed of two hierarchically branched vessel network: the blood and the lymphatic vasculatures. Despite the vascular system appeared later than the nervous system evolutionary (Carmeliet and Tessier-Lavigne, 2005), vasculature is one of the earliest organs to appear developmentally (Hirschi et al., 1998). Its principal role is to establish a systemic circulation to allow gases, nutrients, hormones and cells distribution, as well as metabolic waste removal (Hirschi et al., 1998; Wener Risau, 1997). Additionally, vascular ECs are highly metabolically active and display physiological roles such as: regulating the proliferation and survival of surrounding cells, establishing systemic innate and adaptative immunity, trafficking blood-born signals, and controlling vascular tone and blood pressure (Aird, 2007).

BV organise as a tree-like structure. Blood travels from the heart to the aorta, the largest axial artery, into smaller arteries and arterioles until distal capillary beds. These allow the exchange between the blood stream and the surrounding tissue. Next, oxygen-depleted blood is drained into small venules, veins and, finally, large axial cardinal veins. Blood is carried via the pulmonary artery to the lungs for re-oxygenation. Functional features, morphology and gene expression of arteries and veins are different (Rocha et al., 2009; Swift and Weinstein, 2009). Arteries transport high pressure and speed blood, while veins do it at low pressure, with valves preventing backflow. There are different types of mural cells covering vessels: the vascular smooth muscle cells (vSMCs), pericytes and hepatic stellate cells. Accordingly with their haemodynamic features, arteries are enwrapped with multiple layers of vSMCs, which are contractile and confer stability to the vessel by depositing matrix and elastic fibres. On the other hand, veins are enveloped by fewer vSMCs. Capillaries are covered by their own specialized supporting cell, the pericytes, which regulate vessel stability and transendothelial transport (Armulik et al., 2005; Bergers and Song, 2005). The endothelium of both arteries and veins is continuous, while in capillaries it can be continuous, fenestrated or discontinuous, depending on the tissue.

3.2 The development of the vasculature

The architecture of the vascular system is the result of the coordinated interaction between the ECM (Haas and Madri, 1999), other cell types (Nguyen and D'Amore, 2001) and growth factors (Adams and Klein, 2000; Jones and Dumont, 2000; Veikkola and Alitalo, 1999). Good reviews on how the vasculature is formed have been written by Potente, Carmeliet and colleagues (Carmeliet, 2000a; Potente and Mäkinen, 2017; Potente et al., 2011).

3.2.1 Vasculogenesis

The assembly of a vascular tube, or BV, occurs through two main mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is the process by which BV are formed de novo. In zebrafish, the neuronal PAS domain-containing protein 4-like protein (Npas4l) is at the top of the gene regulatory network orchestrating the formation of the endothelial and haematopoietic lineages (Reischauer et al., 2016). Multipotent cells in the posterior and anterior lateral plate mesoderm differentiate into endothelial progenitors known as angioblasts and assemble into the large axial and cranial vessels, as well as the transient pharyngeal arch arteries (Carmeliet, 2000b; Etchevers et al., 2002; Mikkola and Orkin, 2002; Paffett-Lugassy et al., 2013; Proulx et al., 2010; Siekmann et al., 2009). This process will give rise to the first major artery and vein: the dorsal aorta and the cardinal vein. It was first though that arterial and venous ECs derived from a common precursor vessel, but recent studies suggest that in fact they derive from different pools of angioblasts that arise at distinct locations (Kohli et al., 2013) (see Fig 9 A). In zebrafish, these precursors receive signals from the notochord (Shh) and the ventral endoderm to become restricted to the aorta and trunk vein respectively (Fouquet et al., 1997; Lawson and Weinstein, 2002a; Lawson et al., 2002; Sumoy et al., 1997; Zhong et al., 2001). Shh produced by the notochord will induce the adjacent somites to secrete VEGF, which in turn will drive artery fate in ECs through Notch signalling pathway (Lawson and Weinstein, 2002b; Lawson et al., 2002) (see BOX 1).

Similarly, in mice the first ECs differentiate from mesodermal progenitors and coalesce into primitive vessel networks at extra- and intra-embryonic sites. In this case, NPAS4, the mammalian homologue for Npas4l, is only transiently expressed but dispensable for vasculature development (Reischauer et al., 2016). During mice vasculogenesis, FGF2

and BMP4s control angioblasts specification and ECs differentiation, and VEGF has a role on ECs propagation and survival (Marcelo et al., 2013).

3.2.2 Regulation of vascular formation

The vascular network uses signalling mechanisms to grow accordingly to tissue architecture and demand. One key signal triggering the formation of new vessels is hypoxia, which leads to the secretion of pro-angiogenic factors and cytokines. The most prominent angiogenic factor is VEGF, which binds to VEGFRs. The most notable VEGFRs are the tyrosine kinases VEGFR2 (Flk-1/Kdrl) and VEGFR3 (Flt4), which are expressed in the surface of ECs. Upon ligand binding, VEGFRs trigger downstream signalling including activation of mitogen-activated kinase pathway, phosphoinositide Kinase 3 (PI3K), and Akt, phospholipase C γ, and small GTPases such as Rac1 (Olsson et al., 2006; Zachary, 2005). Selective activation translates into biological responses as diverse as proliferation and differentiation or angiogenesis and lymphogenesis, but the underlying mechanisms are yet not fully understood. Differential receptors' expression might explain part of them. VEGFR3 is present in all vessels in the early embryo, but gradually becomes restricted to lymphatic vessels (Kaipainen et al., 1995). VEGFR2, on the other hand, is only expressed in vascular vessels. The VEGF coreceptor Nrp-1 is only expressed in arteries, and the Nrp-2 in veins and, later in development, in lymphatic vessels (Herzog et al., 2001; Moyon et al., 2001; Mukouyama et al., 2002).

3.2.3 Arterial and venous specification

Arteries and venous ECs are different at the molecular level (Adams and Alitalo, 2007; Swift and Weinstein, 2009). Notch pathway is high in arteries and low in veins. The Notch pathway regulates Eph/ephrin expression, which configures artery-vein boundary. Notch upregulates ephrinB2a, which is a marker for arteries; while it downregulates EphB4, which will be expressed in veins (Adams et al., 1999; Wang et al., 1998).

3.2.4 Angiogenesis

Angiogenesis is the process by which a new vessel is formed from a pre-existing one through EC delamination and migration. Angiogenesis appears to be the main processes for the formation of most vessels during development, tissue repair and disease processes. New sprouts are formed by two cell types: stalk and tip cells. Tip cells sense the environment and guide the sprout until it eventually fuses to another vessel in a process called anastomosis, establishing a network of perfused vasculature (Potente et al., 2011; De Smet et al., 2009; Wacker and Gerhardt, 2011; Wälchli et al., 2015). Tip cells are followed by stalk cells, which proliferate supporting the elongation of the new vessel and forming the lumen, while tip cells almost do not proliferate (Carmeliet and Jain, 2011; Hasan et al., 2017) (see Fig 9 B).

Recent studies suggest that the Notch pathway, which is well known for its roles in cell fate determination and differentiation processes, regulates the tip-stalk cell decision in a dynamic feed-back loop between VEGF-VEGFR and the Notch pathway (Hasan et al., 2017; Phng and Gerhardt, 2009; Roca and Adams, 2007; Thurston and Kitajewski, 2008). When a new sprout has to grow, all the ECs from the pre-existing vessel are stimulated with VEGF-A, this will make the EC to become activated and to up-regulate the expression of VEGFR1-3 and Nrp-1 as well as the Notch ligand Delta-like protein 4 (Dll4) (Jakobsson et al., 2010). The EC that express more quickly higher amounts of Dll4 will take the competitive advantage of becoming the tip cell. Dll4 induces the neighbouring ECs to become specified as stalk cell by activating Notch1 signalling, which results in the down-regulation of VEGFR2, 3 and Nrp-1 and the up-regulation of VEGFR1 - a decoy receptor that sequesters VEGF -, altogether becoming less sensitive to VEGF (Blanco and Gerhardt, 2013; Hellström et al., 2007; Suchting et al., 2007; Tammela et al., 2008). Dll4-Notch signalling, thus, restricts the number of tip cells and the filopodia of tip cells through cell-cell contact-dependent lateral inhibition. Alteration of this pathway leads to hyper-sprouting and filopodia, increase in EC number and blood flow alterations (Hasan et al., 2017; Leslie et al., 2007; Siekmann and Lawson, 2007). In contrast to Dll4, the Notch ligand Jagged-1 (Jag1) is mainly expressed by stalk cells. Given that some Dll4 is detectable in stalk cells, Jag1 helps maintain a differential Notch activity by antagonizing Dll4, which signals back to tip cells (Eilken and Adams, 2010). The described signalling pathway can be influenced by other signals such as BMP9 and 10 provided by blood flow (Larrivée et al., 2012; Moya et al., 2012) and ECM interactions (Germain et al., 2010; Stenzel et al., 2011) (see Fig 9 C).

Compared to tip cells, stalk cells produce fewer filopodia, proliferate more and can produce a lumen. They also establish junctions with neighbouring cells and produce a basement membrane to ensure the integrity of the new vessel (Phng and Gerhardt, 2009). It is important to note that tip and stalk cell phenotype is a transient cell fate, allowing dynamic position changes (Hasan et al., 2017; Jakobsson et al., 2010).

Tip cell guidance, anastomosis, lumen formation and vessel maturation

The vascular system must be properly patterned for optimal oxygen and nutrients delivery. Emerging vessels will use tip cells to guide sprouts correctly. The new sprouts pattern will be determined by the expression of receptors in tip cells and the attractive and repulsive cues that the sprout will encounter while growing.

The establishment of the lumen occurs by different mechanisms that depend on the type of vessel (Iruela-Arispe and Davis, 2009; Zeeb et al., 2010). One of these mechanisms is cell hollowing, which occurs by the fusion of pinocytic vacuoles. Whereas in the chord hollowing ECs adjust their shape, define an apico-basal polarity and rearrange their junctions to open up a lumen (Strilić et al., 2009; Zeeb et al., 2010).

Eventually tip cells will fuse to other tip cells or vessels in a process called anastomosis. Blood flow onset will also shape vessels connections, modifying EC transcriptome by shear-stress responsive transcription factors such as Krüppel-like factor 2 in zebrafish (Nicoli et al., 2010). Responses to physiological laminar shear include actin cytoskeleton and focal adhesions reorganization (Baeyens et al., 2015). Perfusion will also supply with oxygen and nutrients, reducing VEGF expression and EC oxygen sensors, altogether shifting the endothelial behaviour towards a quiescent state. Otherwise, non-perfused segments regress (Korn and Augustin, 2015).

The last steps of vascular mophogenesis will include pruning and remodelling of the vascular network to meet the specific tissue needs (Baluk et al., 2004; Rocha et al., 2009). After vascular networks are established, ECs enter quiescence, a reversible state where ECs do not divide or migrate (Serra et al., 2015; Wilhelm et al., 2016). Pericytes and vSMCs are thought to stabilize BV, together with the deposition of extracellular matrix, and to promote a mature non-angiogenic state of vasculature (Armulik et al., 2005; Bergers and Song, 2005; Betsholtz et al., 2005). Through iterative cycles of angiogenesis, ECs progressively expand the primary vascular network.

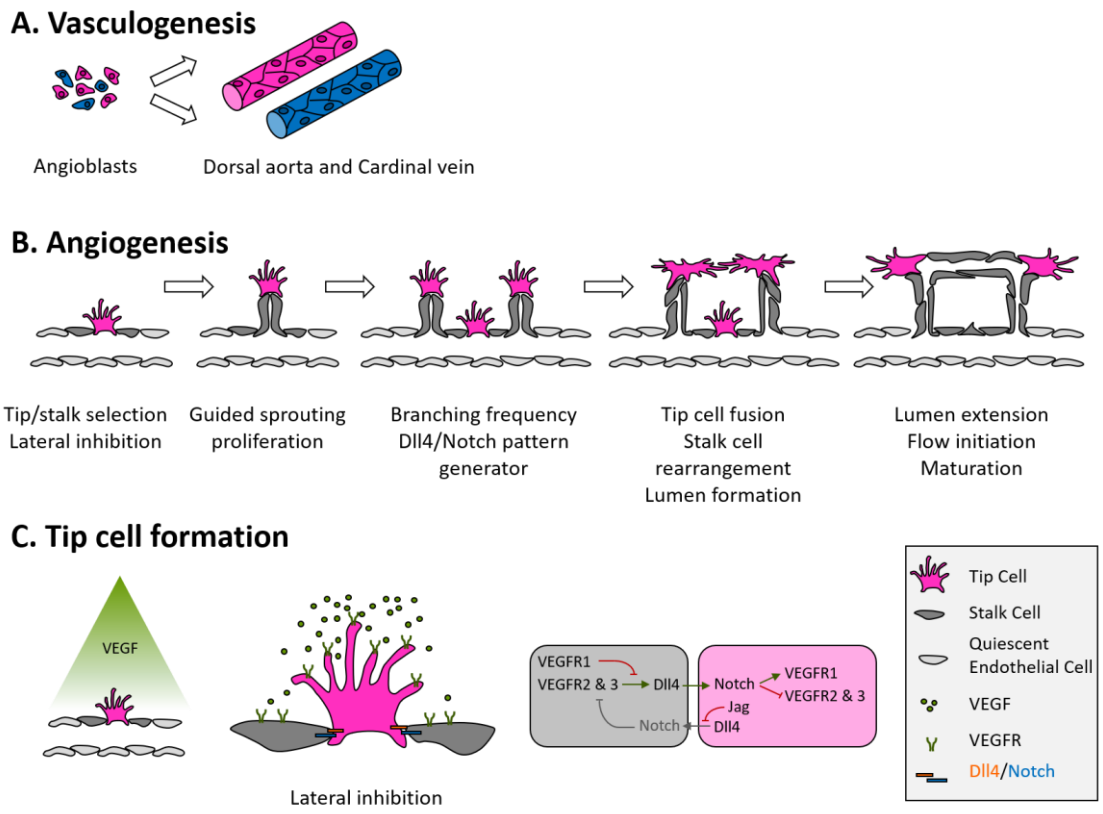

Figure 9. Hallmarks of vessel formation.

A. Vasculogenesis: endothelial progenitor cells, known as angioblasts, differentiate from mesoderm, acquire arterial or venous fate, and assemble into the first embryonic blood vessels: the dorsal aorta and the cardinal vein. **B.** Angiogenesis: i) tip/stalk cell selection, ii) tip cell migration and stalk cell proliferation, iii) branching coordination, iv) stalk cell elongation, tip cell fusion and lumen formation, v) perfusion and vessel maturation. C. Tip cell formation: in response to VEGF and the Notch pathway ECs are selected as tip or stalk cells.

Modified from Potente and Mäkinen, 2017; Potente et al., 2011.

3.3 The development of the vasculature in the CNS

During mouse embryogenesis, the perineural vascular plexus (PNVP) forms around the neural tube via vasculogenesis at embryonic 8.5 (E8.5) regulated by VEGF-A secreted by the neural tube (Mancuso et al., 2008). In the brain, vascularization occurs mainly by angiogenesis (Pardanaud et al., 1989), as a response to the metabolic demands of the expanding nervous system (Tuor et al., 1994). The PNVP will give rise to the arteries and veins of the leptomeninges. At E9.5, angiogenic sprouts will grow from the PNVP to penetrate the CNS parenchyma towards the SVZ, under the regulation of VEGF-A-VEGFR-Nrp-1, forming the intraneural vascular plexus (INVP) (Daneman et al., 2010; Mackenzie and Ruhrberg, 2012). Research over the last years supports that vascularization of the CNS is controlled by CNS-specific vascular cues (Ruhrberg and Bautch, 2013). One of the key angiogenic factors in this process is VEGF (Hogan et al., 2004; Mackenzie and Ruhrberg, 2012).

VEGF is synthesized and released by the ventricular neuroectoderm (Breier et al., 1992), while VEGFR2 and Nrp-1 is expressed on invading ECs (Gu et al., 2003). Wnt7a and 7b, which are expressed by the neuroepithelium coincident with vascular invasion, and the G protein-coupled receptor GPR124 also participate in the proper development of INVP and CNS-specific properties in certain compartments of the CNS (e.g. hind- and forebrain) (Anderson et al., 2011; Cullen et al., 2011; Daneman et al., 2009; Kuhnert et al., 2010; Stenman et al., 2008). Wnt/β-catenin signalling has been demonstrated to interact with the VEGF-VEGFR-Dll4-Jag-Notch pathways both in mice and zebrafish (Corada et al., 2010; Phng et al., 2009). Other signals that participate in the sprouting inside the CNS are VEGFs, Dll4-Notch, Angiopoietins, Integrins, Slits and Dr6/TROY, as well as relative hypoxia (Jeansson et al., 2011; Mancuso et al., 2008; Stenzel et al., 2011; Tam et al., 2012). At a cellular level, the newly formed ECs will interact with neurons, astrocytes, pericytes and, postnatally, also with oligodendrocytes and NSCs (Eichmann and Thomas, 2013; Mancuso et al., 2008; Quaegebeur et al., 2011).

In general, brain vascularization is completed entirely during development. However, the CNS vasculature can continue to remodel postnatally, but the mechanisms governing these angiogenic sprouting are poorly understood further from VEGF-A (Ogunshola et al., 2000; Wälchli et al., 2015).

Composition of blood vessels in the brain

In the head, BV are formed, in general terms, by two cell types: ECs form the inner luminal lining, and mural cells form the outer contractile layer. An additional outer layer exists in larger vessels, such as big arteries, and it is composed of fibroblast, ECM and perivascular nerves (Hirschi et al., 1999).

Large cerebral arteries branch into smaller pial arteries and arterioles. The pial arterioles travel through the surface of the brain in the subarachinoid space and produce the first arteries and arterioles that will penetrate the brain. As arterioles progress into the brain they lose the perivascular space that separate them from the brain, allowing direct contact between astrocytic end-feet and vascular cells basement membrane. When going even deeper into the brain, arterioles become capillaries (Girouard and Iadecola, 2005), they are variably surrounded by pericytes and ECM. The minimal composition of capillaries allows for a unique interface for communication with the surrounding tissue (Aird, 2007).

3.4 The development of the vasculature in zebrafish

For this study, we have used zebrafish embryos. Their external fertilization, small size, rapid embryonic development and optical transparency, together with the amenability to genetic and pharmacological manipulation, have made zebrafish a very popular animal model to study organogenesis in vivo.

The vascular anatomy of zebrafish during development has been described in detail. Early studies were performed by injection of small fluorescent microspheres (Isogai et al., 2001), and thus missed non-lumenizated vessels. The later generation of vascular specific reporter transgenic lines complemented previous studies and allowed the observation of cellular activities such as cell migration, division and cytoskeletal rearrangements as they occur during BV formation (Lawson and Weinstein, 2002b; Phng et al., 2013; Ulrich et al., 2011). Also, it possesses a very interesting feature: zebrafish embryos develop independent of the circulation for oxygen supply until 6 dpf, this makes it a very suitable model to study the effect of endothelial vascular cells and blood cells in cardiovascular mutant embryos, without inducing hypoxia.

One of the most broadly used avascular models in zebrafish is the *cloche* mutant (*clo*), named after its bell-shaped heart. This mutation leads to the loss of most cells of the

endothelial and haematopoietic lineages (Liao et al., 1998, 1997, 2000; Stainier et al., 1995), and an expansion of the cardiomyocytes number (Schoenebeck et al., 2007) (see Fig 10 A). For this reason, it became very popular on the study of mesoderm diversification and differentiation.

The gene defective in this mutant has just very recently been identified by the same group that first described the *clo* mutant (Reischauer et al., 2016). The telomeric location and transient early expression during embryogenesis made this discovery to be delayed for 20 years. The novel gene has been named *npas4*-like (*npas4l*), because of the limited homology shared with the mammalian gene NPAS4 transcription factor, which is involved in the development of inhibitory synapses (Lin et al., 2008). *Npas4l* encodes for a PAS-domain-containing bHLH transcription factor that functions upstream of the earliest endothelial and haematopoietic transcription factors identified to date, *etv2* and *tal1* respectively (Reischauer et al., 2016). Positioned at the top of the transcriptional cascade, Cloche/Npas4l drives the commitment of the multipotent mesodermal cells to the endothelial and haematopoietic lineages (see Fig 10 B).

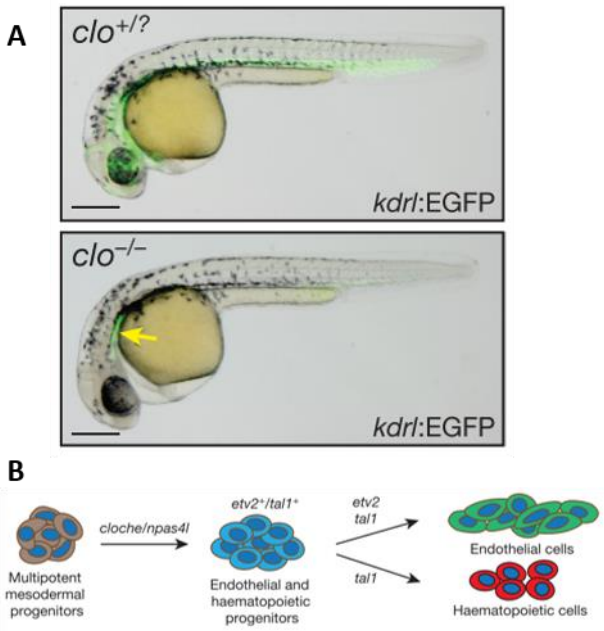

Figure 10. The cloche mutation.

