

Nervous vascular parallels: axon guidance and beyond

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ABSTRACT The vascular and nervous systems are organized with well defined and accurate networks, which represent the anatomical structure enabling their functions. In recent years, it has been clearly demonstrated that these two systems share in common several mechanisms and specificities. For instance, the networking properties of the nervous and vascular systems are governed by common cues that in the brain regulate axon connections and in the vasculature remodel the primitive plexus towards the vascular tree. Here, we summarize the role of semaphorins as a paradigmatic example of the role of axon guidance molecules in physiological and pathological angiogenesis. Finally, we discuss the presence in blood vessels of neurexin and neuroligin, two proteins that finely modulate synaptic activity in the brain. This observation is suggestive of an intriguing new class of molecular and functional parallels between neurons and vascular cells.

KEY WORDS: *semaphorin, neurexin, neuroligin*

Introduction

The development of the nervous system involves progressive and regressive actions. Progressive events like neural proliferation, neurite outgrowth, and synapse formation set up a broad pattern of neural connectivity. Later in development, however, regressive events such as cell death, axon pruning, and synapse elimination are necessary to refine the pattern to more precise and mature circuitry (Low and Cheng, 2006). Vascular development displays a number of similar features. The primitive vascular plexus originated from the migrating angioblasts undergoes a profound remodeling leading to the mature vascular trees. These morphogenetic events are characterized by pruning that enables removal of redundant or misguided capillaries and by generation of new vessels programmed to differentiate into veins and arteries (Jain, 2003). The major driving force leading to these vascular rearrangements is oxygen delivery. Therefore arteries, veins and capillaries have specialized anatomical properties that allow the proper blood pressures along the vascular tree and the exchanges of nutrients and catabolites with tissues. Finally, the intercapillary distances in different organs are programmed accordingly to their partial oxygen pressure and the oxygen diffusion coefficient (~150 μm) (Pittman, 1995).

The similarities between vascular and nervous organogenesis appear indeed at different levels. A first and well established example is the role exerted by biogenic amines in neuron communication and vascular tone. More recently, common mechanisms have been

envisaged at the level of cell commitment and differentiation. *In vitro*, cells purified from bone marrow and cord blood can originate neurons and glia (Goolsby *et al.*, 2003; Reali *et al.*, 2006) whereas mouse neural stem cells acquire an endothelial phenotype when co-cultured with human mature endothelial cells (Wurmser *et al.*, 2004). *In vivo*, bone marrow-derived cells enter the brain and differentiate into neural cells not only in a mouse model, but also in patients that underwent bone marrow transplantation (Mezey *et al.*, 2003). Moreover, murine neural stem cells can engraft into the hematopoietic system of irradiated hosts to produce blood (Bjornson *et al.*, 1999) while in the quail-chick chimera model the avian cranial neuroectoderm originates smooth muscle cells (Korn *et al.*, 2002). In addition, embryonic stem cell-derived Neuropilin-1 positive cell population differentiates *in vitro* towards endothelial or neural phenotypes depending upon microenvironmental cues (Gualandris *et al.*, 2009; Noghero *et al.*, 2010).

Finally, in the last 10 years several studies have thoroughly demonstrated that vascular cells and neurons share molecular tools and strategies during their networking (Adams and Alitalo, 2007; Carmeliet, 2003; Eichmann *et al.*, 2005; Serini and Bussolino, 2004). Several and exhaustive reviews have focused the role of Netrin, Slit and Ephrin as axon guidance cues that function during vascular

Abbreviations used in this paper: LNS, laminin; neurexin, sex-hormone-binding protein domain; SEMA, semaphorin; EC, endothelial cells.

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Final, author-corrected PDF published online: 27 July 2011.

assembly (Adams and Alitalo, 2007; Adams and Eichmann, 2010; Carmeliet, 2003; Eichmann et al., 2005; Gelfand et al., 2009; Serini and Bussolino, 2004) and that of angiogenic inducers in neuronal behavior (Greenberg and Jin, 2005; Zacchigna et al., 2008). Here we summarize the more recent aspects of semaphorins (SEMA) activities in vascular remodeling and envisage a new family of common features between neurons and vascular cells based on the role exerted by the synaptic proteins neurexin and neuroligin in the vascular system.