A. 30 hpf sibling and *clo*^{5} mutant embryos with the *Tg(kdrl:GFP)*^{1a116} reporter of endothelial cells, which are missing in the *clo* mutant. Yellow arrow, kdrl:EGFP expression in pharyngeal arch endoderm observed in both wild-type and *clo* mutant embryos. Scale bars, 200 μm. **B.** Schematic illustration of Cloche/Npas4l function; *cloche/npas4l* is necessary and sufficient for the expression of early endothelial and haematopoietic markers including *etv2* and *tal1*.

Modified from Reischauer et al., 2016.

The basic frameworks of vasculature development have been demonstrated to be conserved among vertebrates, making it possible to assign homologies and compare the formation of vessels in different species (Isogai et al., 2001). Not only this, but functional studies of forward and reverse genetics have shown that the molecular mechanisms governing vascular development are conserved between mammals and fish (Beis and Stainier, 2006; Lawson and Weinstein, 2002b; Thisse and Zon, 2002). As an example, transcription factors of the ETS, GATA and LMO families control the specification of the haematopoietic and EC lineages both in mammals and in zebrafish (Detrich et al., 1995; Feng and Roger, 2008; Thompson et al., 1998; Val et al., 2008; Zon et al., 1991).

Therefore, the zebrafish embryo has become a unique system where live imaging can be combined with functional studies to gain a complete view on the molecular and morphogenetic mechanisms regulating the vascular tree formation.

Description of the cranial vascular anatomy of the developing zebrafish

The primary circulatory loop forms after 1 day post fertilization (dpf) and is comprised by a two-chamber heart connected to the aortic arches (AAs) that empty the blood into the right and left lateral dorsal aortas (LDA), which run caudally until they coalesce into a single dorsal aorta (DA). The DA continues into the tail and, at it most caudal end, turns 180º to empty into the posterior cardinal vein (PCV). The PCV splits into the bilateral cardinal veins, and finally empties into the common cardinal veins (CCVs), which runs across the yolk on both sides, coming together back to the heart.

Shortly after this first circulatory loop, a second one is built around 1.5 dpf. The AAs connect caudally to the LDAs and rostrally to the primitive internal carotid arteries (PICAs), each one will divide into two vessels one caudally and another rostrally. The cranial one will empty into the primordial midbrain channel (PMBC), which will continue caudally to the primordial hindbrain channel (PHBC). The PHBC is located medial to the cranial nerve and the otic vesicle. From the PMBC-PHBC junction, the midcerebral veins (MCeV) will take off extending along the midbrain-hindbrain boundary. In the caudal end of the OV a sprout grows at 2.5 dpf from the PHBC building the primary head sinus (PHS), which runs ventrolateral to the OV and will fuse to the rostral portion of the anterior cardinal vein (ACV). Finally, at 2 dpf sprouts from the PHBC grow to form the central arteries (CtAs), which will fuse with the basilar artery (BA) that extends caudally along

the base of the medulla oblongata (Isogai et al., 2001; Lawson and Weinstein, 2002b; Ulrich et al., 2011) (see Fig 11).

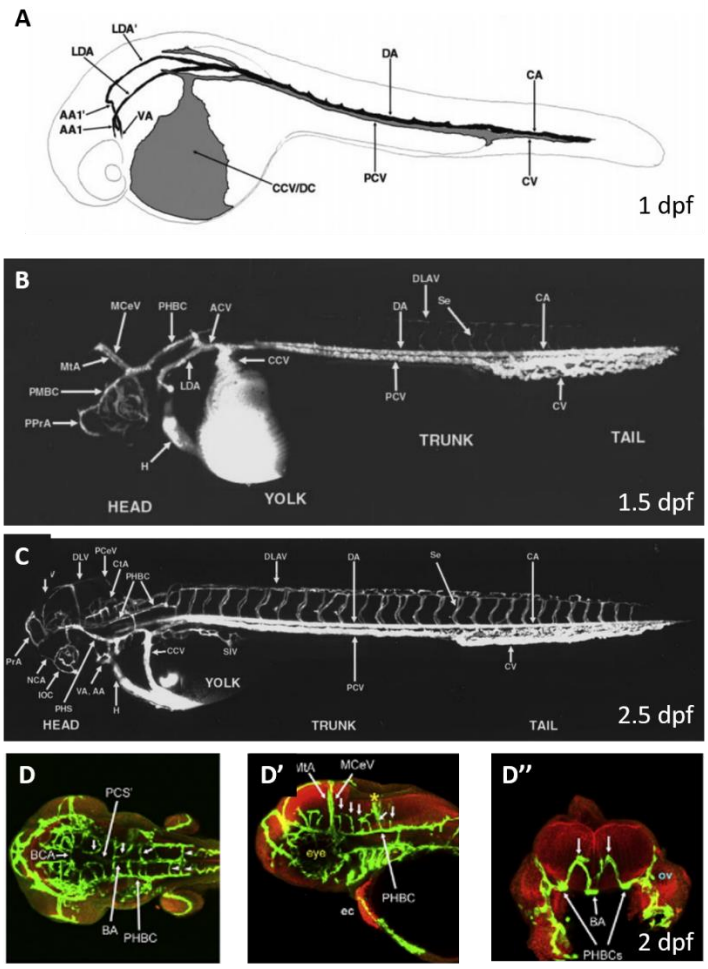

Figure 11. Zebrafish vascular system.

A. Drawing of active vessels in the zebrafish embryo just after the initiation of circulation, at approximately 24 hpf. **B**. Angiogram of a developing zebrafish at approximately 1.5 dpf. Lateral views. **C**. Circulation in the developing zebrafish at approximately 2.5 dpf. **D**. Maximum intensity confocal projections of *Tg(kdrl:GFP)*^{1a116} embryos showing the vascular development of the head. Endothelium, green (GFP). Cell outlines, red (β-catenin). Small white arrows, CtAs. Dorsal view, ventral level. **D'** Lateral view. **D''** Transverse cross-sections of the posterior hindbrain at approximately the r5-r6 level.

Modified from Isogai et al., 2001; Ulrich et al., 2011.

BOX 1. Notch/Delta signalling in neuronal and vascular development

The Notch signalling pathway is highly conserved among all metazoan species. It governs the development of different cell types, including endothelial and neural cells, through cell-cell contacts (Artavanis-Tsakonas et al., 1999) (see Fig BOX 1.1). It regulates different cellular processes as diverse as cell fate decision, quiescence, proliferation, apoptosis, migration and plasticity.

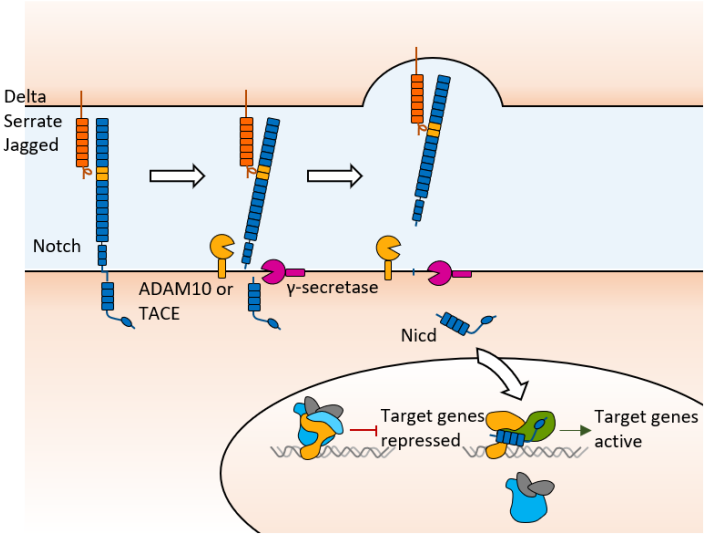

Figure BOX 1.1. The core Notch pathway.

The signalling pathway is initiated when the receptor binds to one of its ligands expressed in the surface of another cell. This binding produces the endocytosis of the Notch-ligand complex by the ligand-expressing cell and the cleavage of the Notch intracellular domain (NICD) by the γ-secretase complex. The NICD translocates to the nucleus and assembles to a transcriptional activation complex that includes DNA binding proteins, which ultimately will relieve the repression of Notch target genes, such as the hairy and enhancer of split (HES). HES, in turn, negatively regulate the expression or activity of differentiation factors such as MASH, MYOD and neurogenin.

Modified from Bray, 2006.

In mammals there are four Notch receptors (Notch1-4), which are a single-pass transmembrane heterodimers, and five ligands that belong to the Delta-Serrate-Lag-2 family and include Jagged1 and 2 (Jag1,2), and Delta-like 1, 3 and 4 (Dll1,3,4) (D'Souza et al., 2010).

The phenotype of Notch haploinsufficiency was one of the first described in *Drosophila*, and results in the failure of the neurogenic ectoderm to segregate neural and epidermal cell lineages and all cells become neuroblasts (Poulson, 1937). Further studies identified a role for Notch signalling in the regulation of NSCs differentiation (Hitoshi et al., 2002; Lütolf et al., 2002; Pompa de la et al., 1997; Yang et al., 2004; Yoon et al., 2004). In the adult brain, Notch signalling also maintains adult NSCs promoting self-renewal and inhibiting cell-cycle exit (Breunig et al., 2007; Givogri et al., 2006; Imayoshi et al., 2010; Irvin et al., 2004; Ottone et al., 2014; Stump et al., 2002).

During angiogenesis Notch signalling is key to determine tip versus stalk cell position, which will regulate migration, branching and lumen formation of new sprouts. Dll4 will have an antiangiogenic function whereas Jag1 will be proangiogenic (Hasan et al., 2017; Pitulescu et al., 2017; Siekmann and Lawson, 2007).

4. The Neural and Vascular systems – Common features and functional interactions

500 years ago, the Belgian anatomist Andreas Versalius already realized that the structure of the nervous system and the vascular system resemble each other (Fig 12 A and B). Evolutionary speaking, albeit less complex, the vascular system arose later than the nervous one. Despite being totally different systems, they have the same functional aim: a transport means over long distances reaching every cell of the body – the nervous system processes electric signals to transfer information, and the vascular system works as a pathway for dissolved messenger molecules, oxygen, nutrients and leukocytes traffic. Thus, it is not surprising that the vascular system co-opted for several of the organizational principles and molecular signals of the nervous system to differentiate, grow and navigate towards their target.

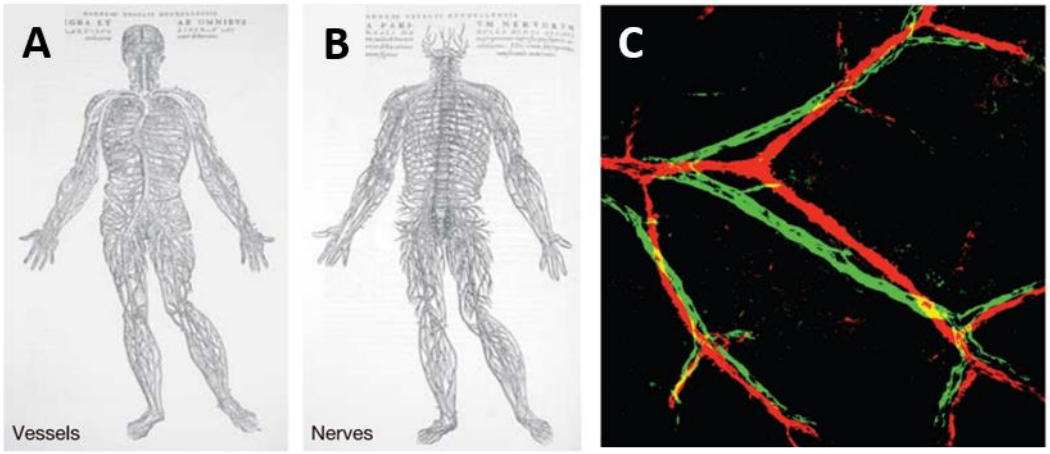

Figure 12. Parallels in vessel and nerve patterning.

A, B, Drawings by the Belgian anatomist Andreas Vesalius, illustrating the similarities in the arborization of the vascular and nervous networks (Aristotle On the Parts of Animals (eBooks@Adelaide, The University of Adelaide Library, University of Adelaide, 2004); hhttp://etext.library.adelaide.edu.au/a/ aristotle/parts/index.htmli). **C**. Coalignment of nerves and vessels in mouse skin. Whole-mount double heterozygous embryos showing arteries (red, ephrinB2) aligned with peripheral sensory nerves (green, neurofilament).

Images taken from Melani and Weinstein, 2010.

4.1 Similar morphological structures

The vascular and nervous systems in vertebrates resemble each other at three different levels. First, they are anatomically similar as they are hierarchically branched structures that form a network that will reach almost every point of an organism. In each system it can be identified an efferent and afferent network -motor and sensory nerves in the nervous system, and veins and arteries in the vascular system. Besides, in the PNS they are often patterned in parallel, a phenomenon called neurovascular congruency (Fig 12 C). This coupling can be explained by different reasons. One example would be for metabolic needs, the nervous system is highly dependent on oxygen and nutrients and thus has to ensure a proper supply from BV. This coordination can be achieved by the two systems following the same signalling cue, or otherwise, by one system directly signalling the other one. These mechanisms also provide for development efficiency: following an already formed "path" can be time efficient and ensure proper patterning. In some cases, axons will crawl on BV to reach a determined point (Makita et al., 2008) or ECs will be called and grouped on top of an already formed axon (Li et al., 2013b). Finally, both systems have a robust patterning and a conservation on their anatomical architecture will be found.

They are also alike at the cellular level. Their most distal structure, being it the axonal growth cone in nerves and the distal tip cell in vessels, are resembling. When neurons are developing, they send a cable-like axon called "pioneer axon" that can travel long distances to reach its target. The leading task of the pioneer axons is performed by the growth cone. The growth cone is a highly motile structure at the axon tip that extends filopodia and lamellipodia protrusions to sense the environment and select the correct path (Goodman and Shatz, 1993; Lowery and Vactor, 2009; Tessier-Lavigne and Goodman, 1996), which was first described by Ramón y Cajal a century ago (Ramon y Cajal, 1890).

During angiogenesis new vessels sprout from already existing ones through the delamination and migration of ECs to colonize avascular areas of the body. In the forefront of this new growing vessels there is a specialized EC called "tip cells" (Gerhardt et al., 2003). The tip cell of vessel sprouts shares many similarities with axonal growth cones. They send and retract filopodia and lamellipodia with the aim to explore the environment and sense signalling cues through the expression of receptors in the cell membrane and respond via regulation of cytoskeleton remodelling (Dickson, 2002; Gerhardt et al., 2003) (see Fig 13).

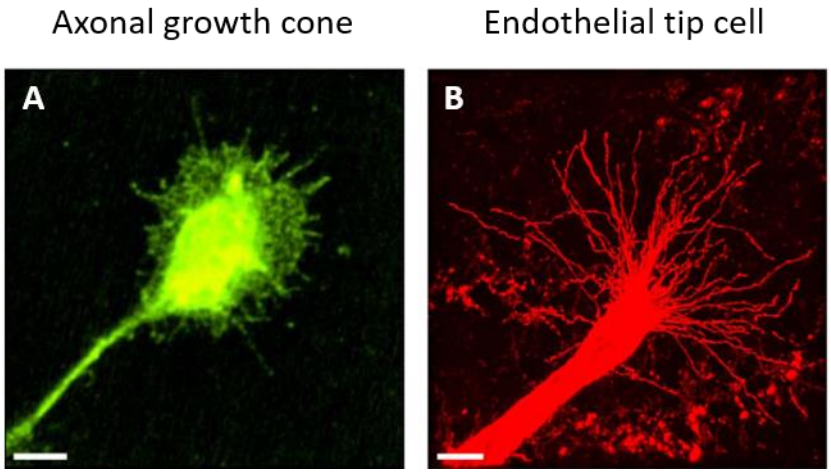

Figure 13. Cellular and molecular similarities between the Neuronal Growth Cone and the Vascular Endothelial Tip Cell.

A. The axonal growth cone at the leading edge of a growing axon is a specialized structure at the tip of an extending neuron, usually far away from its cell body. Actin-based structures (brown) such as lamellipodia and filopodia are used to sense and integrate guidance cues in the local tissue microenvironment in order to guide the extending axon to its appropriate target, where it forms a synapse. **B**. The endothelial tip cell is a specialized vascular endothelial cell type at the tip of the newly forming blood vessel, followed by stalk cells, another specialized cell type. The endothelial tip cell uses actin-based lamellipodia and filopodia sens cues in the local tissue microenvironment. Thereby, the extending blood vessel reaches its target, for example another developing blood vessel constituting a fusion partner (anastomosis).

Modified from Wälchli et al., 2015.

As seen, the development and patterning of the nervous and the vascular system are regulated by the presence of attractive and repulsive cues. Unexpectedly, in the late 90s it was discovered that the molecular signals that they use for specification, differentiation and patterning are in some cases shared, as it is for the case of ephrins, Semaphorins, Netrins and Slit signalling pathways. These signals have also been called “angioneurins” [reviewed in (Klagsbrun and Eichmann, 2005; Melani and Weinstein, 2010)].

Besides sharing the aforementioned guiding cues, morphogens and growth factors typical of each system have been lately been described to play roles on the other system as will be described in the following sections. This is the case for wingless-type proteins (Wnts), Sonic Hedgehog (Shh) and Bone Morphogenic Protein (BMPs) (Charron and Tessier-Lavigne, 2007; Zacchigna et al., 2007), as well as VEGF-A, FGF-2, Endothelin-3 and artemin (Honma et al., 2002; Quaegebeur et al., 2011).

4.2 Neurovascular congruency

The congruence in the peripheral nerves and BV anatomical patterns observed in vertebrate adults is established during embryogenesis (Bates et al., 2002). It has mainly been studied in the forelimb of different vertebrates (Bates et al., 2003; Mukouyama et al., 2005) and occurs through more or less direct cell-cell interactions that result in a mutual crosstalk or co-patterning in which neurogenesis and angiogenesis occur concomitantly, a phenomenon called “neurovascular congruency” (Martin and Lewis, 1989; Taylor et al., 1994). Through it, neural cells ensure a proper vascularization to meet their high metabolic demands or one system uses the other as a scaffold for its patterning. Neurovascular congruency can be achieved through two different mechanisms: independently, in which both systems share the same patterning mechanism; or co-ordinately, where either neurons or vessels pattern the other system. Examples of independent neurovascular congruency occur when both nerves and vessels respond to a common guidance cue, and can be found in the quail forelimb (Bates et al., 2002, 2003) or mice whisker system (Oh and Gu, 2013).

In other cases, these systems follow a one-patterns-the-other model. In it, one of the two systems precedes developmentally, and then instructs the second to form, using an already established architecture as a template. There are cases where peripheral nerves regulate vasculature formation, as happens in the mouse developing limb skin (Li et al., 2013b; Mukouyama et al., 2002, 2005) (Fig 14 A). And also, there other cases in which vessels express signals to attract and pattern axons as they project toward their final target tissues (Honma et al., 2002; Kuruvilla et al., 2004; Makita et al., 2008) (Fig 14 B).

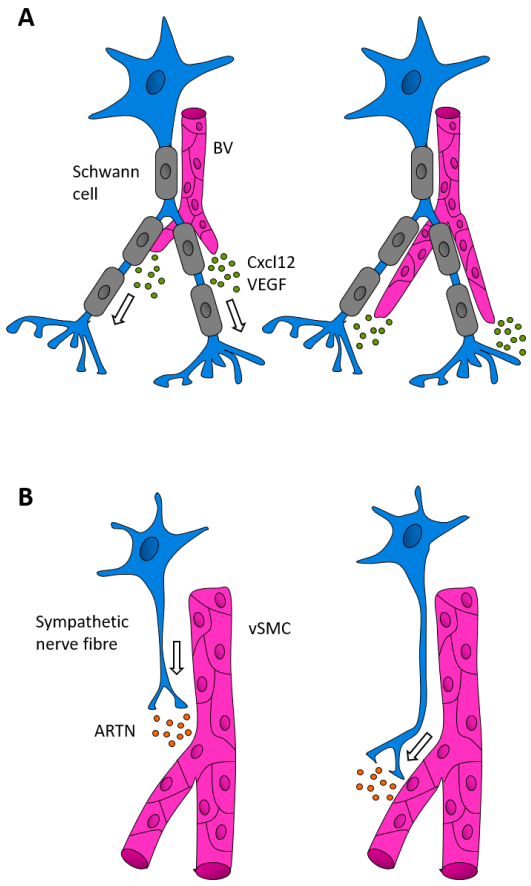

Figure 14. Coordinated patterning of nerves and vessels.

A. Nerve-derived Cxcl12 and VEGF-A control patterns of vascular branching and arterial differentiation. **B.** Vascular smooth muscle cells secrete artemin (ARTN) which is a neurotropic guidance signal sensed by sympathetic nerve axons, and guide the axons to its final target.

Modified from Carmeliet, 2003.

In the PNS, only simple neurovascular interactions have been described, which result in the formation of parallel structures. However, a much more sophisticated type of communication has been described in the CNS, where, unlike in the periphery, the microvascular topology does not match with the neural architecture (Blinder et al., 2013; Lacoste et al., 2014; Tsai et al., 2009; Woolsey et al., 1996).

4.3 Neurons instruct Vessels formation

4.3.1 Neural metabolic requirements and cerebrovascular patterning by neural activity

The brain is highly demanding on oxygen and glucose supply from BV. The brain, despite only representing the 2% of the body mass, consumes the 20% of the body energy at rest. Therefore, an adequate matching between metabolic needs and blood supply is needed (Attwell and Laughlin, 2001; Peters et al., 2004). In fact, the progress from G1 to S phase of NSC is regulated by a checkpoint dependent on nutrient and metabolite support. In the V-SVZ of postnatal rats, NSC cell cycle entry is accompanied by a local increase of blood flow to meet this metabolic requirement (Lacar et al., 2012a). To meet this demand, neurons will instruct vessels growth at its proximities as well as cerebral blood flow (CBF) according to its activity.

Greenough et al. were the first to suggest that the brain adapts the vasculature to its increased metabolic demands postnatally (Black et al., 1987, 1990, 1991). It was observed that requirements of neural tissues were influencing the maturation of the underlying capillary networks (Black et al., 1987; Sirevaag et al., 1988) and that high metabolic activity correlated with higher vascular density (Riddle et al., 1993). A recent study found that vascular density and branching, as well as endothelial proliferation, were decreased in the cortex when sensory information was decreased in mouse pups; whereas increased sensory stimulation resulted in a vascular network with a higher density and branching (Lacoste et al., 2014). These findings suggest that, additionally to the genetic programs regulating vascular angiogenesis, sensory-related neural activity also regulates vasculature refinement in the early brain postnatal development.

Neural cells can also regulate the vascular tone and CBF both during development and at adult stages. Pyramidal neurons (excitatory), inhibitory interneurons and astrocytes are recruited during sensory stimulation (Lecrux and Hamel, 2011) and release vasoactive signals that control vascular tone and CBF (Cauli and Hamel, 2010; Drake and Iadecola, 2007). Astrocytes are the favourite candidates to be the main players regulating the coupling of neural activity to vascular growth because of their physical position contacting neuronal synapses and cerebral microvessels. In fact, astrocytes have already been proven to regulate CBF (Attwell et al., 2010; Iadecola and Nedergaard, 2007; Lind et al., 2013), to secrete the proangiogenic signal VEGF in

response to glutamate (Munzenmaier and Harder, 2000; Potente et al., 2003; Pozzi et al., 2005; Zhang and Harder, 2002), and to be essential for the normal development of postnatal cortical vasculature (Ma et al., 2012). Finally, NSPCs can also regulate CBF in the postnatal V-SVZ (Lacar et al., 2012b). Adjustments of blood flow in response to neuronal activity are known as “functional hyperemia” (Attwell et al., 2010).

4.4 Vessels instruct on neurons behaviour and functions

During growth and regeneration, SCs from different tissues -including the NSCs- reside in close proximity to BV, which supply oxygen and nutrients to meet the high metabolic demands of SCs. Additionally, BV can also exert a role on SCs’ properties by BV-derived molecules (Rafii et al., 2016). While the potential of ECs to modulate NSCs has been well described in adult neurogenesis (see **4.5.2 Vascular control of neuronal function during the adult CNS**), much less is known during development. In the following section, the few studies addressing this topic will be explained.

4.4.1 Vascular control of neuronal function during the developing CNS

During embryo development, there are many cases where neurogenesis occurs in association with vasculature. In fact, angiogenesis of the CNS coincides with the timing when neurogenesis expands in the forebrain (Vasudevan et al., 2008), the hindbrain (Tata et al., 2016; Ulrich et al., 2011) and the spinal cord (Hogan et al., 2004; Takahashi et al., 2015). Beyond a coincidence in time, the first signalling mechanisms of vasculature regulating neurogenesis have started to be described during development. Surprisingly, there does not seem to be a characteristic mechanism, but rather it varies depending on the system.

<u>Vasculature regulates embryonic neuronal expansion</u>

Early studies showed that IPCs divide near BV branch points, suggesting an importance of BV for the creation of a neurogenic niche in mice embryonic cortex (Javaherian and Kriegstein, 2009; Nie et al., 2010; Stubbs et al., 2009). Additionally, since these vessels do not yet have lumen, the interaction of IPCs with vasculature is probably due to ECs signals rather than factors supplied by the blood. Interestingly, the entrance of the first

sprouts in the mouse hindbrain is required for the neural progenitors to proliferate and to hold up cell cycle exit to ensure a correct number of final neurons (Tata et al., 2016). IPC in the ventral telencephalon, but not in the dorsal telencephalon, also depend on vasculature to proliferate (Tan et al., 2016). In these two cases where vessels exert a role on progenitors' proliferation, neurons are physically contacting ECs -via integrin β1- or enwrapping them. When this contact is prevented (by protein of anchorage mutation or whole vascular system abrogation), cell proliferation is disrupted and, as a consequence, there is a decrease in the descendant more differentiated cells (Tan et al., 2016; Tata et al., 2016).

Another study showed that conditional deletion of *vegf* from ECs produces an overexpansion of neural progenitors in abnormal places of the cerebral cortex, as well as problems in migration and axonal tracks development. These observation demonstrated that endothelial-derived VEGF works as a stop signal for neuronal proliferation (Li et al., 2013a). These conditional mutant mice, however, also present defects in the density of the brain vasculature and thus the participation of other signals apart from VEGF should not be excluded (see Table 1).

Vasculature regulates embryonic neuronal differentiation

There are other examples where the vascular system is involved in neuronal differentiation.

In zebrafish, sympathetic ganglia neurons differentiate into noradrenergic neurons when they reach the vicinity of the DA, just at the same time when blood flow onset starts. Blood flow onset is thought to produce the maturation of this artery and the recruitment of vascular mural cells (VMC). VMCs are the ones that direct neuronal cell cycle exit (Fortuna et al., 2015). VMC have, however, been demonstrated not to have a hematopoietic or endothelial origin (Santoro et al., 2009).

Finally, there is an interesting study where the vascular system development regulates the balance between precursors' expansion and differentiation. In mouse cerebral cortex, the lack of vessels produces a hypoxic condition that permits the expansion of neural progenitors. After the ingrowth of BV, the oxygen supply will induce neuroblasts differentiation, possibly through a change in cell metabolism to oxidative phosphorylation (OxPhos). Thus, the ingression of the vascular system indicates the time for the expansion-to-differentiation shift (Lange et al., 2016) (see Fig 15 and Table 1).

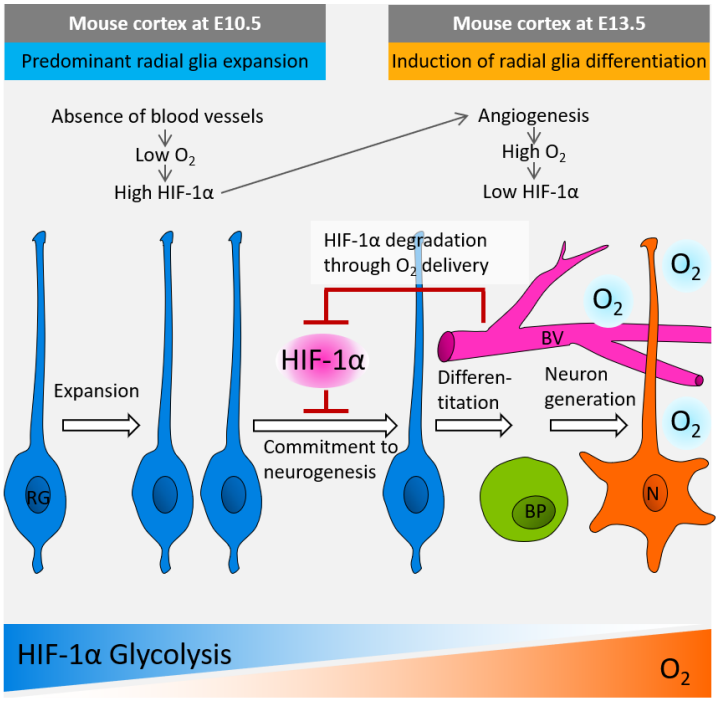

Figure 15. The switch from RG expansion to neurogenesis depends on angiogenesis and hypoxia relief in the mammalian cortical development.

At E10.5 the absence of BV produces a hypoxic environment that maintains high levels of HIF-1α, a protein that prevents the premature differentiation of RG. At 13.5, however, the ingrowth of BV produces the oxygenation of the developing cortex and thus the HIF-1α degradation, relieving its inhibitory pressure on neurogenesis.

RG, radial glia; BP, basal progenitor; N, neuron; BV, blood vessel; O_2, oxygen.

Modified from Lange et al., 2016.

Vasculature regulates embryonic neuronal migration

Finally, a role for BV in neuronal migration during developmental stages has also been described. The angiogenic growth factor Angiopoietin-2 (Ang2) is required for IPCs to migrate from the cortical ventricular zone to the cortical plate (Marteau et al., 2010), following brain capillaries (Stubbs et al., 2009). Another neural cell type that will use BV to move are the oligodendrocyte precursor cells, a glial cell type that gives rise to mature oligodendrocyte. These cells have to migrate extensively through the compact and developing brain and spinal cord to achieve a uniform distribution to develop their

NEURONAL BEHAVIOUR	VASCULAR SIGNAL	NEURONAL RECEPTOR	TYPE OF INTERACTION	REFERENCES
PROLIFERATION	?	?	cell-cell contact	Javaherian and Kriegstein, 2009; Nie et al., 2010; Stubbs et al., 2009; Tan et al., 2016
	Integrin β1	?	cell-cell contact	Tata et al., 2016
	VEGF	?	?	Li et al., 2013
DIFFERENTIATION	VMC-derived factor	?	?	Fortuna et al., 2015
	Oxygen	HIF-1α	Secreted signal	Lange et al., 2016
MIGRATION	Angiopoietin-2	?	Secreted signal	Marteau et al., 2010
	Wnt7a/7b, CXCR4	GRP124	?	Tsai et al., 2016

Table 1. Summary of vascular-to-neurons interactions in the developing brain

VEGF, vascular endothelial growth factor; CXCR4, C-X-C chemokine receptor type.

function as myelinating cells of the CNS. To do so, they use vasculature as a migrating scaffold via Wnt7a and 7b and CXCR4 signalling (Tsai et al., 2016) (see Table 1).

The vascular-to-neurons communication is not exclusive to developmental stages but continues in the adult brain. A lot of studies have investigated the vascular-to-neurons communication in the adult brain and thus more information about the underlying mechanisms is available.

4.4.2 Vascular control of neuronal function during the adult CNS

The neurovascular interaction was first discovered in the adult NSC niche, where neurogenesis takes place outside the embryonic development. As previously seen in section "**1.2. Neurogenesis during adulthood**", adult neurogenic niches harbour a unique population of NSCs that are intimately associated with BV. Their relationship has been proven to be such important that it has been given a its own name as "the vascular niche for neurogenesis".

Special Physical relationship between adult NSC and BV

The vascular architecture in the V-SVZ is different from the one present in non-neurogenic regions such as the cortex. In the V-SVZ an extensive planar network of vessels runs parallel to the ventricles, while in the cortex seems to be disorganized (Tavazoie et al., 2008). Also, it is more densely vascularized (Kazanis et al., 2010). Finally, it has been demonstrated to present a heterogeneity at the level of laminin (Kazanis et al., 2010; Tavazoie et al., 2008). Apart from the vasculature, the blood-brain-barrier also presents differences in the neurogenic regions. In the V-SVZ BV are more permeable due to a reduction in endothelial tight junctions and pericyte and astrocyte coverage (Tavazoie et al., 2008). Finally, blood flow is slower close to V-SVZ (Culver et al., 2013). It has been demonstrated that shear stress can alter ECs gene expression (Chiu and Chien, 2011; Korn and Augustin, 2015; Nicoli et al., 2010; Weijts et al., 2018), thus, changes in blood flow could be regulating NSCs. It is therefore tempting to think that all these are adaptations to permit neurovascular communication.

Despite all these differences, a study that analysed isolated ECs and pericytes from the V-SVZ and cortex could not find intrinsic differences between the vasculature in neurogenic and not neurogenic regions. Suggesting that, if they are not inherently different, external stimuli would be required to break the homogeneity (Crouch et al., 2015).

The vasculature of the neurovascular niche is composed of cellular and acellular components related to BV, which will be ECs, a basal lamina rich in laminin, collagen IV and fibronectin (Kazanis et al., 2010; Mercier et al., 2002; Tavazoie et al., 2008), and pericytes and astrocytes that enwrap and contact BV to regulate their permeability and signalling (Abbott, 2002; Armulik et al., 2010). BV are capable to recruit or "home" NSC through the expression of SDF1, which binds to its receptor CXCR4 in SVZ cells. This will upregulate α6integrins in NSCs, thus promoting their binding to laminin-rich ECs (Kokovay et al., 2010).

Besides, BV interact differently with the different NSCs. C-cells and B-cells lie adjacent to BV, whereas A-cells (migrating NB) are more distantly found. C and B cells directly contact BV that are not covered by astrocyte endfeet (40%) or/nor pericytes (70%). These contacts occur through the neurons' soma, long processes of neurons contact laminin^{+} fractons extending from BV; both during homeostasis and regeneration. NSCs are not only close to vessels but they have a greater access to signals coming from BV than do cells outside the SVZ (Tavazoie et al., 2008). The first *in vitro* studies that cultured V-SVZ explants with ECs showed increased growth and maturation of neurites as well as neuronal migration, already suggesting a potential role for EC regulation of neural cells (Leventhal et al., 1999). Later, clusters of dividing cells were found close to vessels in the SGZ (Palmer et al., 2000). Finally, Shen et al. first demonstrated that EC-secreted factors could promote V-SVZ progenitors' self-renewal and differentiation *in vivo* (Shen, 2004). Since these studies on, increasing literature has demonstrated that vasculature has a function on neuronal behaviour.

As seen during development, in the adult brain, vasculature will also communicate with NSCs through direct contacts, secreted factors and circulating hormones to regulate NSC quiescence, proliferation, migration and differentiation.

Vasculature Regulates Adult NSCs Quiescence maintenance

One beautiful example on how vasculature signals the NSC was described by Ottone and colleges. In this study they show -*in vitro* and *in vivo*- that anchorage of vasculature exposes B1 cells to endothelial-expressed ephrinB2 and Jag1 ligands, which restrain their proliferation and differentiation, maintaining NSCs in a quiescent state. When the niche is activated, precursor cells loose contact with BV, terminating Eph and Notch signalling and allowing these cells to progress through the lineage (Ottone et al., 2014).

ECs in the V-SVZ -and CP capillaries- also maintain NSC quiescence through the production and secretion of neurotrophin-3 (NT-3). NT-3 is bound to its receptor TrkC in NSCs, activating eNOS enzyme and leading to NO production, which eventually acts as a cytostatic signal in NSC (Delgado et al., 2014) (see Fig 16 and Table 2).

Vasculature Regulates Adult NSCs Renewal and Expansion

Several EC-derived factors have been shown to regulate NSC proliferation. Pigmented epithelium-derived factor (PEDF; Serpinf1), secreted by endothelial and ependymal cells, promotes the renewal of NSCs (or type-B cells) and induces them to an undifferentiated state both in neurospheres culture and *in vivo*, probably through Notch, Hes1, Hes5 and Sox2 expression (Andreu-Agulló et al., 2009; Gomez-Gaviro et al., 2012; Ramírez-Castillejo et al., 2006).

Laminins and integrins are known EC-derived secreted ECM proteins that act as SC anchors to increase proximity to vasculature-derived signals (Tanentzapf et al., 2007; Del Zoppo and Milner, 2006). In the subependymal zone laminin is present all over the niche (Mercier et al., 2002) but it is specially enriched in the surface of BV. When NSCs enter cell cycle they will start expressing the laminin receptor β1-integrin. The interaction between these two molecules will negatively control the levels of proliferation of NSCs and progenitors (Kazanis et al., 2010). However, in the cerebellum cortex the binding of β1-integrin of granule cell precursors to laminin seems to produce the opposite effect, promoting proliferation and preventing premature differentiation, through the mediation of Shh – although this study did not consider BV as the source of laminin (Blaess et al., 2004).

Betacellulin is another factor secreted by BVs that promotes proliferation via EGFR/ErbB4/ERK signalling, while inhibiting differentiation of neuroblasts (Gomez-Gaviro et al., 2012). FGF-2 is a potent angiogenic factor (Biro et al., 1994) secreted by BV to regulate ECs' proliferation, migration and differentiation. But, at the same time, type B cells also express the corresponding receptor (Doetsch et al., 2002; Zheng et al., 2004), being able to respond to FGF-2 (Ciccolini and Svendsen, 1998; Gritti et al., 1996; Kilpatrick and Bartlett, 1995; Palmer et al., 1997). It has been demonstrated that this signal promotes neurogenesis and proliferation in cortical progenitors (Alzheimer and Werner, 2002; Raballo et al., 2000; Vaccarino et al., 1999).

In the V-SVZ, BMP2 and -4 are expressed by astrocytic glia and ECs. Its expression reduces NSPCs proliferation while promoting cell-cycle exit when EGF and FGF-2 are also present (Mathieu et al., 2008), probably favouring astrocyte formation (Gross et al., 1996; Lim et al., 2000).

VEGF, a part from being a well-known angiogenic factor, also promotes neurogenesis when infused in the V-SVZ (Jin et al., 2002). Another study, however, pointed to a role of VEGF-A promoting NSC survival rather than affecting the proliferation of these cells (Wada et al., 2006). It is difficult to differentiate if these effects are a response to a larger vasculature. However, VEGFR3 is only expressed in NSC -and not in EC- and its ligand VEGF-C stimulates adult neurogenesis but not angiogenesis (Calvo et al., 2011) (see Fig 16).

Other important angiogenic signals are Ang1 and Ang2, which bind to their receptor Tie2 (Maisonpierre et al., 1997; Suri et al., 1996). Tie2 was found to be expressed in NSCs and TACs both in the V-SVZ and SGZ (Androutsellis-Theotokis et al., 2009), suggesting that angiopoietins may have an effect in neurogenesis. Indeed, injections of Ang2 activate precursors in the adult brain (Androutsellis-Theotokis et al., 2009) and Ang-1 in cell culture (Rosa et al., 2010).

Circulating factors can also regulate neurogenesis in special cases. One example was found is pregnant mice females, in which the production of neural precursors is increased through the effect of prolactin hormone in the V-SVZ. The corresponding increase in new olfactory interneurons is thought to be important for maternal behaviour as mother will recognize and rear their offspring thanks to odour discrimination (Shingo et al., 2003).

Finally, exercise-induced neurogenesis is mediated through vascular-derived signals. Exercise stimulate IGF-1 uptake from bloodstream to the hippocampus, producing an increase in hippocampal neurons proliferation and BDNF mRNA and protein (Ding et al., 2006; Trejo et al., 2001; Vaynman et al., 2003) (see Table 2).

Vasculature Regulates Adult NSCs Differentiation

ECs in adult V-SVZ actively secrete BDNF to the local environment promoting neuronal maturation, migration and survival in culture (Leventhal et al., 1999). However, B cells would be protected from the differentiating signalling of BDNF as astrocytes express the high affinity receptor TrkB, sequestering BDNF and making it inaccessible for NSCs

(Klein et al., 1990; Leventhal et al., 1999). Additionally, B and C-cells only express the low-affinity neurotrophin receptor p75 ($p75^{NTR}$) (Young et al., 2007). It is not only until neuroblast leave the niche and start migrating towards the OB (A cells) that they start expressing the high-affinity BDNF receptor TrkB (Chiaramello et al., 2007; Galvao et al., 2008). As such, BDNF is limited in areas where NSCs must remain as so, and gradually becomes accessible as neuroblasts leave the niche and signalling for differentiation, maturation and post-mitotic survival become necessary. Additionally, it has been proposed that BDNF acts in a positive feed-back loop to reduce proliferation and increase neuroblast differentiation through the release of NO by type B and C cells (Cheng et al., 2003; Packer et al., 2003).

Collagen IV expressed in the outer surface of BV has been shown *in vitro* to inhibit proliferation while promotes differentiation of NSPCs into neurons in rats (Ali et al., 1998).

Finally, a recent study demonstrated that BV are also involved in neuronal maturation and functional integration, through plasticity remodelling. Sema3G secreted by ECs positively regulates excitatory synapse density in hippocampal neurons through its binding to Nrp2/PlexinA4 receptor complex. Besides, researchers have shown how vasculature signalling have consequences on synaptic plasticity and cognitive behaviour (Tan et al., 2019) (see Fig 16 and Table 2).

Vasculature Regulates Adult NSCs Migration

New-born neurons from the SVZ are continuously added to the OB. During development, neuroblasts use RGC projections to reach their final destination, but RGCs are absent in the adult brain. Neuroblasts, thus, need a different strategy to migrate. These cells migrate radially using vessels as a scaffold and trophic support. It is hypothesised that it works though a contact-mediated and humoral signals (Bovetti et al., 2007; Emsley and Hagg, 2003; Kokovay et al., 2010; Snapyan et al., 2009).