Semaphorins and vascular development

SEMA constitute a wide family of membrane bound and secreted guidance cues originally characterized for their ability to steer axon growth cones and then found to regulate vascular development as well (Ruiz de Almodovar et al., 2009). In vertebrates, seven different secreted class 3 SEMA (SEMA3) exist, each SEMA3 protein being assigned a letter from A to G (Committee, 1999). SEMA3 signal through a holoreceptor complex formed by neuropilin (Nrp)1 or 2 and type A or D plexin, which respectively constitute the ligand binding and signal transducing subunit (Neufeld and Kessler, 2008). The multifaceted SEMA-elicited plexin signaling is a key regulator of cell adhesion and cytoskeletal dynamics in motile cells during neural, vascular, and epithelial morphogenesis (Jackson and Eickholt, 2009; Serini and Bussolino, 2004). Different SEMA3 can preferentially bind either Nrp1, e.g. SEMA3A, or Nrp2, for example SEMA3F, while others, such as SEMA3C, bind both Nrp1 and Nrp2 (Neufeld and Kessler, 2008). Moreover, distinct SEMA3 employ different type A or type D plexins to transduce their signals; for example, in neurons SEMA3A and SEMA3F principally use plexinA4 and A3 respectively (Yaron et al., 2005). PlexinD1 is the major endothelial plexin and among the different SEMA3, SEMA3E is a notable exception, since it can bind with high affinity plexinD1 (Gu et al., 2005). However, plexinD1 can also combine with either Nrp1 or Nrp2 to give rise to SEMA3 holoreceptor complexes endowed with a higher affinity than the corresponding plexinA-containing holoreceptors (Gitler et al., 2004). Furthermore, recent mutational studies (Merte et al., 2010; Nogi et al., 2010) suggest that, similarly to SEMA3E, the collapsing activity of Nrp1-bound SEMA3A also relies on its ability to interact, albeit at lower affinity, with plexins.

After a decade of studies, we are just starting to understand the mechanisms by which SEMA3 signaling can control vascular development. Indeed, this issue is complicated by the species-specific usage of different SEMA3 and by the high degree of redundancy and compensation. Zebrafish *sema3a1* or *sema3a2* or *plxnd1* morphants display a qualitatively similar aberrant and unrestrained morphogenesis of intersegmental blood vessels, suggesting that SEMA3A via plexinD1 can exert an inhibitory effect on endothelial cell migration during vascular development (Torres-Vazquez et al., 2004). On the contrary, in *sema3e* morphants the sprouting of intersomitic blood vessels is severely delayed; indeed, autocrine/paracrine Sema3e promotes endothelial cell (EC) motility likely through plexinB2 receptor (Lamont et al., 2009).

In mice, the regulation of vascular development by SEMA3 signaling appears to be different and more complex than in Zebrafish. Murine *Sema3a* and *Sema3f* mRNAs are enriched in endothelial tip cells of sprouting blood vessels (Strasser et al., 2010) and knockdown of *Sema3a* gene resulted in a defective embryonic vascular remodeling of head and trunk blood vessels in the outbred