Vessel-supported migration has been found to be part of neural progenitors movement in the granular cell layer (Sun et al., 2015), along the RMS and within the OB (Bovetti et al., 2007). Even, oligodendroglial progenitors use it to migrate from the V-SVZ to the corpus callosum (Cayre et al., 2013).

ECs express SDF1, which chemoattracts CXCR4-expressing activated type B cells and C cells on one hand, while on the other hand upregulates α6 integrin in NSCs (Kokovay

et al., 2010; Shen et al., 2008; Tavazoie et al., 2008). The expression of α6β1 integrin in neuroblasts will favour their migration through the RMS, thanks to their binding to the laminin present in the basement membrane of BV (Emsley and Hagg, 2003). Finally, as neuroblast leave the niche, endothelial-derived BDNF will serve both as a chemoattractant and survival factor for the migration of neuroblasts expressing cognate p75NTR (Snapyan et al., 2009), which will eventually differentiate (see Fig 16).

Other candidate signals to play a role in RMS neuroblasts migration are EphB1-2/EphA4 and ephrin-B2/3. EphA4 is widely expressed in the endothelium at developing stages, its expression becomes progressively restricted to the RMS and V-SVZ vasculature at early post-natal live and finally to only V-SVZ vasculature at adult stages (Colín-Castelán et al., 2011; Conover et al., 2000).

Finally, in the adult songbird higher vocal centre (HVC) neurogenesis proceeds throughout life. Testosterone circulating in BV increases VEGF in neural cells and VEGFR-2 in ECs, stimulating angiogenesis. Then, the expanded vasculature creates a permissive environment for neurogenesis through the expression of BDNF, which promotes the migration of neurons towards the HVC (Louissaint et al., 2002) (see Table 2).

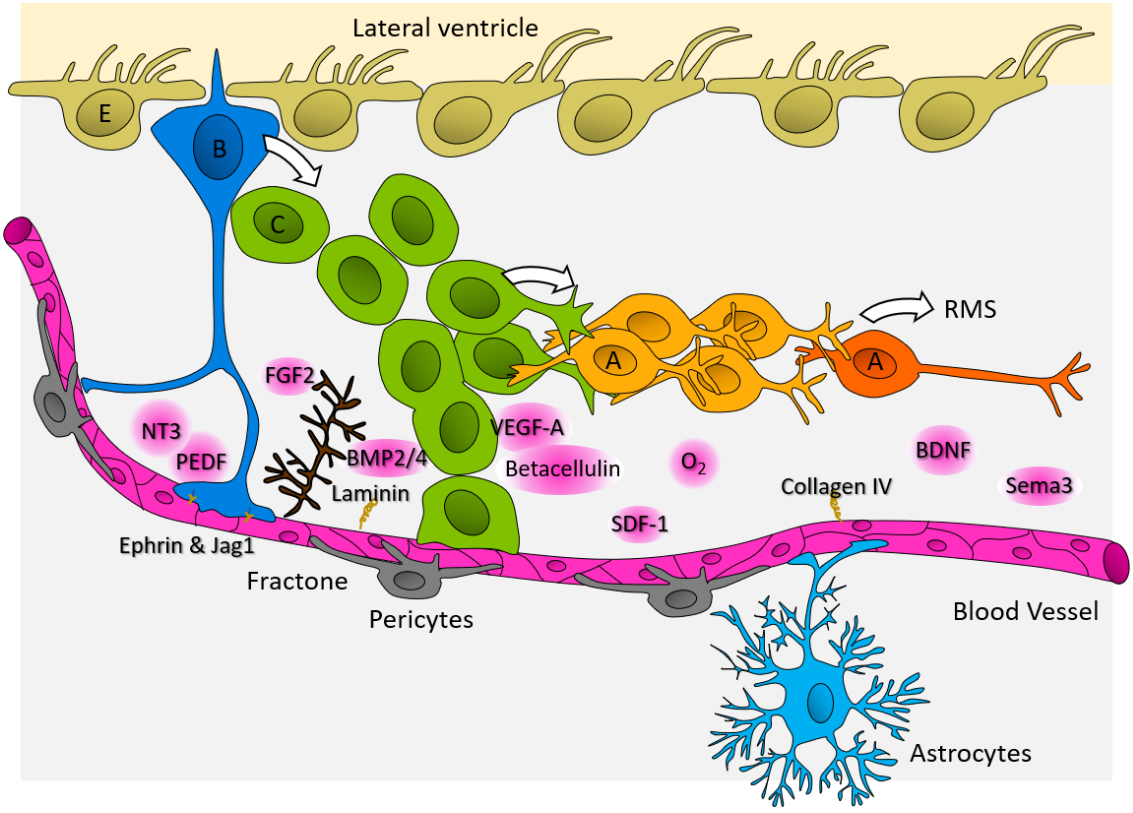

Figure 16. Major signals involved in the vascular interactions with NSCs in the V-SVZ.

As developed in the text, ECs secrete or express in their membrane different molecules that promote quiescence, proliferation, differentiation and migration of the NSCs in the adult brain.

E, ependymal cell; B, B cell; C, C cell; A, A cell; RMS, rostral migratory stream; NT3, neurotrophin-3; PEDF, pigmented epithelium-derived factor; FGF2, fibroblast growth factor 2; BMP2/4, bone morphogenetic protein 2/4; VEGF-A, vascular endothelial growth factor A; SDF-1, stromal cell-derived factor 1; O_2, oxygen; BDNF, brain-derived neurotrophic factor; Sema3, semaphorin 3.

Modified from Goldman and Chen, 2011.

NEURONAL BEHAVIOUR	VASCULAR SIGNAL	NEURONAL RECEPTOR	TYPE OF INTERACTION	REFERENCES
QUIESCENCE	ephrinB2 & Jagged1	Eph & Notch	Cell-cell contact	Ottone et al., 2014
	Neurotrophin-3	TrkC	Secreted signal	Delgado et al., 2014
SELF-RENEWAL/SURVIVAL	VEGF-A	VEGFR	Secreted signal	Wada et al., 2006
	PEDF	probably Notch, Hes1, Hes5 and Sox2 (?)	Secreted signal	Ramírez-Castillejo et al., 2006; Gómez-Gaviro et al., 2012; Andreu-Agulló et al., 2009
PROLIFERATION	Laminin	β1-integrin	Cell-cell contact	Kazanis et al., 2010; Blaess et al., 2004
	Betacellulin	EGFR/ErbB4/ERK	Secreted signal	Gomez-Gaviro et al., 2012
	FGF-2	FGFR	Secreted signal	Biro et al., 1994;Alzheimer and Werner, 2002; Raballo et al., 2000; Vaccarino et al., 1999
	BDNF	?	Secreted signal	Louissaint et al., 2002; Vaynman et al., 2003; Trejo et al., 2001; Ding et al., 2006
	FGF-2	?	Secreted signal	Vaccarino et al., 1999; Raballo et al., 2000; Alzheimer et al., 2002
	BMP2 and -4	?	Secreted signal	Mathieu et al., 2008
	Eph/ephrin	?	Cell-cell contact	Holmberg et al., 2005; Conover et al., 2000
	VEGF-A & C	VEGFR3	Secreted signal	Jin et al., 2002; Calvo et al., 2011; Cao et al., 2004
	Angiopoietin-1 and 2	Tie2	Secreted signal	Maisonpierre et al., 1997; Suri et al., 1996; Androutsellis-Theotokis 2009; Rosa et al., 2010
	Prolactin	?	Circulating factor	Shingo et al., 2003
	IGF-1	?	Circulating factor	Lethinen et al., 2011
	Oxygen	HIF-1α ?	Circulating factor	Arvidsson et al., 2002; Tomita et al., 2003

NEURONAL BEHAVIOUR	VASCULAR SIGNAL	NEURONAL RECEPTOR	TYPE OF INTERACTION	REFERENCES
DIFFERENTIATION	BDNF	TrkB	Secreted signal	Leventhal et al., 1999; Klein et al., 1990; Young et al., 2007; Chiaramello et al., 2007; Galvao et al., 2008
	Collagen IV	?	Cell-cell contact	Ali et al., 1998
	Semaphorin-3G	Nrp2/PlexinA4 receptor complex	Secreted signal	Tan et al., 2019
MIGRATION	BDNF	?	Secreted signal	Snapyan et al., 2009
	Laminin	α6β1 integrin	Cell-cell contact	Emsley and Hagg, 2003
	SDF-1	CXCR4	Secreted signal	Kokovay et al., 2010; Shen et al., 2008; Tavazoie et al., 2008
	Eph/ephrins	?		Conover et al., 2000
	Testosterone	?	Secreted signal	Louissaint et al., 2002; Hartog et al., 2009

Table 2. Summary of vascular-to-neurons interactions in the adult brain

VEGF, vascular endothelial growth factor; PEDF, pigment epithelium-derived factor; FGF, fibroblast growth factor; BDNF, brain-derived neurotrophic factor; BMP, bone morphogenic protein; IGF-1, insulin-like growth factor 1; SDF-1, stromal cell-derived factor.

On summary, vasculature can signal NSCs through three different ways: direct contacts, secreted diffusible cues, and hormones. Until now, there is only one study that has demonstrated direct contact *in vivo*, in which ephrinB2 and Jag1 in EC maintain NSC quiescence (Ottone et al., 2014). On the other hand, EC-secreted signals have a diversity of effects, from promotion to reduction of both neurogenesis and neuronal differentiation, as well as neuroblasts' migration regulation. Finally, circulating factors can also regulate NSC activity in certain cases, such as IGF-2 and prolactin favouring neurogenesis (Ding et al., 2006; Shingo et al., 2003; Trejo et al., 2001; Vaynman et al., 2003). Together these findings reveal an unexpected level of complexity in the system and the presence of multiple co-existing regulatory mechanisms that jointly orchestrate quiescence, survival, proliferation, migration and differentiation of NS and progenitor cells. However, BV have never been reported to play a role on neuronal specification, which matches with the hypothesis that vasculature becomes crucial for NSCs once they are established in the niche. These findings on the vascular regulation of neural functions and behaviour have led to a new appreciation of the structural plasticity in the adult mammalian brain.

Further research needed

Most of the studies on vasculature-to-neurons communication have been conducted in the adult CNS. However, the first articles on this interaction during embryonic stages have started to appear recently and still little is known about the influence that BV have on neurogenesis during development

Understanding how this communication occurs at developmental stages is also of great interest since neurogenesis at adult and embryonic stages differ greatly, and thus, it is likely that its regulation by BV also changes. Basically, at adult stages NSC proliferate rarely and there is scant vasculogenesis. Whereas, at embryonic neurogenesis there is a high rate of proliferation of progenitor cells, that will gradually loose potency, while at the same time the vascular system is being formed.

Almost everything we know about this topic has been studied in the CNS, while we know nothing on the vascular regulation of neuronal behaviour in the developing PNS.

4.5 The role of Hypoxia in neurogenesis and angiogenesis

Hypoxia causes cellular dysfunction and even cell death. During evolution, organisms developed mechanisms to sense and restore oxygenation to hypoxic areas. The cellular response to oxygen tension is mainly mediated by the Hypoxia-Inducible Factor-1 (HIF-1). During normoxia, some proteins use oxygen to hydroxylate HIF-1α, targeting it for proteasomal degradation. But, when conditions change to hypoxia, the degradation pathway is circumvented. As a consequence, HIF-1α accumulates, translocates to the nucleus and heterodimerizes with HIF-1β, leading to the transcription of downstream

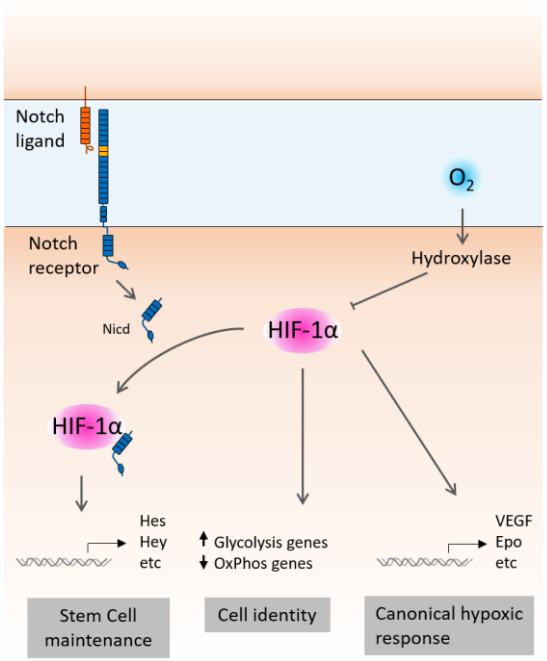

Figure 17. Crosstalk between Notch and Hypoxia.

HIF-1a activity is inversely proportional to oxygen tension. Increasing oxygen tension induce proteins that hydroxylate HIF-1α, leading to its degradation.

In this drawing three modes of hypoxic responses are shown: i) the canonical hypoxic signalling leading to the activation of downstream genes such as VEGF and Epo, through the HIF-1α binding in the corresponding promoters; ii) maintenance of the stem cell state through interaction with the Notch signalling pathway. HIF-1α and NICD form a point of convergence leading to the stabilization of NICD, recruitment of HIF-1α to Notch-responsive promoters and activation of Notch downstream genes such as Hes and Hey. And iii) HIF-1α shifts metabolic balance from oxidative phosphorylation toward glycolysis.

Modified from Panchision, 2009 and Gustafsson et al., 2005.

genes. Its targets include genes that code for molecules that participate in vasomotor control, angiogenesis, erythropoiesis, iron metabolism, cell proliferation and cell cycle control, cell death, and energy metabolism (Semenza, 2006; Sharp and Bernaudin, 2004) (see Fig 17).

4.5.1. Hypoxia regulating angiogenesis

Initially, it was thought that the brain vasculature was fixed structurally at adulthood, except for pathological deterioration. Surprisingly, upon hypoxic stimulus, CBF and glucose consumption increase (Beck and Krieglstein, 1987; Sun and Reis, 1994), as well as the capillary density (Lauro and LaManna, 1997). During the course of hypoxia-induced angiogenesis, capillary density begins to increase after 1 week of hypoxia exposure, and it completes after 2 or 3 weeks (Harik et al., 1996, 1995). Several pro-angiogenic factors, including VEGF, have hypoxia responding elements in their promoters, making hypoxia a strong inducer of angiogenesis (Chávez et al., 2000; Pichiule and LaManna, 2002) (see Fig 17). Also, Ang2, which is not expressed in normoxic conditions, will be induced in ECs following hypoxia (Pichiule and LaManna, 2002). In the presence of growth factors such as VEGF, angiogenesis will start, otherwise capillaries will undergo apoptotic regression, a process known as de-adaptation, in case hypoxia lasts for more than 3 weeks (Harik et al., 1996; Pichiule and LaManna, 2002).

Therefore, there are physiological mechanisms for capillary density increase and decrease in order to balance capillary density and structure with tissue oxygen balance and activity.

4.5.2. Hypoxia regulating neurogenesis

Less is known about the effects of hypoxia on adult neurogenesis. Ischaemia-induced brain damage can be caused by stroke, cerebral artery occlusion, cardiac arrest, or by coronary artery occlusion. In these cases, NSCs both in the CNS and the PNS show increased neuronal proliferation and differentiation in response to low oxygen tension (Morrison et al., 2000; Studer et al., 2000a). In hypoxia conditions the body responds increasing the expression of erythropoietin (EPO), which promotes the proliferation and differentiation of erythroid progenitors as well as maturing erythroid cells survival (Youssoufian et al., 1993). EPO receptors are also found in the developing CNS (Liu et

al., 1997, 1994) and it was later proven that EPO expression is increased in the brain as an hypoxia-response to increase neural progenitors in the adult CNS (Shingo et al., 2001). This hypoxia-regulating neurogenesis might explain a strategy of self-repair of the CNS after injury or disease.

Oxygen levels function as a signalling mechanism also during brain development, regulating the balance between NSC maintenance, proliferation and differentiation, in a context dependent way (Chen et al., 1999; Mohyeldin et al., 2010; Simon and Keith, 2008; Studer et al., 2000b). Interestingly, embryonic stem and progenitor cells frequently occupy hypoxic niches (Mohyeldin et al., 2010; Panchision, 2009). HIFs interact with the Notch and Wnts stem cell signalling pathways (see Fig 17) (Gustafsson et al., 2005; Kaidi et al., 2007; Mazumdar et al., 2010; Wang et al., 2006). A reduction of the atmospheric oxygen concentration from 21% to 3% promotes proliferation and stem cell maintenance of isolated neural progenitor cells (Storch et al., 2001; Studer et al., 2000a). Similar to their predecessors, adult NSCs also reside in a hypoxic niche (Mohyeldin et al., 2010), and numerous studies have demonstrated that low oxygen levels promote their proliferation and survival (Li et al., 2014) (see Fig 17). Of course, there is a threshold of beneficial hypoxia below which proliferation and neurogenesis is hampered due to cell death.

4.5.3. Oxygen as a cell metabolism regulator

Oxygen levels regulate cells metabolic state from glycolysis to OxPhos via HIF-1α (Iyer et al., 198). Some evidences suggest that this metabolism changes have a role on NSC fate regulation. For example, in *Drosophila*, switch from anaerobic glycolysis to OxPhos is required for cell cycle exit and differentiation in neuroblasts (Homem et al., 2014). This also happens in vertebrates: embryonic neural progenitors use aerobic glycolysis, but they switch to OxPhos during neuronal differentiation (O'Brien et al., 2015; Zheng et al., 2016). Lange and colleagues show this phenomenon *in vivo*. During cerebral cortex development, NP proliferate until vessels ingress, changing the oxygen tension in the NSC niche, which produces the inactivation of HIF-1α and results in NP differentiation (Lange et al., 2016). This mechanism is maintained in adult NSCs. In their quiescent state utilize glycolysis to support their energy needs, whereas highly proliferative activated NSCs and their differentiated progeny uses mitochondrial OxPhos (Ito and Suda, 2014; Llorens-Bobadilla et al., 2015; Shin et al., 2015) (see Fig 18).

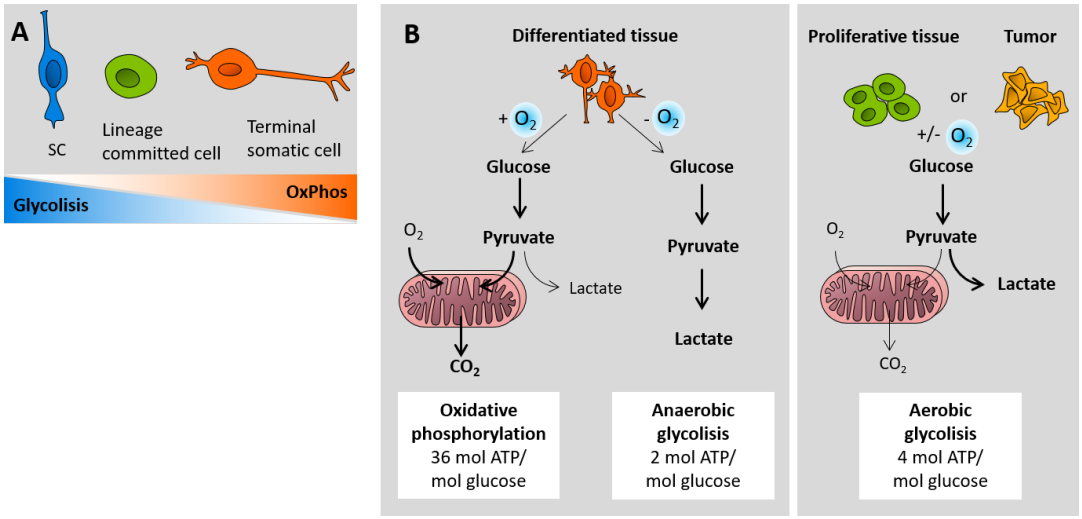

Figure 18. Metabolic pathways used by cells at different differentiation states.

A. Stem cells and differentiated cells use different metabolic pathways, the first preferentially use glycolysis, while the latest use oxidative phosphorylation (OsPhos). **B.** Schematic representation of the differences between OxPhos, anaerobic glycolysis, and aerobic glycolysis. When oxygen is present, differentiated tissues first metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO_2 during the process of oxidative phosphorylation. Because oxygen is required as the final electron acceptor to completely oxidize the glucose, oxygen is essential for this process. When oxygen is limiting, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate (anaerobic glycolysis). This generation of lactate during anaerobic glycolysis allows glycolysis to continue (by cycling NADH back to NAD^+), but results in minimal ATP production when compared with oxidative phosphorylation. Cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis). This property is shared by normal proliferative tissues. Mitochondria remain functional and some oxidative phosphorylation continues in both cancer cells and normal proliferating cells. Nevertheless, aerobic glycolysis is less efficient than oxidative phosphorylation for generating ATP.

Modified from Hu et al., 2016 and Vander Heiden, 2009.

5. Mechanisms of cell signalling: Filopodia and cytonemes

In the previous section, we have seen that ECs and neuronal cells communicate with each other. Cell-cell communication can be achieved through different signalling mechanism: via direct cell-cell contact or juxtracrine signalling, over relatively short distances or paracrine signalling, and over large distances or endocrine signalling. Cell-cell direct contacts is probably the most precise spatial and temporal mechanism for the transference of signalling molecules.

Recently, some cell communication events thought to be occurring via diffusion of signalling molecules have been demonstrated to be actually via direct cell-cell contacts. It has been demonstrated, thanks to the advance of live imaging and staining and reporter tools, that in some cases signals are driven by thin cytoplasmatic protrusions called signalling filopodia or cytonemes.

5.1 Filopodia

Filopodia are thin, actin-made plasma membrane protrusions that function as antennae to sense the mechanical and chemical environment of a cell. They have been described in a multitude of cell types and to have a role on cell migration, ECM adhesion, neurite outgrowth, tip cell guidance and wound healing.

Two different types of protrusive structures are found in the leading edge of motile cells: lamellipodia and filopodia. While lamellipodia are sheet-like protrusions, filopodia are finger-like structures, but both are thin (0.1-0.2 µm and 0.1-0.3 µm, respectively) and filled with filamentous (F)-actin (Chhabra and Higgs, 2007; Pollard and Borisy, 2003). Filopodia length ranges from 1 to 100 µm. The actin filaments that compose filopodia are polarized, there is a rapidly growing end called barbed end and a slowly one called pointed end. Filopodial protrusions grow and retract rapidly through the actin polymerization at barbed ends and retrograde flow of the actin filament bundle (Mallavarapu and Mitchison, 1999). Filopodia have been described in different organisms and systems with different dynamics, lengths and positions (Welch and Mullins, 2002).

Filopodia formation mechanisms

There are two models that explain how filopodia is formed. The “convergent elongation model” propose that branched filaments from the lamellipodia cytoskeleton network form filopodia via the ARP2/3 complex (Gupton and Gertler, 2007). The other model named “*de novo* filament nucleation” proposes that filopodial actin filaments are newly formed by formins (Schirenbeck et al., 2006). Most probably different mechanisms regulate the formation of actin filaments to produce filopodia elongation, and the relative importance of each mechanism varies depending on the organism or cell type (Yang et al., 2007). Despite the growing list of proteins interacting with signalling pathways leading to the formation of filopodia (Jacquemet et al., 2019), the exact sequence of events is far from being fully unveiled.

Cellular roles of filopodia

For many years filopodia roles remained unproven because it was not possible to selectively remove them without compromising the whole cell. Initially, filopodia were described as protrusions sensing or exploring the environment, but the roles of filopodial processes are diverse and yet not fully understood (see BOX 2).

Filopodial processes are formed during directed cell migration. Filopodia tips contain receptors for signalling molecules and ECM molecules, probing the environment and working as “sticky fingers” that will adhere to the ECM or other cells to migrate by cell body translocation (Galbraith et al., 2007). Filopodia also allow cell-cell adhesion during embryonic development and wound healing. Opposing cells send filopodia to facilitate cell-cell matching, to align and adhere together, a process known as “zippering”, for example during the closure of the neural tube (Millard and Martin, 2008; Vasioukhin et al., 2000). Filopodia are part of neuronal growth cones and of endothelial tip cells to guide axons and new sprouts to their proper targets respectively, thanks to the sensing of chemoattractants and repulsive cues as previously described (see **3.1. Similar morphological structures**) (Dickson, 2002; Gerhardt et al., 2003). More recently filopodia have been described to play a role on cell-cell signalling mediation. This specialized filopodia or signalling filopodia have also been named “cytonemes”.

BOX 2 – Methods used to inhibit filopodia formation

Different methods have been used to study cell membrane dynamics. Cytochalasin-D is the classic drug, with an origin in fungi, used to inhibit actin polymerization by many laboratories (Lidke et al., 2005). But it has been supplanted by a more effective, specific and rapid drug, called Latrunculin (Spector et al., 1983). Latrunculin (A and B) derive from a toxic secreted by a sea sponge that, upon binding to actin monomers prevents polymerization in a reversible way (Morton et al., 2000). In zebrafish, low concentrations of Latrunculin B (LatB) have been used to inhibit filopodia formation without affecting cell morphology or proliferation (Phng et al., 2013). ML141 is another drug that inhibits filopodia formation by blocking Cdc42/Rac1 GTPase (Stanganello et al., 2015). Transient knockdown and overexpression of genes have also been carried out for Cdc42, N-WASP, MyoX, IRSp53 and DN IRSp53 (Lee et al., 2010; Millard et al., 2005; Nalbant et al., 2004; Stanganello et al., 2015). Finally, optogenetic tools are starting to be developed (Wu et al., 2009). Of special importance are the functional studies *in vivo*, as in *in vitro* studies cells are removed from their physiological environment and lack features such as the ECM, mechanical inputs and signals from the surrounding tissue.

5.2 Cytonemes

During the development of an organism, cell-cell communication through signals is required to induce cellular behaviours and organize tissues. This can occur between closely adjacent cells, but also between cells further separated by interstitial fluid, ECM or other cells. The understanding of developmental patterning is based on positional information that indicates cells to differentiate accordingly (Wolpert, 1969). The classical morphogen model, explained by the French Flag model, proposes that cells (or organizers) secrete diffusible signals creating gradients that are sensed by receiving cells in a concentration-dependent manner (Crick, 1970; Mathison, 1952; Wolpert, 1969). In the last years morphogens have been intensely investigated and their functional importance demonstrated (Driever and Nüsslein-Volhard, 1988; Green and Smith, 1990; Rogers and Schier, 2011). However, increasing experimental evidences also challenged this model (Kornberg and Roy, 2014; Wolpert, 2016). The morphogen model cannot explain how signals spread through complex 3D structures, only some

cells are targeted by different signals acquiring different fates or physically separated by other tissues.

Quite recently a new model has been proposed thanks to the advance of imaging technology and fluorescent staining and reporter lines (see BOX 3). In this model, cell communication is mediated by filopodia transporting morphogens and establishes patterns of gene expression and cell differentiation (Ramírez-Weber and Kornberg, 1999). This signalling filopodia have also been called “cytonemes”, which can be regarded as a subclass of canonical filopodia (Sanders et al., 2013).

Cytonemes were first observed in sea urchin gastrulation (Miller et al., 1995), but the term “cytoneme” was coined for signalling filopodia in *Drosophila* imaginal discs (Ramírez-Weber and Kornberg, 1999). This signalling mechanism has been documented in flies (Bischoff et al., 2013; Callejo et al., 2011; Chen et al., 2017; Cohen et al., 2010; Du et al., 2018; Fuwa et al., 2015; González-Méndez et al., 2017; Gradilla et al., 2014; Huang et al., 2019; Huang and Kornberg, 2015; Inaba et al., 2015; Iwaki et al., 2005; de Joussineau et al., 2003; Mandal et al., 2007; Peng et al., 2012; Ramírez-Weber and Kornberg, 1999; Rojas-Ríos et al., 2012; Roy et al., 2014; Sohr et al., 2019), chick (Sagar et al., 2015; Sanders et al., 2013), and zebrafish (Caneparo et al., 2011; Eom et al., 2015; Hamada et al., 2014; Luz et al., 2014; Mattes et al., 2018; Stanganello et al., 2015). Cytonemes are highly dynamic structures that allow spatially restricted signalling. Different lengths have been observed depending on the distance to the target cells, ranging from 1 to 380 µm (Caneparo et al., 2011; Luz et al., 2014; Rojas-Ríos et al., 2012; Sagar et al., 2015; Sanders et al., 2013) (see Table 3). They also have different velocities of extension and retraction, but also have been described as stable. This opposite characteristic might be in fact two different states of the same structure. Dynamic cytonemes correspond to protrusions that have not yet contacted the target

BOX 3 – Technical challenges of filopodia and cytonemes detection

Filopodia extensions are difficult to detect because most of them do not survive fixation, and those that do, do not retain their normal shape. Antibody staining is thus problematic. Filopodia can be visualized by cytoplasmatic fluorescence staining, membrane-tethered GFP or fluorescent proteins fused to filopodia components such as actin or signalling proteins (in the case of cytonemes). Besides, fluorescence quenching, phototoxicity and 3D growth through tissues limit or prohibit real-time imaging.

cell, while stable cytonemes have established the contact and are actively signalling (Sagar et al., 2015). Cytonemes are emerging as a general mechanism for intercellular crosstalk, next to the classical diffusion models.

5.2.1 Roles of cytonemes

Cytonemes have been described as signalling structures in *Drosophila* and vertebrates (amphibians, zebrafish, chick and mouse). They transport fundamental signalling molecules such as Notch, EGF, FGF, BMP, Wnt and Shh. Additionally, cytonemes can transport the ligand or the receptor, always achieving a spatially restricted distribution of the signals. Cytonemes are a mechanism of cell signalling used during development that allows the selective distribution of signalling molecules to create gradients and patterns for cell differentiation, and maintaining SCs in their niches (see Fig 19 and Table 3). They are also involved in processes such as tissue regeneration and cancer progression.

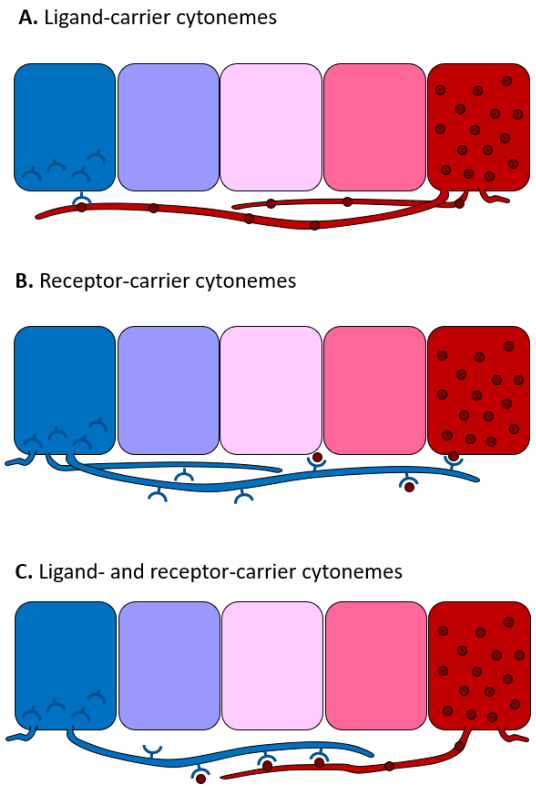

Figure 19. Cytonemes transport ligands and receptors to create morphogen gradients.

The signal-sending cell (red) and the signal-receiving cell (blue) communicate with each other through cytonemes. Graded distribution of the signal is achieved by the number of contacts and distance between cells. Additionally, cytonemes allow for the specialization of a single cell without affecting neighboring cells. Different cytoneme contact types are possible: **A.** Cytonemes extending from the signal-producing cell contact with specific receptors in the receiving cell. B. Cytonemes carrying specific receptors extend from receiving cells to capture the ligand of the signal-producing cell. **C.** Cytonemes from both the signal source and the receptor cells contact each other for the signal transfer.

Modified from González-Méndez et al., 2019.

Cytonemes can produce a graded dispersion of morphogens depending on their length and contact frequency. This function has been shown in the ligand delivery of Wnt during neural plate patterning in zebrafish (Luz et al., 2014; Mattes et al., 2018; Stanganello et al., 2015), in Hh in *Drosophila* and its homologue Shh in chick limb bud (Bischoff et al., 2013; Chen et al., 2017; González-Méndez et al., 2017; Gradilla and Guerrero, 2013; Sanders et al., 2013), and in the receptor delivery of Btl in *Drosophila* (FGF receptor homologue) (Du et al., 2018) (see Fig 20 and Table 3).

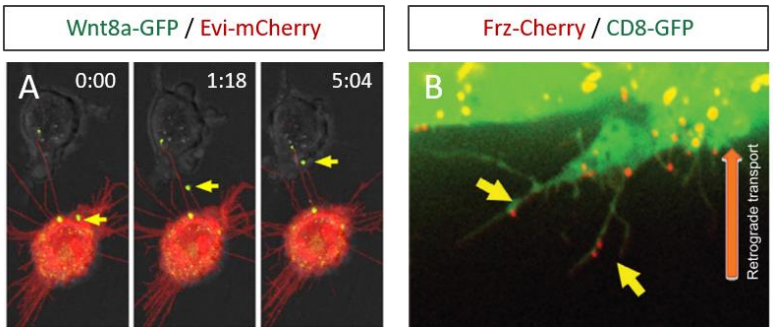

Figure 20. Cytonemes transporting signals of the Wnt/Frz pathway in different organisms.

A. PAC2 zebrafish fibroblast transfected with Wnt8a-GFP and Evi-mCherry. Multiple filopodia and Wnt8a at the distal tip (arrow) can be seen forming cell-cell contact with another PAC2 fibroblast (min:s). **B**. Myoblasts labeled with CD8-GFP express Frz-Cherry to contact Wnt-producing air sac cells in *Drosophila*.

Modified from Stanganello and Scholpp, 2016; Stanganello et al., 2015.

Cytonemes can also generate spatially patterned cell fates. This role has been described in the formation of bract cells (a type of thick hair) in *Drosophila* via Spitz/EGF distribution (Peng et al., 2012), in the organization of the bristles in the notum via Notch signalling , also in *Drosophila* (Cohen et al., 2010; de Joussineau et al., 2003), in the colours pattern of butterfly wings (Iwasaki et al., 2017; Ohno and Otaki, 2015), and the pigmented stripped pattern of zebrafish through Delta/Notch signalling (Eom and Parichy, 2017; Eom et al., 2015; Hamada et al., 2014) (see Table 3).

It has also been shown that cytonemes can coordinate morphogenesis bridging the space of physically separated tissues. It is the case of the air sac primordium (ASP) in *Drosophila* wing disc. Cells from the ASP send cytonemes to ligand-producing cells (Dpp

and FGF), to promote cell migration and patterning (Du et al., 2018; Huang and Kornberg, 2015; Huang et al., 2019; Roy et al., 2014; Sohr et al., 2019) (see Table 3).

Finally, cytonemes have also been described in the maintenance of SC niche. For example, in the ovary, testis and haematopoietic SCN of *Drosophila* signals carried by cytonemes regulate undifferentiated cells (Fuwa et al., 2015; Inaba et al., 2015; Mandal et al., 2007; Rojas-Ríos et al., 2012) (see Table 3).

5.2.2 Signal trafficking by cytonemes

Several membrane-bound signalling proteins have been observed travelling through cytonemes, and an increasing number of studies are reporting that this transport of morphogens is associated with vesicles such as argosomes (Greco et al., 2001), lipoprotein particles (Panáková et al., 2005), exosomes (Beckett et al., 2013; Gradilla et al., 2014; Koles et al., 2012) and exosome-like particles (Danilchik et al., 2013; Matusek et al., 2014).

5.2.3 Signal delivery by cytonemes

Scientists are still trying to understand how cytonemes recognize and contact their target cell for signal transfer. Two types of contacts have been identified: the first is the cytoneme-cell body contact, for example found in cytonemes transporting the Wnt8a ligand in the zebrafish embryo (Mattes et al., 2018; Stanganello et al., 2015) and the cytonemes carrying the Fz receptor from the ASP of *Drosophila* wing disc (Huang and Kornberg, 2015). The second mechanism is the cytoneme-cytoneme contact, for examples cytonemes carrying receptors for Dpp and FGF in *Drosophila* wing disc (Du et al., 2018; Roy et al., 2014), and myoblasts carrying Delta to contact the ASP (Huang and Kornberg, 2015), in the Hh distribution within the *Drosophila* epithelia (González-Méndez et al., 2017) and possibly in Shh signalling in the developing chick limb bud (Sanders et al., 2013) (see Fig 21).

5.2.4 Cytonemes as synaptic contacts

A synapse is a mechanism of intracellular communication in which the secreting cell by extending a process can, after crossing other intervening cells, reach the target cell in a

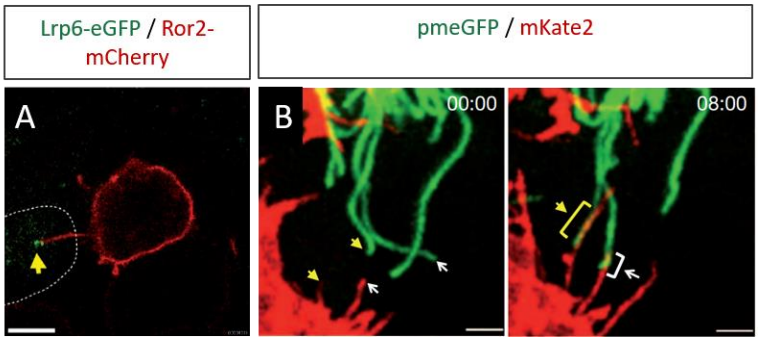

Figure 21. Types of cytoneme contacts.

A. Cytoneme-cell body contact. Single-plane image of a Ror2-mCherry/Wnt8a producing cell leading to Lrp6-GFP clustering of the responding cell clone, showing the cytoneme contact site. Scale bars 10 mm. **B**. Cytoneme-cytoneme contact of mesenchymal cells of the developing limb bud in chick. Mosaic labelling through Cre-Lox system generates membrane-palmitoylated mKate2 (a far-red fluorescent protein) or pmeGFP. Scale bar 3 mm.

Image modified from Mattes et al., 2018; Sanders et al., 2013.

highly precise way. These two cells present pre- and postsynaptic in specific membrane areas. Cytoneme signalling features resemble to synaptic contacts in some aspects. Cytonemes allow for specific and direct contacts, saving distances between cells, the signalling is directed from sending to receiving cells, the ligand presentation would be the parallel to the presynaptic molecules, and the receptor to the postsynaptic molecules. For these reasons, the new term “morphogenetic synapses” is been used to describe cytonemes’ contacts. Also, the contact sites at membranes between cytonemes are similar to the synaptic buttons, with membrane swellings and vesicles release (González-Méndez et al., 2017; Huang et al., 2019), as well as the space between the cells (Roy et al., 2014). Of course, differences must not be underestimated, as the complexity of molecules involved, contact duration and the link to the propagating electric impulse (reviewed in Kornberg, 2017).

5.2.5 Cytonemes’ contacts establishment and regulation

Finally, cytoneme signalling will depend on the frequency and the stability of these contacts. Therefore, membrane bound proteins must regulate the establishment of cytonemes (or cytoneme-cell body) contacts. Some of them have already been identified,

they are adhesion molecules such as integrins and synaptic adhesion molecules such as Neuroglian that guide cytonemes sensing the ECM and cell membranes to recognize and establish physical contacts with the correct cell (Bischoff et al., 2013; González-Méndez et al., 2017; Huang and Kornberg, 2015; Huang et al., 2019; Roy et al., 2014).

The spatial and temporal establishment of cytonemes must be regulated. Some studies propose a self-regulatory mechanism between the signalling pathway and the filopodia formation. For example, FGF in the *Drosophila* ASP promotes protrusion enhancement, while low levels of signalling repress protrusion extension (Du et al., 2018) and Wnt8a cytoneme transport in zebrafish leads to the activation of actin polymerization process (Mattes et al., 2018; Stanganello et al., 2015). Interestingly, these feed-back loops do not always function the same way. In the Drosophila ovary stem cell niche cytonemes grow until they reach the ligand, and impairment of ligand reception causes cytonemes to grow further (Rojas-Ríos et al., 2012). However, autoregulatory feed-back loops are not always present: In the wing imaginal disc and abdominal histoblasts (cuticle-producing cells) cytonemes formation and dynamics are independent of the signalling by Hh (González-Méndez et al., 2017). Thus, still further work is needed to fully understand the mechanisms of cytonemes formation and the links to the signalling pathways.

Other cell protrusions with signalling capacities have been described as tunnelling nanotubes or intracellular bridges (Caneparo et al., 2011; Gerdes et al., 2012).