CD-1 strain (Serini et al., 2003). Ostensibly because of genetic heterogeneity due to different maintenance this was not observed in another colony of *Sema3a* null CD-1 mice (Vieira et al., 2007). However, in further support for a role of SEMA3A in vascular remodeling, it has been recently reported that *Sema3a* deletion in an inbred strain results in defective renal vascular patterning (Reidy et al., 2009). Notably, in the same *Sema3a* null mice both *Sema3b* and *Sema3e* mRNA were found to be up-regulated (Reidy et al., 2009), thus providing a molecular mechanism by which compensation can occur in *Sema3a* null mice (Vieira et al., 2007). Indeed, both *Sema3e* and *Plxnd1* null embryos have defects in intersomitic vessel patterning (Gitler et al., 2004; Gu et al., 2005). Nevertheless, while all *Plxnd1*^{-/-} pups die shortly after birth because of life-threatening cardiovascular defects (Gitler et al., 2004; Gu et al., 2005), *Sema3e* null mice are viable and healthy (Bellon et al., 2010; Chauvet et al., 2007; Gu et al., 2005); suggesting that in ECs plexinD1 might convey signals not only from SEMA3E, but also from other Nrp-binding SEMA3, such as SEMA3A. Accordingly, plexinD1 is required for SEMA3A-mediated EC migration *in vitro* (Zhang et al., 2009) and both *Sema3a* (Behar et al., 1996) and *Plxnd1* (Kanda et al., 2007) null embryos display similar defects in axial skeletal morphogenesis, such as rib fusion and vertebral split. Recently, a *Tie2Cre*-mediated gene inactivation approach showed endothelial knock down of *Plxnd1* in mice causes skeletal malformations that are associated to a severe reduction of the bone marrow microvasculature (Zhang et al., 2009), strongly supporting the hypothesis that skeletal defects of *Plxnd1* null mice are most likely secondary to vascular abnormalities. Since *Sema3a* gene deletion causes similar abnormalities in the axial skeleton (Behar et al., 1996), it will be worth studying the bone vasculature of *Sema3a*^{-/-} mouse embryos as well.

A knock-in mouse strain harboring mutations that altered the SEMA3 binding ability of Nrp1 was generated (*Nrp1Sema-*) and reported to display neural, but not vascular patterning defects (Gu et al., 2003). However, a careful analysis of some peripheral nerve projections, such as those of the ophthalmic nerve, shows that the phenotype of *Nrp1Sema-* knock-in mice (Gu et al., 2003) is significantly less severe than that of both *Sema3a* (Taniguchi et al., 1997) and *Nrp1* (Gu et al., 2003; Kitsukawa et al., 1997) knockout mice. The latter observation, together with the fact that plexinD1, i.e. the main endothelial plexin, is significantly more efficient than type A plexins in increasing the affinity of Nrps for SEMA3 (Gitler et al., 2004), raises the possibility that the lack of vascular defects in *Nrp1Sema-* knockin mice could be due to the persistence of responsiveness of ECs to SEMA3, albeit reduced if compared to that of wild type Nrp1. It will be therefore important to test the ability of *Nrp1Sema-* ECs to respond to Nrp1-binding SEMA3 *in vitro*.

Semaphorins and tumor angiogenesis

The capacity of semaphorins to regulate vessel patterning is particularly relevant to cancer progression and in general to the altered vascularization occurring, for example, in chronic inflammation. SEMA3 emerge as promising targets for cancer therapy. Indeed, molecules belonging to this class of chemorepulsive agents can display a direct effect of both on tumor cells; e.g. SEMA3F inhibits the growth of a wide range of xenograft cancer models (Neufeld and Kessler, 2008; Potiron et al., 2007), and on ECs, e.g. SEMA3A and SEMA3F repel ECs and inhibit their migration promoted by