ORGANISM	TISSUE	LIGAND OR RECEPTOR CARRIER	SIGNAL	FUNCTION	LENGTH	REFERENCES
DROSOPHILA	Wing disc & adbdominal epithelia	Ligand	Hh (Shh)	Morphogen gradient	40 µm wing disc; 8-12 µm histoblasts	Bischoff et al., 2013; Gradilla et al., 2014; González-Méndez et al., 2017
		Receptor	Ptc Rc (Hh signalling)	Morphogen gradient	41 µm wing disc; 8-12 µm histoblasts	Chen et al., 2017; González-Méndez et al., 2017
	Wing disc epithelium	Receptor	Tkv Rc (Dpp signalling)	Morphogen gradient	20-80 µm	Ramirez-Weber and Kornberg 1999
	ASP epithelium	Receptor	Btl Rc (FGFR)	Morphogen gradient	< 40 µm	Roy et al., 2014; Du et al., 2018; Huang et al., 2019; Sohr et al., 2019
		Receptor	Tkv Rc (Dpp signalling)	Signalling across tissues	< 40 µm	Roy et al., 2014; Huang et al., 2019
	Eye disc	Receptor	EGFR	Signalling across tissues	< 30 µm	Roy et al., 2011
	Flight muscle progenitors	Receptor	Fz Rc (Wnt signalling)	Signalling across tissues	25 µm	Huang and Kornberg, 2015
		Ligand	Delta (Notch signalling)	Signalling across tissues	25 µm	Huang and Kornberg, 2015
	Notum cuticle	Ligand	Delta (Notch signalling)	Pattern formation	< 10 µm	De Joussineau et al., 2003; Cohen et al., 2010; Hadjivasiliou et al., 2016
	Leg disc epithelium	Ligand	Spi	Pattern formation	< 9.5 µm	Peng et al., 2012

ORGANISM	TISSUE	LIGAND OR RECEPTOR CARRIER	SIGNAL	FUNCTION	LENGTH	REFERENCES
DROSOPHILA	Ovary	Ligand	Hh (Shh)	Stem cell niche	0.25 - 1.11 µm	Rojas-Rios et al., 2012
	Testis	Receptor	Tkv Rc (Dpp signalling)	Stem cell niche maintenance	< 4 µm	Inaba et al., 2015
	Lymph gland	Unknown	Unknown	Stem cell niche maintenance	Unknown	Mandal et al., 2007; Fuwa et al., 2015
BUTTERFLY	wings	Unknown	Unknown	Pattern formation	Unknown	Ohno and Otaki, 2015; Iwasaki et al., 2017
SEA URCHIN	Embryo gastrulation	Unknown	Unknown	Pattern formation	< 80 µm	Miller et al., 1995
ZEBRAFISH	Neural plate	Ligand	Wnt8a	Morphogen gradient	< 50 µm	Stanganello et al., 2015; Mattes et al., 2018; Luz et al., 2014
	Embryo blastula	Unknown	Unknown	Signalling across tissues	60 - 380 µm	Caneparo et al., 2011
	Pigment cells	Ligand	Delta (Notch signalling)	Pattern formation	60 - 250 µm	Hamada et al., 2014; Eom et al., 2015
XENOPUS	Embryo blastocyst	Unknown	Unknown	Morphogen gradient	10 - 250 µm	Danilchik et al., 2013
	Fibroblasts	Ligand	Wnt2a	Unknown	Unknown	Holzer et al., 2012

ORGANISM	TISSUE	LIGAND OR RECEPTOR CARRIER	SIGNAL	FUNCTION	LENGTH	REFERENCES
CHICKEN	Embryo somites	Receptor	Fz Rc (Wnt signalling)	Signalling across tissues	< 20 µm	Sagar et al., 2015
	Limb bud	Receptor	Shh	Morphogen gradient	< 150 µm	Sanders et al., 2013
		Ligand	Cdo and Boc co-receptors (Hh signalling)	Morphogen gradient	< 150 µm	Sanders et al., 2013
MOUSE	Embryo blastocyst	Unknown	Unknown	Signalling across tissues	< 34 µm	Salas-Vidal and Lomeli, 2004
HUMAN	Stem cell line	Unknown	Unknown	Stem cell niche maintenance	< 80 µm	Snyder et al., 2015

Table 3. Table summarizing structural and functional characteristics of cytonemes described in different organisms

ASP, air sac primordium; BMP, bone morphogenic protein; Bnl, branchless; Boc, brother of CDO; BTl, breathless; Cdo, Cam-related/downregulated by oncogenes; Dpp, decapentaplegic; EGF, epidermal growth factor; FGF, fibroblast growth factor; Fz, frizzled, Hh, hedgehog, Ptc, patched; Shh, sonic hedgehog; Spi, spitz; Tkv, Thick-Vein; Wg, wingless. Extra references not included in the text but present in the table: (Holzer et al., 2012)

Table modified from: González-Méndez et al., 2019; Pröls et al., 2016.

II. AIMS OF THE THESIS

Cells within an organism develop in association with other cell types around them, which will constitute the so-called “niche” and with which they communicate to build functioning organs and tissues. This communication also occurs in the development of neural cells. As seen in the introduction, BV have emerged as key members of neurogenic niches. However, only the CNS has been investigated yet.

The aim of this work was to explore the niche in which the cranial ganglia is formed, focusing in the putative function of BV in neurogenesis, to shed light into the unknown BV functional role in the developing PNS neurons. My investigations were mainly done in the SAG, while principal results were also searched in other sensory ganglia.

The specific issues addressed were:

[1] To describe the anatomical relationship between the head vasculature and cranial ganglia during their development.

[2] To explore a functional role of vasculature on cranial sensory neural development, focusing in the SAG.

[3] To investigate a functional role of neurogenesis on vascular development.

[4] To search for the mechanisms involved in the neurovascular interaction.

[5] To identify the signalling cues responsible for the identified neurovascular communication.

III. RESULTS

Article 1. Anatomical map of the cranial vasculature and sensory ganglia

Taberner L, Bañón A, Alsina B. Anatomical map of the cranial vasculature and sensory ganglia. J Anat. 2018 Mar 1;232(3):431–9. DOI: 10.1111/joa.12762

Article 2. Sensory neurogenesis depends on vascular-neuronal cytoneme contacts and blood flow

Taberner L, Bañón A, Alsina B. Sensory Neuroblast Quiescence Depends on Vascular Cytoneme Contacts and Sensory Neuronal Differentiation Requires Initiation of Blood Flow. Cell Rep. 2020 Jul 14;32(2). DOI: 10.1016/j.celrep.2020.107903

IV. DISCUSSION

During neurogenesis, both in embryo and adult, each of the NSCs behaviours – quiescence, proliferation, self-renewal and differentiation– has to be tightly balanced. Otherwise pathologies may arise. Increased self-renewal without differentiation could result in brain cancer (Palm and Schwamborn, 2010). On the other hand, premature differentiation at the expense of progenitors' proliferation can deplete the NSC pool producing phenotypes of aging.

The development of the nervous system highly depends on the developing vasculature. The nascent vasculature regulates NSC behaviour and neurogenesis by providing oxygen but also specific signalling cues. This relationship is maintained in adult neurogenic regions. ECs and neural cells have evolved to be able to communicate and ensure their proper development and maintenance.

Despite the importance of the neurovascular crosstalk, we are just beginning to understand the underlaying mechanisms responsible of it and their implications. Research on the neurovascular interaction has mainly focused in the adult, while the first studies during development are starting to emerge. Still, in both cases reports are focused in different regions of the CNS. Thus, previous studies left a totally unexplored terrain: the possible regulation of ECs in neurons' behaviour has remained totally elusive in the developing PNS.

This thesis explores the vascular regulation of NSCs' properties in the developmental PNS, beyond the axonal patterning.

Description of cranial sensory ganglia in a vascular niche

My first work consisted of studying the relative position of sensory ganglia with respect to head vessels, as other studies did before trying to understand any functional role between the vascular and the neuronal system (Bates et al., 2002; Javaherian and Kriegstein, 2009; Mukouyama et al., 2002; Tata et al., 2016). The development and precise architecture of the zebrafish vasculature have been studied in detail (Isogai et al., 2001; Ulrich et al., 2011), but whether they develop in close proximity with cranial ganglia and how had not been addressed before.

Thanks to the use of reporter transgenic lines for cranial ganglia and vessels, we found that all sensory ganglia develop in close proximity with at least one BV. We described the complex 3D interactions in space and time (24, 30, 48 and 72 hpf) between these two systems. The Tg develops on top of the PHBC in its most anterior part, the aLL and the pLL relate with the PHBC in their dorso-medial edges, all the epibranchial ganglia grow on top of an AAs, and the Xg follows the PHS. In particular, we found that the SAG was in contact with a higher number of BV: two veins, the PHBC and the PHS, and one artery, the LDA. The analysis of these interactions in 3D allowed us to identify different domains in cranial ganglia depending on whether cells are in close contact or further from BV, which might be translated to different cells' behaviour – for instance more proliferative. Finally, as we studied the physical interaction over time, we can state that the contacts perdure, and thus, they might not be something coincidental at some time points but actively sustained.

Discovering that cranial sensory ganglia were developing in a rich vascular environment gave us the first hint that BV might be part of the sensory neuronal niche.

Vasculature promotes neural quiescence in cranial sensory ganglia

The function of a niche is to both maintain SCs in quiescent or undifferentiated state and to control proliferation and differentiation into the correct lineages. Much effort is put, therefore, on understanding how the same environment can conduct such opposite effects on SCs.

To functionally evaluate the putative regulation of vasculature on sensory ganglia development, the avascular *clo* mutant transgenic line has been used (Stainier et al., 1995). We analysed the growth dynamics of the SAG in *clo* mutant versus control embryos. The results of this project identified that the SAG and Tg volume and cell number were increased in the avascular mutants compared to control embryos, meaning that BV play a role in negatively regulating neuroblasts' proliferation or promoting their quiescence.

Interestingly, there does not seem to be a common mechanism by which BV regulate neural behaviour. A good collection of reports demonstrate that BV are required for neuronal precursors to proliferate, both in the adult and developing brain (Biro et al., 1994; Gomez-Gaviro et al., 2012; Louissaint et al., 2002; Tan et al., 2016; Tata et al., 2016). However, very few examples are found in literature where BV are required to

restrict neuronal growth, probably promoting quiescence (Delgado et al., 2014; Ottone et al., 2014). These cases of quiescence promotion have only been described in adult stages. Thus, our results are the first ones to demonstrate that during neuronal development BV can also promote quiescence.

Out of these investigations, we also gained a detailed temporal information on the SAG growth dynamics. These type of quantifications in the cranial ganglia had been hampered due to their loss of an epithelial character and its complicated 3D organization. Here, by quantifying overtime the number of neurod cells (neuroblasts) and also islet 2 cells (postmitotic and differentiated neurons) we have drawn at high resolution the growth dynamics of the SAG in zebrafish from 30 hpf to 96 hpf. We believe that the quantitative data provided can be useful for other projects in which growth defects might be taking place upon specific signalling pathways manipulations in the SAG.

Cranial vasculature development does not require neural cells

Neurovascular communication does not only occur from vessels to neurons, but also from neurons to vessels. ECs have been reported to sense neural-derived signalling cues for their patterning and maturation in the limb skin (Li et al., 2013b; Mukouyama et al., 2002, 2005), and to vascularize the retina (Okabe et al., 2014), cortex (Ma et al., 2012) and spinal cord (Himmels et al., 2017). In this project we also wanted to assess the possible requirement of neural cells for the proper development of the cranial vasculature, but our analysis demonstrated that it develops correctly independently of the presence of cranial sensory neurons. Therefore, in the cranial system, neurovascular communication occurs only in one direction, from vessels to neurons.

A possible reason why might be because of developing times: the PHBC is formed (two initial sprouts merge) at 24 hpf, the time point at which neuroblasts peak in their delamination from the OV to form the SAG. Thus, the development of the vasculature is preceding the one of the ganglion. Also, cranial ganglia present complex 3D morphologies, not “straight paths” that vascular sprouts could use to follow for a correct patterning. Also, no small vessels penetrate the ganglia, so neural cells do not need to promote the formation of new sprouts. Finally, and further speculating, if BV are going to regulate fine decisions such how many neural cells will proliferate or differentiate it is “safer” -evolutionary speaking- if the first system forms correctly independently of the system they are going to signal to.

Signalling Filopodia (Cytonemes) mediate neurovascular cross-talk

When we took a closer look to our neurovascular interacting system, we found that, despite endothelial and neural cells were separated by a small space, they establish direct physical contacts through filopodia. Multiple studies have described the close physical relationship between endothelial and neural cells both during development and in adult neurogenic niches (Delgado et al., 2014; Ottone et al., 2014; Tan et al., 2016; Tata et al., 2016; Tavazoie et al., 2008). In some cases, a small distance between BV and dividing neurons is described and measured, which still makes a significant difference with the further located non-diving neurons (Tavazoie et al., 2008). In these cases, communication might occur through diffusible cues. Direct physical contacts had been described before both in adult and developmental stages, consisting in end-food projections of neurons enwrapping BV, (Kacem et al., 1998; Tan et al., 2016), but never through filopodial protrusions.

Further, pharmacological inhibition of filopodia formation demonstrated that these filopodia are required to transmit the signal that promotes neuroblasts' quiescence in the SAG. This signal is not secreted, because BV are still present in the filopodia inhibition experiment. When filopodia formation is inhibited, the BV-derived signal cannot reach the SAG. Thus, we demonstrate that these cytoplasmatic protrusions are indeed signalling filopodia, also named as cytonemes (Ramírez-Weber and Kornberg, 1999).

Signalling filopodia (or cytonemes) between SCs and the cells of their niche participate in the maintenance of SCs in an undifferentiated state in the ovaries, testis and haematopoietic stem cell niche, but they have only been described in *Drosophila* (Fuwa et al., 2015; Inaba et al., 2015; Mandal et al., 2007; Rojas-Ríos et al., 2012). Thus, our study is the first one to describe cytonemes in a vertebrate niche. Specifically, we observe cytonemes in a neurogenic niche between NSCs and ECs. Tavazoie and colleagues observed that the contact sites between NSC and BV were often small, and suggested that it might be occurring dynamically *in vivo*. This hypothesis could not be assessed as their observations were based in fixed tissues whole mounted or sectioned (Tavazoie et al., 2008). The use of zebrafish embryos, otherwise, allow for *in vivo* imaging, which permitted cytonemes observation, opening the possibility that neurovascular communication is also occurring through a cytoneme-based mechanism in other organisms and contexts.

Dll4/Notch1 are required for maintenance of neural quiescence

Despite the growing number of reports describing functional roles for ECs on neurons, the EC-derived molecular cues required for these interactions still remain elusive in many cases.

Notch signalling pathway play key roles in balancing NSC quiescence and proliferation (Borghese et al., 2010; Shimojo et al., 2008, 2011), where high Notch activation induces growth arrest and low Notch cell proliferation, in adult NSC niches. On the other hand, the two Notch ligands Dll4 and Jag1 are highly expressed in ECs (Benedito et al., 2009), thus it could be expectable that ECs may also be signalling to Notch-expressing embryonic and adult NSCs.

Only one study identified ECs regulating Notch in NSCs, through the expression of Jag1. The Jag1-Notch ligand-receptor binding, together with the Eph-ephrinB2a interaction, promotes NSC quiescence in the V-SVZ in adult mice (Ottone et al., 2014). In our study, though, it is not Jag1 but Dll4 the EC ligand binding to Notch. Interestingly, EC-derived Dll4 has already been linked to SC quiescence maintenance, but in the adult muscle SC niche (Verma et al., 2018).

Dll4 has been widely studied in the formation and guidance of new angiogenic sprouts. High amounts of this protein are expressed in tip cells, while low quantities are present in stalk cells (Hasan et al., 2017). Signalling to otic sensory neuroblasts come from stalk and not tip ECs. We propose that maybe, small quantities but spatially concentrated in specialized regions of the cytoplasm -or filopodia tips- are sufficient to signal to another cell type, the neuroblasts.

In summary, our results support the hypothesis that the Notch ligand Dll4 expressed in ECs regulate NSCs quiescence, and suggests that, if it is true for adult NSCs in the CNS and in the developing PNS, it might be a general signalling mechanism present in other neurogenic regions and even other SC niches.

It is worth mentioning that neuroblasts have been proven to be less dependent on Notch signalling than NSCs and more responsive to environmental cues for the regulation of their proliferation (Ables et al., 2010). These findings match with previous studies carried out in the SAG where other signals, such as IGFs, are also important to regulate neuroblasts' proliferation (Alsina et al., 2003; Camarero et al., 2003). Our results add one piece of information in the canvas of all the signalling cues regulating the SAG development, where vasculature is now a new player.

Cytonemes carrying Dll4 and Notch1

An increasing amount of reports show cytoneme signalling associated with vesicle transport. In our study we did not investigate this feature in detail, but still it is worth mentioning that rounded thickenings travelling to the distal edges of neuroblasts' filopodia can be observed in some of the videos. This type of signal transportation has been described in *Drosophila* (González-Méndez et al., 2017; Gradilla et al., 2014; Matusek et al., 2014), chick (Sanders et al., 2013) and zebrafish (Eom and Parichy, 2017; Eom et al., 2015; Luz et al., 2014; Stanganello et al., 2015). Thus, it is not risky to hypothesise that ECs and neuroblasts might use membranous vesicles containing Dll4 and Notch1 to reach each other.

Cytonemes deliver their signals to target cells through two different ways, some studies state that cytonemes contact the cell body of their target cells (Huang and Kornberg, 2015; Stanganello et al., 2015), while in others cytoneme-cytoneme contacts have been observed (Du et al., 2018; González-Méndez et al., 2017; Huang and Kornberg, 2015; Roy et al., 2014; Sanders et al., 2013). In our particular system, we have been able to identify cytoneme-cytoneme direct contacts thanks to the physical separation of both tissues and the different colours of the two transgenic lines used. In other cases, these direct contacts were not seen. These observations can be explained in different ways, one possibility is that all cases are cytoneme-cytoneme contact but, in some cases, the "missing cytoneme" is inside one of the tissues and cannot be seen as all the cells are stained with the same fluorophore. The other possibility is that the two types of contacts are occurring at the same time, enhancing the number of signalling contacts. Maybe, single-cell labelling techniques could be used to solve this question in future research.

The signalling though cytonemes depends on their frequency and stability. In our system, dynamic and stable filopodia have been observed, being the first probably those that have not correctly contacted with their target cell yet, and the second ones those that have and are actively signalling (Sagar et al., 2015). The establishment of cytonemes' contacts is mediated by adhesion molecules (Bischoff et al., 2013; González-Méndez et al., 2017; Huang and Kornberg, 2015; Huang et al., 2019; Roy et al., 2014). In some cases the signalling pathways, the adhesion molecules and the the filopodia formation machinery are connected via feedback loops in some cases but do not in others (Du et al., 2018; González-Méndez et al., 2017; Mattes et al., 2018; Rojas-Ríos et al., 2012; Stanganello et al., 2015). The adhesion molecules involved in the neuroblast-EC contact in the SAG have not been explored, nor the filopodia formation machinery. However, we

can state that they function independently on the Dll4-Notch1 signalling, since filopodial processes are still formed when Dll4 is downregulated, despite they not being signalling. On the contrary, it is clear that some signal located in ECs is required for NB to form filopodia, as absence of BV produces a lack of filopodia in neuroblasts. To explore deeper in the interdependent mechanisms of the PHBC-SAG cytoneme contacts, the dynamics/speed of filopodial processes could have been measured in the absence of Dll4 and neuroblasts (*neurog1* $^{-/-}$), as well as performing gain and loss-of function experiments for already described adhesion and filopodia formation molecules.

Some researchers state that cytonemes are a new type of "synapsis", and called them morphogenetic synapsis, due to their multiple common features such as cell polarization, directed secretion for communication and membrane domains specialized with receptors and adhesion molecules (Kornberg, 2017). Increasing evidence shows the relevance of cytonemes, or signalling filopodia, as a cell-cell communication mechanism, which could be a general feature of signalling processes in cells that allows a precisely spatio-temporal regulation, and not only as a sophistication of neuronal synapses. The plasticity of the cytoneme mode of signalling is especially important in our system: it probably allows some neuroblasts to be maintained in quiescence, those specific cells that have been contacted; while others are not touched by cytonemes, and thus can continue to proliferate, achieving the correct SAG growth dynamics. This selective signalling could not have been possible with other signalling mechanisms such as signal diffusion.

Blood flow requirement to trigger neuronal differentiation

We have shown that blood flow onset is required for neuronal differentiation in the SAG at specific developmental stage (from 54 to 60 hpf). Only one previous study described a similar observation. Fortuna et al. demonstrated that blood flow onset was required for VMC to be recruited around BV, once there, SMCs drive neuronal differentiation through PDGFR secretion (Fortuna et al., 2015). However, SMC are not ECs (they have a different developmental origin) and are not present around the PHBC (Santoro et al., 2009). Another mechanism must, therefore, be responsible of otic sensory neuronal differentiation.

Upon blood flow onset, ECs lining the inner surface of BV sense two types of mechanical stimuli: shear stress, the frictional force tangential to the vessel, and mechanical tension, the force perpendicular to the flow direction. Shear stress can modify the patterning of

BV (Chen et al., 2012a), be detected by ECs through the mechanosensitive transcription factor *klf2a* (Nicoli et al., 2010), and also produce changes in the ECs gene expression through the activation of transcription factors, making ECs to “mature” (Chiu and Chien, 2011; Korn and Augustin, 2015; Nicoli et al., 2010; Weijts et al., 2018). Shear stress has been described to activate Notch signalling in arterial ISVs (Weijts et al., 2018), and to allow the temporal translocation of Yap1 into the EC nucleus in zebrafish (Nakajima et al., 2017). Then, it is possible that the transcriptional changes happening on ECs after blood flow onset promote the expression of a diffusible signalling cue that prompts neuroblasts to exit cell cycle and terminally differentiate. A signalling mechanism in which ECs translates blood flow and mechanotransduction into secreted signals for growth or differentiation of another cell type has been described in a beautiful study in the mouse liver. Liver ECs sense the blood flow mechanical forces through β-integrin and VEGFR3, which in turn promote the secretion of growth factors that promote hepatocyte proliferation (Lorenz et al., 2018).

On the other hand, blood flow onset will also transport multiple molecules that could also have a signalling function on neuronal differentiation. Unfortunately, it is technically difficult to separate these two factors *in vivo*.

Finally, still one last possibility should be noted. BV or blood flow-derived signals might not be essential for instructing cell-fate or differentiation, but rather might just provide survival cues for a certain type of new-born neuron (Kirschenbaum and Goldman, 1995; Leventhal et al., 1999), a selection role that could give the illusion of an effect on differentiation instruction. To discard this possibility, a cell death essay (TUNEL or caspase 3) could be done from 54 to 72 hpf. If the EC-derived signal is a survival cue, Nifedipine treated and avascular mutant embryos should present more apoptosis than control embryos, while proliferation would be occurring in all conditions. However, we consider this not to be very plausible as the neurod reporter (*Tg(neurod:egfp)* ^{nl1}) labels also the differentiated population (Islet2^{+} cells are also GFP^{+}) and we do not see this population to decrease at 72 hpf or apoptotic bodies in the confocal images.