angiogenic inducers (Bielenberg *et al.*, 2004; Futamura *et al.*, 2007; Kessler *et al.*, 2004; Serini *et al.*, 2003). Several SEMA3, including SEMA3A and SEMA3F, are expressed by ECs, suggesting that they may function in an autocrine fashion. This autocrine production of chemorepulsive SEMA3 molecules is balanced by the autocrine production of angiogenic inducers, with the final results of a proper vessel navigation and shape (Serini *et al.*, 2008). An imbalance in the ratio of autocrine VEGF-A/SEMA3 in ECs might happen during tumor progression and contribute to the structural and functional defects observed in tumor blood vessels (Jain, 2008). By studying two mouse transgenic models of carcinogenesis, namely Rip-Tag insulinoma model and K14-HPV16/E2 uterine cervix carcinoma as well as patients affected by aggressive multiple myelomas (Maione *et al.*, 2009; Vacca *et al.*, 2006), we observed that such a homeostatic balance can be disrupted due to a dramatic drop in the production of SEMA3 by tumor ECs. Importantly, we found that the reintroduction of SEMA3A caused marked endothelial apoptosis, significant reduction in vessels density and branching, enhancement of pericyte coverage and reduction of tumor tissue hypoxia (Maione *et al.*, 2009). Of note, the normalizing activity exerted by SEMA3A on tumor blood vessels is similar, but apparently longer lasting than that observed upon VEGF-A inhibition (Jain, 2005). Interestingly, another member of SEMA class 3, SEMA3E, inhibits tumor angiogenesis and the growth of primary tumor, but it favors metastasis spreading (Casazza *et al.*, 2010). In addition, SEMAs other than SEMA3 has been found to modulate tumor angiogenesis. For instance, SEMA4D behaves as a pro-angiogenic factor (Basile *et al.*, 2005; Basile *et al.*, 2007; Conrotto *et al.*, 2005; Sierra *et al.*, 2008) which is also involved in tumor progression. In particular, it has been reported that tumor growth is significantly impaired in mice lacking Sema4D (Sierra *et al.*, 2008); tumor associated macrophages constitutes a major source of Sema4D that is required for proper vessel maturation in tumors.

Neurexin and neuroligin: synaptic proteins tackle vasculature

It was recently discovered (Bottos *et al.*, 2009) that various isoforms of the essential synaptic proteins neurexins and neuroligins are expressed by cells of the vascular wall (endothelial and smooth muscle cells). In this context, in analogy to the brain, they are modified by alternative splicing and reciprocally interact. Moreover, an antibody against a specific isoform of neurexin (anti- β NRXN) inhibits vascular remodeling/angiogenesis, whereas the overexpression of neuroligin in ECs placed in a tumorigenic environment induces vessel growth in the chicken chorioallantoic membrane. As an insight into its mechanism of action it was demonstrated that anti- β NRXN modulates vascular tone of isolated arteries by reducing vessel tension induced by noradrenalin.

Neurexins and neuroligins are transmembrane proteins that are codified respectively by three and five genes in humans (Bolliger *et al.*, 2001; Tabuchi and Sudhof, 2002). Both gene families present a high degree of evolutionary conservation that can be explained by the action of a positive selective pressure which in turn suggests a conservation of their functions across vertebrate evolution (Rissone *et al.*, 2007; Rissone *et al.*, 2010)

Neurexins, which are produced as a long (α) and a short (β) forms by each of their genes, have been widely studied because of their exceptionally extended alternative splicing (Rissone *et al.*, 2007; Tabuchi and Sudhof, 2002) and have been indirectly localized at the pre-synaptic membrane (Sudhof, 2001). Thousands of different isoforms were predicted to be produced by the three mammalian neurexin genes, so that they were proposed as molecular codes for the specific reciprocal neuronal recognition (Missler and Sudhof, 1998). Neuroligins are less subjected to alternative splicing, are localized at the post-synaptic membrane (Song *et al.*, 1999) and interact with neurexins from the opposite

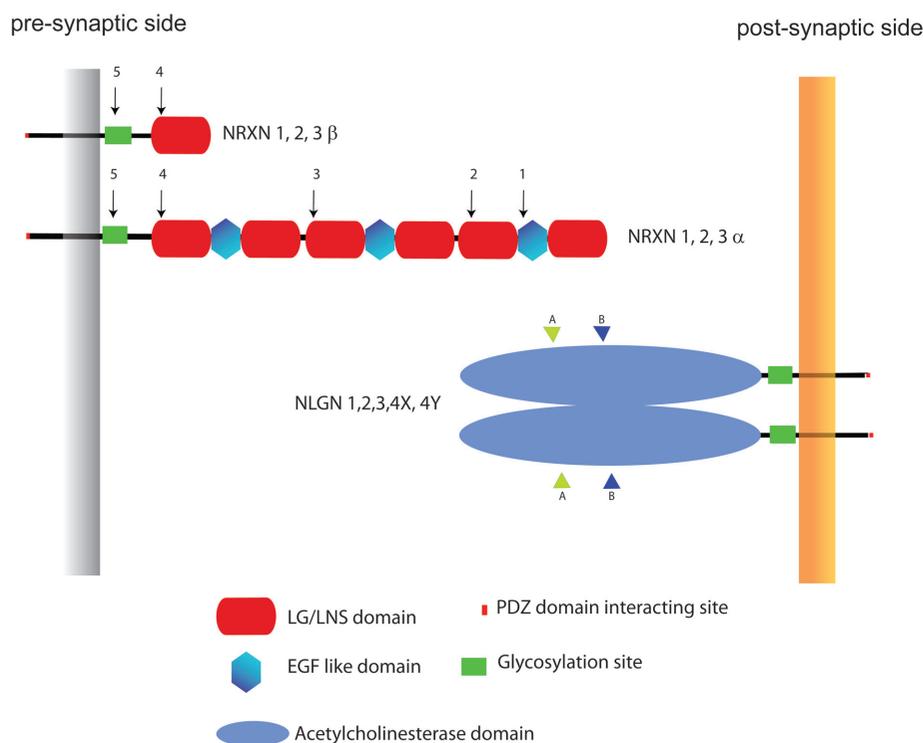


Fig. 1. Domain structures and synaptic arrangement of neurexin and neuroligin. There are three neurexin genes in mammals, each of which has both an upstream promoter that is used to generate the larger α -neurexins and a downstream promoter that is used to generate the β -neurexins. Alternative splicing at five sites (arrows 1 to 5) and N- and O-glycosylation contribute additional diversity. β -Neurexins contain a single LNS domain (laminin, neurexin, sex-hormone-binding protein domain; also known as a Laminin G, LG, domain), whereas α -neurexins contain six LNS domains organized into modules with three EGF-like domains. There are five neuroligin genes in humans: NLGN1, NLGN2, NLGN3, NLGN4 and NLGN4Y. The major extracellular domain of neuroligins, which contains two potential splicing sites (A and B), is homologous to acetylcholinesterase but lacks cholinesterase activity and mediates binding to neurexins. Cytosolic tail of both neurexin and neuroligin contains a PDZ (Post synaptic density protein (PSD95), Disc large tumor suppressor, DlgA and Zonula occludens-1 protein, zo-1, domain) binding site. Neuroligin is presented as a dimer, which represents its normal functional conformation.

side (*in trans*) of the synaptic cleft in a calcium-dependent manner (Nguyen and Sudhof, 1997) (Fig. 1).

Both neurexins and neuroligins display a strong and selective synapse formation-promoting activity *in vitro* (Graf et al., 2004; Scheiffele et al., 2000). Nonetheless, knocking down the expression of the three α forms of neurexins (Missler, 2003) or neuroligins 1-3 (Varoqueaux, 2006) in mouse shows that these proteins have fundamental functions that reside in the modulation of synaptic transmission more than in the early adhesive steps of synapse formation. The same studies reveal a redundancy of function between isoforms of the same gene family. Altogether, neurexin and neuroligin are part of a large set of Synaptic Cell Adhesion Molecules (SCAMs) whose elimination in mice surprisingly results in an overall maintenance of synaptic structure (Piechotta et al., 2006), while affecting their functions.

Albeit with a number of open questions, both neurexin and neuroligin have been involved in the pathogenesis of autism spectrum disorders (ASDs) (Sudhof, 2008). Neuroligins were the first to be implicated in this disease when it was found that the X linked genes NLGL4 and NLGN3 contained point mutations in affected individuals (Jamain, 2003). The role of neurexins in autism susceptibility was widely reported in the following years (Feng, 2006; Kim, 2008; Szatmari et al., 2007). Finally, SHANK3, a downstream intracellular scaffolding protein that binds indirectly to neuroligin through PSD95 and GKAP (Sheng and Hoogenraad, 2007) has been involved in ASDs (Durand, 2007; Marshall, 2008; Wilson, 2003).