We conclude that a signal carried by or secreted upon blood flow is promoting neuronal differentiation at late stages and that this mechanism is independent from the first one as no filopodia is observed at late stages.

Blood vessels-derived cues upon blood flow onset mediating neuronal differentiation

BV can exert their roles on NSC niches via angiocrine signalling or via delivery of nutrients or oxygen (Cleaver and Dor, 2012; Ramasamy et al., 2015).

The transcriptome analysis of SAG neuroblasts in *clo* mutant vs control embryos allowed us, first of all, to demonstrate that neuronal cells were actually sensing signals from BV and that this was translated into changes in their transcriptional profile. More specifically, what we observed is that neuroblasts of the avascular mutant embryos presented far more downregulated than upregulated genes when compared to neuroblasts of control embryos. This may indicate that a whole signalling cascade is being activated in the control case, while it is not in the mutant, rather than different signalling pathways are acting in each condition. This also matches with a cell differentiation arrest in the avascular mutant.

Among the differentially expressed genes we identified some related to oxygen tension and mitochondria metabolism. *egln2* (Egl nine homolog 2) is a cellular oxygen sensor involved in the degradation of HIF (Epstein et al., 2001), *rdh8a* (retinol dehydrogenase 8a) is involved in redox processes (Haller et al., 2010), and *abat* (4-aminobutyrate aminotransferase) and *rmnd1* (Required for meiotic nuclear division protein 1 homolog) in mitochondrial metabolism, translation and OxPhos (Besse et al., 2015; Janer et al., 2012, 2015). Therefore, this means that control embryos are sensing oxygen, high levels of Egln2 are degrading proteins and a shift to OxPhos metabolism is occurring, while this in not happening in the avascular mutants.

Our results provide evidences that, at 2 dpf, zebrafish larvae start to depend on BV for a better oxygen perfusion to produce the correct number of otic sensory differentiated neurons. Oxygen tension increase is required for cranial ganglia sensory neurons differentiation, while reduced levels of oxygen -either by complete lack of vasculature or by blood flow inhibition- prevent the switch to neuronal differentiation. Importantly, this does not imply an increase in the neuroblasts' proliferation at late stages, demonstrating that the mechanisms for proliferation control and differentiation are independent in the cranial sensory ganglia system. Also, as already discussed, it is not related to cell death, as the number of neurod^{+} cells does not decrease at 72 hpf.

Current evidence has demonstrated that, in addition to growth factors, the metabolic pathways of glycolysis and OxPhos provide important signals for stem-cell renewal and

differentiation, respectively. Oxygen tension impinges on the shift from glycolysis to OxPhos, through the regulation of HIF-1α. Absence of OxPhos is a defining feature of embryonic SCs, and it is required to maintain their pluripotency (Harvey et al., 2016; Ryall et al., 2015). In fact, certain SCs reside in hypoxic niches (Mohyeldin et al., 2010; Panchision, 2009). Some cases where the metabolic switch from aerobic glycolysis to OxPhos regulates NSC differentiation have been described both in invertebrates and vertebrates through the regulation of HIF-1α (Homem et al., 2014; Lange et al., 2016), despite it is yet a rather unexplored terrain. Osteogenic and adipogenic differentiation of mesenchymal SCs is also regulated by the loss of aerobic glycolysis and increase of OxPhos and HIF-1α downregulation (Palomäki et al., 2013; Shum et al., 2015; Zhang et al., 2013). In other cases, the switch is done by the regulation of epigenetics, redox and reactive oxygen species, a part from HIF-1α (Hawkins et al., 2016; Kida et al., 2015; Ryall et al., 2015). Thus, we think that a possible mechanism by which neuroblasts need blood flow is for oxygen tension to increase and switch their metabolism to OxPhos, whose transcriptional changes will ultimately lead to neuronal differentiation.

A direct demonstration of this mechanism could be gained with additional experiments such as growing avascular mutant and control embryos in hypoxic or hyperoxia chambers (Khaliullina-skultety et al., 2017; Rouhi et al., 2010), reproducing the phenotype of differentiation arrest in control embryos and rescuing the phenotype with *egln2* overexpression in *clo* mutant embryos, respectively. Also, by doing loss-of-function experiments of *egln2* in WT embryos (by MO or CRISPR), or assessing the differential transcriptional levels of HIF target genes involved in cell cycle control and energy metabolism (such as IGF2, p21, or GLUT1) (Sharp and Bernaudin, 2004) between *clo* mutant and control embryos.

Finally, our results do not exclude that BV also participate in the otic sensory neurons' differentiation via angiocrine factors as suggested by previous work *in vitro* (Shen et al., 2008), and *in vivo* in adult NSCs (Delgado et al., 2014; Ottone et al., 2014; Tan et al., 2019).

Vasculature as a key component of is part of the stem cell niche

In the previous sections we have seen that vasculature, in addition to its well established role of oxygen and nutrients transport, also regulates neural expansion and differentiation. But this new feature is not exclusive in the nervous system, few studies

have started to demonstrate a role for vascular cells in stem cell niches of other organs such as the liver, bone, kidney and pancreas during development (Bjarnegård et al., 2004; Han et al., 2011; Lammert et al., 2001; Lorenz et al., 2018; Ramasamy et al., 2015; Serluca et al., 2002; Yoshitomi and Zaret, 2004), and at adult stages in the lungs, skin, spermatogonia and muscle (Paquet-Fifield et al., 2009; Rafii et al., 2015; Verma et al., 2018; Yoshida et al., 2007).

The vascular system is, thus, emerging as a signalling centre with functions that involve the hosting and regulation of stem and progenitor cells, in many different organs systems.

Neurovascular linked diseases

As widely described in the introduction, the nervous and vascular system share similarities at the morphological, cellular and molecular levels. Additionally, they are also functionally interdependent. It is, therefore, not surprising that both neuronal and vascular defects are also involved in the pathogenesis of neurodegenerative disorders.

Owing to the aging of the world population and lack of effective treatments, neurodegenerative diseases are predicted to grow to epidemic proportions in the following decades (Gammon, 2014).

Traditionally, neurological diseases have been thought to arise primarily from an intrinsic malfunctioning of the neurons themselves. However, alterations of the neurovascular crosstalk can also contribute to numerous neurological disorders (Zlokovic, 2008). Conditions where vascular abnormalities affect the nervous system include stroke, vascular dementia, hypertension and diabetes. Thus, there is a growing interest in exploring the potential contribution of neurovascular dysfunction to neurodegeneration (de la Torre, 2017).

Vascular defects also affect certain neurodegenerative diseases. Patients suffering Alzheimer disease present profound changes in the cerebrovascular structure. A hallmark of this disease is the deposition of β-amyloid peptide in the brain parenchyma (Zlokovic, 2005). This protein also accumulates in BV producing vasoconstriction, reduced blood flow, vascular resistance and EC damage increase (Kalaria, 2002). Certain studies document that the vascular alterations are present before the disease onset, suggesting that they might contribute to its initiation (Zlokovic, 2008).

Frontotemporal dementia is the leading cause of dementia in the middle age (Olney et al., 2017). Pre-symptomatic patients present reduced CFB in the frontal, parietal and temporal cortex (Dopper et al., 2016) as well as alterations in the BBB (Janelidze et al., 2017). Another example is found in patients suffering Parkinson disease, who show capillary damage and fragmentation (Guan et al., 2013), reduced CBF (Borghammer et al., 2010) and increased VEGF in the CSF (Janelidze et al., 2017). Vascular alterations are also present in dementia with Lewy bodies, where CBF is reduced in several parts of the cortex (Galasko, 2017). These changes in the CBF appear just before the onset of the disease (Roquet et al., 2016) and are probably due to reduced energy metabolism (Ishii et al., 2015). Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a syndrome that leads to cognitive decline and dementia. This condition is due to a progressive degeneration of cerebral arterioles which produces arterial narrowing and hypoperfusion, caused by mutations in Notch3 (Kalimo et al., 2002). Finally, in diabetic neuropathies, endoneural perfusion deficits and chronic ischaemia is caused by several vascular abnormalities such as the thickening of the basement membrane and EC hyperplasia.

Subtle mutations in the VEGF promoter have been identified to produce lower levels of VEGF protein. As a consequence, people with these VEGF gene variations present deficits in spinal cord perfusion and chronic ischaemia, this specially affects motor neurons due to their active metabolism (Oosthuyse et al., 2001). They also have a higher risk of suffering amyotrophic lateral sclerosis (ALS).

Other neurological disorders, such as brain tumours are characterized by excessive vascularization. Some studies suggest the existence of a cancer stem cell-vascular niche complex, where ECs promote brain tumour survival (Calabrese et al., 2007; Klagsbrun et al., 1976).

Therapeutic implications of the neurovascular interaction

We are gaining evidence on the importance of BV in NSC behaviour control. Elucidating the signals and mechanisms by which ECs participate in the regulation of neural cells behaviour *in vivo* will shed light into the first tools to target neurovasculature therapeutically and to build SC-based therapies to treat neural diseases and disorders. However, we first need a solid understanding of the intrinsic and extrinsic interactions of NSC regulation. For example, the realization that angioneurins have multitasking

properties in angiogenesis, but also in neuroregeneration and neuroprotection, has created opportunities to explore novel therapeutic opportunities. Some examples of clinical trials are the use of VEGF and EPO for neurotrophic support and angiogenesis in ALS (Azzouz et al., 2004; Lambrechts et al., 2003; Storkebaum et al., 2004, 2005), Parkinson's Disease (Chen et al., 2007; Tian et al., 2007), diabetic neuropathies (Hobson et al., 2000; Isner et al., 2001; Schratzberger et al., 2001; Sondell et al., 2000) and ischemic injuries (Nishijima et al., 2007); and VEGF inhibitors for brain tumours (Loges et al., 2009; Miletic et al., 2009).

Another therapeutic option is to take profit of the potential of NSCs to home into injured brain regions (Imitola et al., 2004; Thored et al., 2006). The use of SCs to repair the nervous system is of great potential, but being able to control the expansion and differentiation of these NSC is still very challenging. Additionally, despite still controversial, some studies claim to detect adult hippocampal neurogenesis (AHN) in humans (Choi and Tanzi, 2019). Therefore, therapies focused on the promotion of AHN will be useful for prevention and treatment of neurodegenerative diseases.

A fundamental understanding of neuronal and vascular interactions will have far-reaching benefits in developing strategies to treat psychological and neurodegenerative diseases, brain tumours and stroke. Understanding how these interactions occur during development and adulthood in physiological circumstances will help to understand whether they become reactivated or dysregulated during pathological conditions. With this thesis I have participated in the expansion of the knowledge on the neurovascular interaction, demonstrating that it is also required for the proper sensory cranial ganglia development. Our work in the SAG might serve as a model system for a general mechanism found in other regions of the nervous system.

Neurovascular linked diseases in the Inner Ear

Hearing loss, resulting from aging, genetic predisposition or environmental exposure to noise, infections or ototoxic drugs, is one of the most prevalent chronic conditions affecting older adults, affecting the 5% of the world population – or 466 million people– according to the World Health Foundation (https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss). Hearing loss impacts in the individual ability to communicate, with social consequences such as loneliness, isolation, and frustration, particularly among older people; and this can be an important driver of morbidity and

mortality. Vertigo, on the other hand, affects between the 20 and 30% of the population, and can cause nausea, vomiting, ataxia, and even anxiety and depression (Karatas, 2008).

Hearing loss and vertigo commonly reflect a loss of sensory HC and auditory synapse degeneration (Kujawa and Liberman, 2009; Spoendlin, 1975). Loss of VIIIth ganglion neurons also significantly contributes to hearing loss. Otic progenitor and SC transplantation is a potential strategy to replace lost neurons (Chen et al., 2012b; Rivolta, 2015). Also, promising advances are being done in the development of inner ear organoids (Koehler et al., 2017; Munnamalai and Fekete, 2017). Thus, understanding the mechanisms and key signals that regulate otic sensory neurons' development will accelerate efforts to a future where replacement therapies are real. The work of this thesis has contributed on it and has put BV derived signals in the scenario. More specifically, this new knowledge may benefit some deficiencies in the auditory and vestibular system that have been described to be due to neurovascular alterations (Jensen et al., 2004; Lago et al., 2018; Paul et al., 2016).

Future perspectives

In summary, an array of cell-cell contact, diffusible and circulating cues from BV have been demonstrated to regulate NSC quiescence, self-renewal, proliferation, differentiation and migration. Key questions would be to understand how neural cells integrate all these signals, especially when they can have overlapping or antagonistic roles; and how BV signalling is integrated with the rest of the information coming from other cells that conform the niche in which NSCs develop. It would also be of particular interest to know how this is coupled in time, in the short term for temporal needs -for instance damage- but also in the long term, since these interactions have been seen from embryogenesis to adult stages. Additionally, ECs do not only regulate neural cells' behaviour, but they also instruct on liver and pancreas formation. This positions the vascular system as a new signalling system that may be responsible of many organs' regulation.

After demonstrating the requirement of BV in the correct development of the SAG in zebrafish, it would be interesting to elucidate whether this interaction is common in all vertebrates and explore whether vasculature is also involved in the vestibular and cochlear ganglion development in mice. Vascular interactions can be supressed by the

use of transgenic lines such as VEGF-A, VEGF-R2 and VEGF-R1 mutants (Carmeliet et al., 1996; Ferrara et al., 1996; Fong et al., 1995; Shalaby et al., 1995), and blood flow can be altered by the use of BDM and epinephrine (Lorenz et al., 2018).

Also, we have positioned zebrafish as an interesting model organism to study the neurovascular interaction. A detailed spatiotemporal study has been carried out for the cranial sensory ganglia found in the PNS. I think it would be interesting to continue exploring the neurovascular interaction in the CNS, both during development and adult stages in this animal model. Zebrafish show some interesting features that could complement the knowledge gained in mouse studies. The vascularization has been deeply studied and thus well known, besides, it is highly stereotypic (Gore et al., 2012; Isogai et al., 2001). On the side of the adult neurogenesis, in contrast to mammals, zebrafish present more NSC niches in the adult brain (Chapouton et al., 2007; Kaslin et al., 2008, 2009). Thus, zebrafish provide invaluable comparative material for extracting core mechanisms in vertebrate adult NSC niche, and to study the role of vasculature in it.

Another interesting line of study could be to explore the possible contribution of BV other organs different to the nervous system. As developed in the introduction, some studies are demonstrating that BV are required for the proper development of kidney, pancreas and liver (Han et al., 2011; Lammert et al., 2001; Serluca et al., 2002). Taking the optical and imaging advantages zebrafish embryos offer, we could study whether communication in these cases also occurs through cytonemes.

Finally, it would also be very exciting to study whether the signalling through Dll4 is also taking place in other species such as the chick and the mouse embryo, where a good Dll4 mutant line is present (Duarte et al., 2004), and thus elucidate if it is a general neurovascular signalling mechanism.

With this thesis I hope to contribute to the knowledge on how sensory neurogenesis is regulated positioning the vasculature as a new and important player.

V. CONCLUSIONS

1. The SAG and the rest of the cranial sensory ganglia develop in a vascular environment.

2. The SAG is surrounded by three main vessels: two veins, the PHBC and the PHS, and one artery, the LDA.

3. Vasculature is required to negatively control neuroblasts proliferation or to maintain neuroblasts quiescent in the SAG, at early stages, and not to regulate specification (*neurog1*$^{+}$) or differentiation (Islet 2^{+}).

4. Signalling from ECs to neuroblasts to negatively control proliferation or to maintain quiescence is also required in other sensory ganglia such as the Tg, at early stages.

5. Direct physical contacts between SAG neuroblasts and PHBC ECs are established through filopodia growing from both cell types.

6. Some of these filopodia protrusions are dynamic while other are stable.

7. Some of these interactions are made through filopodia-filopodia contacts.

8. Neurovascular interaction through filopodia contacts is occurring in other sensory ganglia such as the Tg and aLL.

9. Filopodia extending in PHBC ECs are not randomly exploring the environment, but spatiotemporally regulated.

10. At early stages, cranial vasculature develops correctly independently of the presence of sensory cranial ganglia neurons.

11. SAG neuroblasts need some signal coming from BV to form filopodia, whereas ECs do not need signals from neuroblasts for filopodia formation.

12. Filopodial contacts are required to deliver the signal that prevents neuroblasts' proliferation or promotes quiescence, and thus are cytonemes.

13. Dll4 is involved in the promotion of neuroblasts' quiescence. This signalling is specific to the presence of blood vessels, as Dll4-MO injected avascular mutants do not present additional effects.

14. Dll4 is not required for filopodia formation in ECs nor in SAG neuroblasts.

15. Blood vessels are required at late stages to trigger Islet2^{+} sensory neuronal differentiation in the SAG. This also affects the axonal density entering the hindbrain.

16. At late stages, vasculature is required for sensory neurons' differentiation, but not for the formation of other cell types in the inner ear such as HC of the maculae and cristae.

17. Blood vessels are also required for Islet2^{+} sensory neuronal differentiation in other ganglia such as the Tg and the Xg, at late stages.

18. Blood flow is required in a specific time window to trigger Islet 2^{+} sensory neuronal differentiation.

19. The neuroblasts transcriptome of avascular mutant and control embryos reveals changes in oxygen sensing and metabolic status.

VI. APPENDIX

As a result of this thesis, other data and tools were generated which are not included in the articles.

Table with the exact number of neurod⁺ and Islet2⁺ cells from 30 to 96 hpf

Number of neurod⁺ cells in the SAG

hpf	30	36	42	48	54	72
E1	77	132	143	109	100	103
E2	81	146	125	108	93	90
E3	105	144	138	100	99	120
E4	107	124	136	131	130	78
E5	105	139	109	145	128	132
E6	110	141	113	111	165	106
E7	92	140	125		148	
E8	75	103	116		109	
E9	115	102			108	
E10	101	108			118	
count	10	10	8	6	10	6
Prom	88,43	127,90	125,63	117,33	119,80	104,83
SD	14,54	17,47	12,49	17,03	23,12	19,56
SEM	4,60	5,52	4,42	6,95	7,31	7,99

Number of Islet 2⁺ cells in the SAG

hpf	30	36	42	48	54	72	96
E1	10	33	28	40	46	56	88
E2	7	40	31	41	62	54	66
E3	11	41	28	58	37	48	75
E4	9	42	31	47	39	40	76
E5	7	29		46	45	56	74
E6	5	31			40	60	62
E7	9	27			40		71
E8	9	27			35		
E9		31			34		
E10		32					
count	8	10	4	5	9	6	7
Prom	8,38	33,30	29,50	46,40	42,00	52,33	73,14
SD	1,92	5,68	1,73	7,16	8,51	7,20	8,30
SEM	0,68	1,80	0,87	3,20	2,84	2,94	3,14

Table 4. Number of Neurod ⁺ and Islet2 ⁺ cells from 30 to 96 hpf. Tables showing the number of quantified cells per embryo of the Neurod ⁺ cell population and Islet 2⁺ cell population. Neurod ⁺ cells were counted using the *TgBAC(neurod:egfp)* ^{nl1} and DAPI staining. Islet2 ⁺ cells were counted using immunostaing with Islet 2 antibody, which is nuclear.

The exact number of cells counted in the neurod^{+} and Islet2^{+} populations per embryo are given in a table with the scope that they may serve as a guidance tool for further studies were the manipulation of the developmental conditions influence on the number of SAG cells.

Dll4 morpholino does not affect filopodia formation in SAG neuroblasts

Apart from assessing the consequences of Dll4 knockdown in filopodia formation in ECs, we also took into consideration its possible effects on SAG neuroblasts. With this aim I counted the number and length of filopodia in Dll4- and random-MO injected embryos and no differences could be found (Fig APP 1).

Therefore, the differences in neurod^{+} cell number in Dll4 morphants (Article 2, Fig 5 C) are not due to problems in filopodia formation in neuroblasts that could imply difficulties in receiving the signal from BV. The phenotype observed in neurod^{+} cell number is solely due to the downregulation of Dll4.

Also, it implies that the mechanism of filopodia formation does not depend on the presence of Dll4. However, when BV are completely absent (*clo* mutants), neuroblasts do not form filopodia at all (Article 2, Fig 3 D). This means that another signal derived from BV -but not Dll4- is responsible for triggering the mechanism of filopodia formation in neuroblasts.

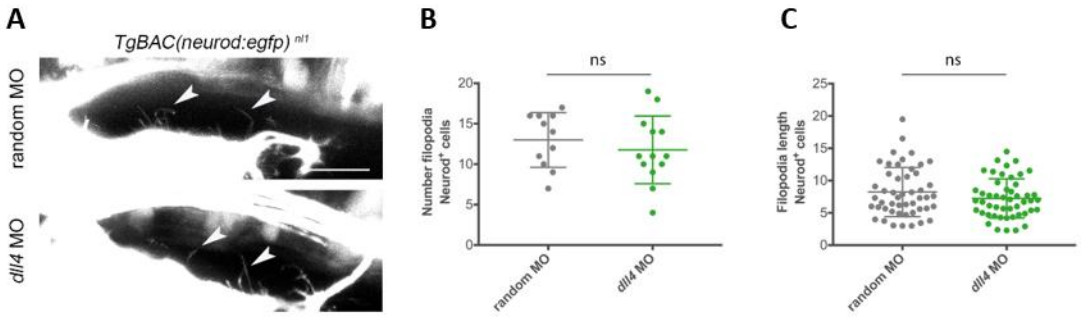

Figure APP 1. Filopodia formation in neuroblasts is not altered in Dll4 morphants.

A. Representative confocal lateral images of the SAG of random- and Dll4-morphants, at 30 hpf. **B.** Graph of neuroblasts filopodia number in random- and Dll4-morphants (n=11 and 13), at 30 hpf. **C.** Graph of neuroblasts filopodia length in random- and Dll4-morphants (n=49), at 30 hpf.

Scale bars, 20 µm. Error bars, mean ± SEM. Unpaired two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, non-significant.

Development of the transgenic line *Tg(UAS:DN Irsp53, IRES:nls-eGFP)*

We generated a new transgenic line with the aim to inhibit filopodia formation in a tissue specific way, through the expression of the dominant-negative (DN) form of the Irsp53 protein.

The Irsp53 (Insulin-receptor substrate p53) is a protein with multiple domains through which it interacts with different proteins and thus develops different activities related to filopodia formation. Through its I-BAR domain it interacts with plasma-membrane lipids inducing membrane deformation (Choi et al., 2005; Henne et al., 2007; Mattila et al., 2007). IRSp53 binds to CDC42, ENA/VASP and WAVE2 proteins via the SH3 domain (Krugmann et al., 2001; Miki and Takenawa, 2002; Scita et al., 2008), and also to EPS8, an actin filament capping protein (Disanza et al., 2006; Funato et al., 2004). The DN form of this protein contains a 4K mutation where four lysines (142, 143, 146 and 147) are altered. This mutation prevents the bundling of actin fibres due to the disruption of the Irsp53 actin-binding sites (Millard et al., 2005).

The construct was built through the Tol2 Gateway system (Kwan et al., 2007). We first amplified the full-sequence of the DN Irsp53 from a plasmid kindly provided by Erez Raz (Meyen et al., 2015), with PRC primers 5'-ATAGGTACCGCTTAGATCCACCATGTCTCG-3' and 5'-ATACTCGAGATCTCACTGTGCAAAGCCTGCCAT-3'. Then, we incorporated the sequence of the DN Irsp53 in a pME-MCS (237), with KpnI and XhoI. Then, the resulting plasmid was subcloned with the p5E-4xrnUAS (Akitake et al., 2011) and p3E-IRES-nlsEGFPpA (391) in a pDestTol2CG2 (395), which contains a cmlc2:egfp transgenesis marker. The final plasmid was co-injected with Tol2 mRNA into 1-cell-stage embryos in the double *TgBAC(neurod:EGFP)*^{nl1}*;(Kdrl:ras-mCherry)*^{s896} background (see Fig APP 2 A and B). A stable transgenic line was established.

In parallel, we grow in our fish facility the *Tg(Flk1:Gal4)*^{bw9}, sent by Julien Bertrand (Kim et al., 2014; Mahony et al., 2016). We expected that the later crossing of these two transgenic lines would prevent the formation of filopodia specifically in ECs, to study their requirement for the correct expansion of SAG neuroblasts (see Fig APP 2 B and C).

Unfortunately, the resulting embryos of the cross between *Tg(Flk1:Gal4)*^{bw9} and *Tg(UAS:DN Irsp53, IRES:nls-eGFP)* showed a very weak expression of the construct (see Fig APP 2 D), as observed by the few number of nuclei expressing eGFP and the presence of filopodia in ECs.

After discussion with Shigetomo Fukuhara, I learned that *Tg(Flk1:Gal4)* ^{bw9} was not a strong promoter of UAS transgenic lines (Wakayama et al., 2015) and asked for a better one, *Tg(fli1:Gal4ff)* ^{ubs3}, to Heinz-Georg Belting (Paatero et al., 2018).

Regrettably, similar results were obtained when *Tg(UAS:DN Irsp53, IRES:nls-eGFP)* was crossed to *Tg(fli1:Gal4ff)* ^{ubs3}, which lead me to think that there must be additional problems with the new transgenic line such as silencing.

As possible solutions the LR reaction can be repeated in a pDestTol2pA2 (394), which contains insulators, to favour the construct expression, and the resulting plasmid will be co-injected in with Tol2 mRNA into 1-cell-stage embryos in the double *TgBAC(neurod:EGFP)* ^{nl1}*; (fli1:Gal4ff)* ^{ubs3}.

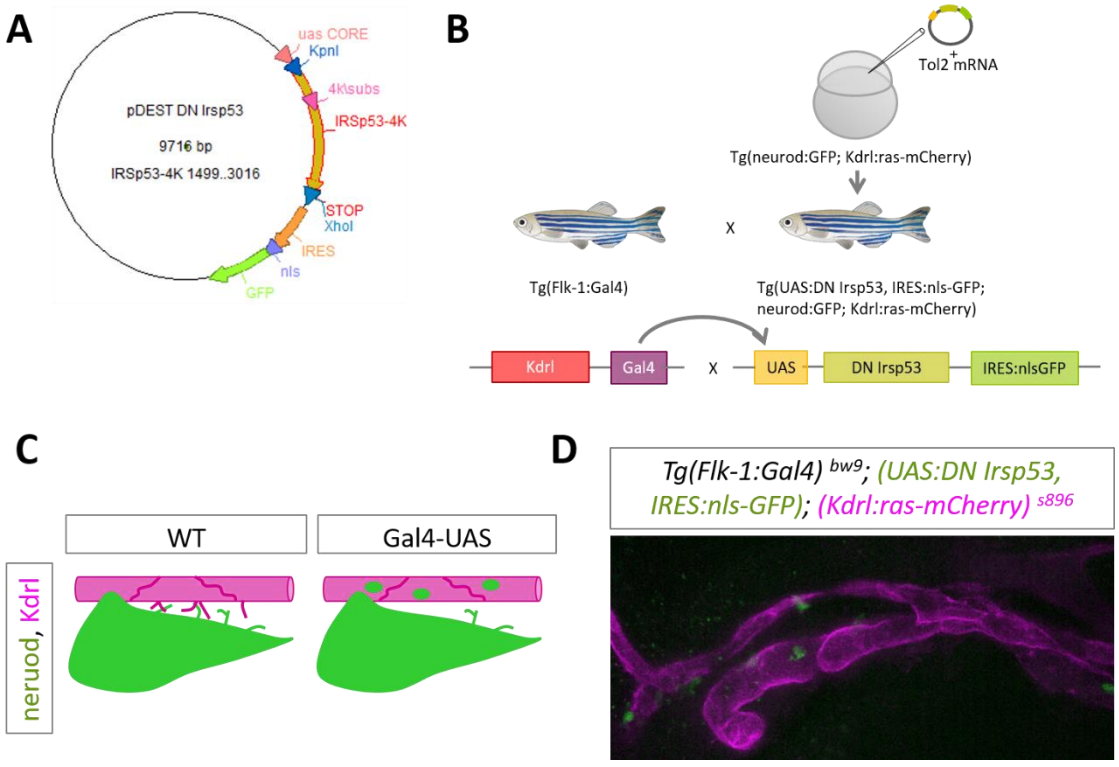

Figure APP 2. Generation of the *Tg(UAS:DN Irsp53, IRES:nls-eGFP)*.

A. Plasmid generated with the Tol2 Gateway system containing the 4xUAS, the IRSp53-4K and the IRES:nls-GFP. **B.** This construct was injected together with Tol2 mRNA in 1-cell stage embryos of the *TgBAC(neurod:gfp)*^{nl1}*; (Kdrl:ras-mCherry)*^{s896} background and grown to adulthood. To be later crossed with *Tg(Flk-1:Gal4)* fish. **C.** Illustration of the expected phenotype. Embryos Gal4^{+} and UAS^{+} express the marker of green nuclei specifically in BV and ECs do not form filopodia. **D.** Lateral confocal image of an embryo resulting of the cross illustrated in B, where some green nuclei can be observed.

Generation of mRNA DN Irsp53

Finally, the injection of DN Irsp53 mRNA could be used as a last solution to produce a genetic alteration of the filopodia formation, to complement the results obtained with the LatB treatment.

The full-length DN Irsp53 cDNA was amplified from the plasmid provided by Erez Raz (Meyen et al., 2015) using the PCR primers 5'-ATAGAATTCGCTTAGATCCACCATGTCTCG-3' and 5'-ATACTCGAGATCTCACTGTGCAAAGCCTGCCAT-3' and subcloned into a pCS2+ with EcoRI and XhoI (see Fig APP 3).

mRNA would transcribed from ApaI or KpnI or NotI -linearized template using SP6 RNA polymerase and the mMessage mMachine kit (Ambion) and injected in *TgBAC(neurod:EGFP)* ^{nl1}*;(Kdrl:ras-mCherry)* ^{s896} embryos to visualize filopodia formation and analyse the phenotype in SAG neuroblasts.

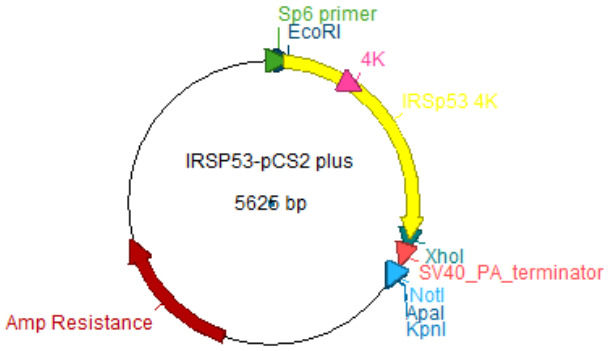

Figure APP 3. Generation of mRNA DN Irsp53.

Illustration of the generated plasmid containing the IRSp53-4K inside the pCS2^{+}.

Generation of mRNA Dll4-eGFP

In other to visualize the location of Dll4 and assess if it is located in EC cytoneme tips, we decided to generate a fusion protein of Dll4 with GFP.

The full-length Dll4 cDNA was first amplified from a plasmid provided by Stefan Schulte-Merker (Hogan et al., 2009), using the PCR primers 5'-ATTTGGTACCACCATGGCAGCTTGGCTCACCTT-3' and 5'-ATTTGGATCCGTACCTCAGTTGCTATGAC-3' to eliminate the STOP codon and

cloned into the pEGFP-N2 vector to obtain a C-terminal eGFP fusion protein, with KpnI and BamHI. Then, it was subcloned into the pCS2+ with KpnI and NotI (Fig APP 4).

mRNA will transcribed from XbaI-linearized template using SP6 RNA polymerase and the mMessage mMachine kit (Ambion) and injected in *TgBAC(neurod:EGFP)* ^{nl1}*;(Kdrl:ras-mCherry)* ^{s896} embryos to visualize Dll4 expression and analyse the phenotype in SAG neuroblasts.

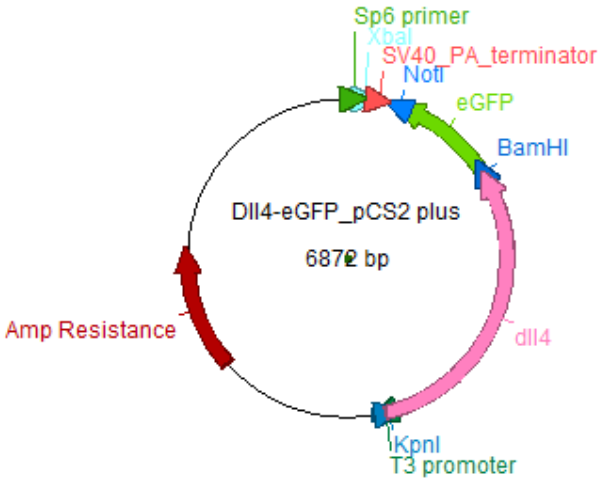

Figure APP 4. Generation of mRNA Dll4-eGFP.

Illustration of the generated plasmid containing the Dll4-eGFP fusion protein inside the pCS2^{+}.

Development of the transgenic line *Tg(UAS:Dll4-eGFP)*

We generated a new transgenic line that, after its cross with *Tg(fli1:Gal4ff)* ^{ubs3} , would allow us to i) visualize the expression Dll4 at a sub-cellular resolution, to ideally see its presence in EC filopodia tips; and ii) produce a gain-of-function EC-specific experiment to then analyse its effect in SAG neuroblasts.

The construct was generated through the Tol2 Gateway system (Kwan et al., 2007) The full-length Dll4 cDNA was amplified from a plasmid provided by Stefan Schulte-Merker (Hogan et al., 2009), using the PCR primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACATGGCAGCTTGGCTCACCTTTCTC-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGTACCTCAGTTGCTATGACACATTCACTC-3' to eliminate the STOP codon and cloned into the pDNOR221 (218) vector, via a BP reaction. Then, the resulting plasmid was subcloned with the p5E-4xrnUAS (Akitake et al., 2011), p3E-EGFPpA (366) in a pDestTol2pA2 (394), which produced a fusion protein of Dll4 with eGFP under the regulation of a UAS promoter (Fig APP 6).

The final plasmid was co-injected with Tol2 mRNA into 1-cell-stage embryos in the double *TgBAC(neurod:EGFP)* ^{nl1}*; (fli1:Gal4ff)* ^{ubs3} background. The resulting embryos are currently growing in the fish facility.

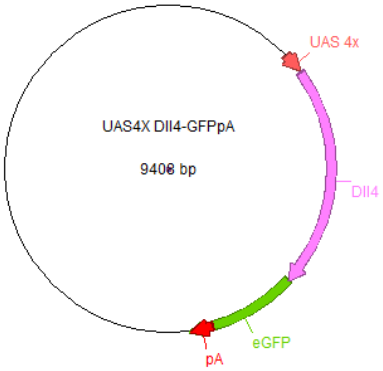

Figure APP 6. Generation of the transgenic line *Tg(UAS:Dll4-eGFP)*.

Illustration of the generated plasmid containing the 4xUAS and the Dll4-eGFP fusion protein.

Development of the transgenic line *Tg(Dll4 KO)* with CRISPR technology

To complement the results obtained by the Dll4 knock-down experiment with MO, we would like to study the effect of a Dll4 knock-out (KO) mutant in the SAG neuroblasts. One *dll4* mutant is already available in the zebrafish community, the *dll4*^{j16e1} (Leslie et al., 2007). This line carries a dominant haplo-insufficient mutation that produces phenotypes similar but less severe than the ones seen in Dll4 morphants (Hasan et al., 2017; Leslie et al., 2007).

With the aim to generate a Dll4 mutation with a stronger phenotype I designed two scRNA to target 2 of the 4 functional domains of Dll4: 5'-AGTATTCCCCCTTCCAGCCA-3' and 5'-TCTGTCGACCTGGCTACACC-3' (Fig APP 7), and co-injected them with trRNA and Cas9 protein in 1-cell-stage embryos in the double *TgBAC(neurod:EGFP)* ^{nl1}*;(Kdrl:ras-mCherry)* ^{s896} background. The resulting CRISPR embryos are currently growing in the fish facility. Some of these embryos were used to obtain genomic DNA

and to assess the efficiency of the scRNA by PCR amplification of the targeted sequences. In 3/5 embryos the scRNA target sequence was altered.

Feature	Direction	Type	Location ↓
MNNL	>>>	misc_feature	66..251
DSL	>>>	misc_feature	444..629
qRNA chop11	>>>	misc_feature	609..628
EGF1 - ch7	>>>	misc_feature	832..944
qRNA chop26	>>>	misc_feature	911..930
EFG2	>>>	misc_feature	1299..1403

```
      *   10    *   20    *   30    *   40    *   50    *   60    *   70    *
   1 ATGGCAGCTTGGCTCACCTTTCTCATCGCATTTTTCACGACCGTTTTGACGCAGATATTTGGATCTGGTGTTTTC
  76 GAACTCGACCTTCACGAATTCAAAAATTTCAAAGGCTTGCTCGCAAATGGCAATTCGTGCACGCCTGACTGCAGG
 151 ACTTTCTTCCGAGTTTGCCTAAAAAACTACCAGGTGGTCGTGTCTCCAGGTGACTGTATCTTTGGAAGTGCCTTA
 226 ACGCCAGTGCTGGGGACAAACTCGTTTAGAATCATGGGAGGTGGCTCTTTCAGCACACCAATTCGACTGCCGTTC
 301 AACTTTGGGTGGCCGGGGTCATTTTCATTGATTATTGAGGCCTGGCACTCACCTTACTCGGATCTACCTGTAGAC
 376 GCATACAACCCTAACATGCAGATAAGCTTTTTTGCCATCCAAAGAAAGTTGGAAGTAGGGGCTGAATGGTCTCAG
 451 GATGTTCAAAGTGGGAAGCAGACAGAGCTAAGGTATTCTTACCGGTTCATCTGCAACGAAAATTACTACGGCGAC
 526 AGTTGTTCCAAAAAATGCACACCCAGGGATGACCGTTTCGGCCACTACACCTGCAACCCAGATGGCCAGTTATCC
 601 TGTCTCCCTGGCTGGAAGGGGGAATACTGCGAAGAACCGATCTGTCTGGAGGGATGCAGTGAGGCGAATGGCAAT
 676 TGCTCCAAACCTGGAGAGTGTGTATGCAGAGAAGGATGGCAGGGTAAATTCTGCACAGAGTGTAAAACATATCCA
 751 GCCTGTAAACACGGCACCTGTCACCTGCCAGGGCAGTGTAACTGTAAAGAGGGCTGGGGTGGACTGTTCTGTGAC
 826 CAAGATTTGAACTTTTGCACACACCATAAACCATGTGTGAATGGAGCCACATGTATGAATACAGGTCAGGGCAGT
 901 TACACCTGCATCTGTCGACCTGGCTACACCGGGGTCAACTGTGAGCTGCAGGTGCGAGAGTGTGACAGCAGCCCA
 976 CGCAAGAATGGAGGTCTTTGCACGGATCATGATAAAAGTTACACATGCACCTGTCTGCCTGACTTTGAGGGAACT
1051 CACTGTGAACACAGTCTGCTCACCTGCGCAGATTCCCCATGCTTTCACAAGGGCAGATGCCACGAGAAGGACAAT
1126 GGACGCAGCTACGCGTGTGAATGTCCTTTAGGCTACACGGGTCTTAACTGCGAGAGAAGGATGGACAAATGCACC
1201 AGTATGCTGTGCGCAAATGATGGCTTGTGTTTGATCCTGGGGGGAAAACGGATATGCAGCTGTCGAGCGGGCTTC
1276 ACTGGCCAGCGCTGTGAGATCAACATTAACGACTGCGCAAACAACCCCTGTGCCAACGGAGGCACGTGCTACGAT
1351 CGCATCAACGAATACGTCTGCTCCTGTCCACCGGGATATAAAGGACGCAACTGCGACCGGCCCTCCTGCTTGTCC
1426 ACACCCTGCCTCAACGGCGGCTCCTGCGTGGGCATCACGGGAAACCCACCAGCCTGCTTCTGCCCCAGTGGCTTC
1501 ACCGGACCCCTCTGTGAATACTACGCCGTCACAGCGCCCGTGACCAGAGGAGAAACTCAAGACCGCTTCCAGTGG
1576 GCTGCTGTTTCTCTGGCCGTTGGCTTAGTGGCTCTTGTGGTCTTACTCTGCATGGTTGTCATTGCCCTCCGCCAC
1651 ATTCACAGGCAGGCATCAGGAGAAAGGACTCGAGGAGAGGCAATGAACAACTTGTCAGAATCACAAAGAGACAAC
1726 TTGATCCCCACCTCCCAGCTGAAGAACACTAATAAACAGGTCAGCCTGGAGGTGGACTGCACACCTGACAAATCC
1801 AATTACATACACAAGAACTGCCACTTGGACTACAATTCATCGAAAGAGTTCAAGGACATCGTGTCCCAAGAGGAC
1876 AAATCAAGTCATAAATACGAAAAGTGTTTAGAAGAGAAAATACCATTGAGTAGAATGTACAGGGAAAAGCCAGAG
1951 TGTAGGATATCAACGATATGTTCCCCAAGGGACTCCGTGTACCAGTCTGTGTTTGTAATAGCAGAGGAGAGGAGT
2026 GAATGTGTCATAGCAACTGAGGTATAA
```

Name	scRNA	Strand	Fw	Rv	Product size
Dll4 scRNA D2	AGTATTCCCCCTTCCAGCCA	-	AAAGAAAGTTGGAAGTAGGGGC	CAAAGCCTCACTCCTCAAGAAT	273
Dll4 scRNA D3	TCTGTCGACCTGGCTACACC	+	TTTTGCACACACCATAAACCAT	CAATTACTCACCGTGCAAAGAC	173

Figure APP 7. Dll4 functional domains and scRNA used to mutate Dll4.

Full sequence of Dll4 and its 4 functional domains: MNNL in blue, DSL in yellow, EGF1 in pale green and EGF2 in pink. The 2 scRNA used to target the sequence of Dll4 with the Cas9 proteins are highlighted in orange (targeting on the functional domain DSL) and in green (targeting the functional domain EGF1). Their strands, FW and RV primers to amplify and sequence the targeted region and its product size are also given.

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