Why synaptic proteins on blood vessels?

Although very appealing, the finding of neurexin and neuroligin in blood vessels poses important conceptual challenges, mostly because synaptic activity appears distant from the vascular functions. As a matter of fact, unlike many of the proteins that mediate axon or vessel guidance, the neuronal role of neurexin and neuroligin cannot easily be linked to any of the most characterized cellular events of angiogenesis (proliferation, adhesion, or migration).

Which are the possible roles of these two synaptic proteins

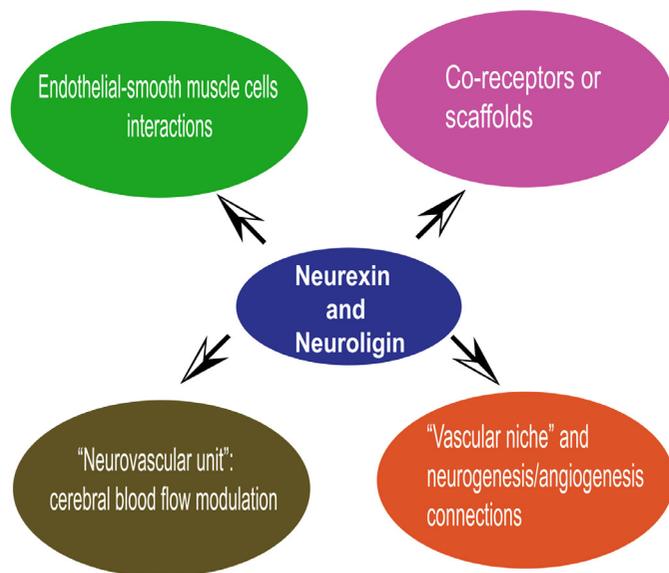


Fig. 2. Working hypotheses regarding the role of neurexins and neuroligins in the vascular systems.

in the vascular context (Fig. 2)? The neurexin/neuroligin protein families are very complex and achieving a gratifying answer to this question will take time and different approaches. However, the data can be looked at from two perspectives.

The first is the role that neurexin and neuroligin can have during the growth and remodeling of the vascular system itself. In this context it should be noted that, although β neurexin null mice are not yet available, no vascular defects have been described for mice carrying null mutations within the three α neurexin (Missler, 2003) or the three neuroligins (Varoqueaux, 2006) genes. Therefore, though dedicated studies are needed, to date, the most direct approaches aimed at illustrating the functions of these proteins have not been informative. The following working hypotheses and reflections can be introduced: i) in analogy to the synaptic environment (Piechotta et al., 2006), a redundant collection of adhesive proteins (that never perform pure “adhesive” functions) including neurexins and neuroligins, is at work to finely regulate blood vessels functions; ii) neurexins and neuroligins, which are crucially found in association in both nervous and vascular systems, could also work separately, because of the much higher amount of neuroligin that is expressed in blood vessels and the fact that neuroligin coprecipitates specifically with β neurexins and not with α neurexins (Bottos et al., 2009), that can remain “orphans”; iii) these proteins, as other neuronal cues (Neufeld and Kessler, 2008), could exploit the molecular machinery of key regulators of vascular physiology (e.g. members of the VEGF/KDR and Angiopoietin/Tie-2 families) to achieve their specific functions, by acting as coreceptors or scaffold proteins; iv) similarly, vascular proteins that are identical or have homologous counterparts to the known nervous protein partners of neurexins and neuroligins (for example dystroglycan (Sugita, 2001), ion channels (Missler, 2003) or intracellular PDZ domain rich proteins) are good candidates for molecular and functional studies. Another possibility stems from the observation that during vascular development both endothelial and vascular smooth muscle cells express neurexin and neuroligin (Fig. 3). In mature vessels, however, neurexin and neuroligin tend to segregate respectively in smooth muscle cells and in endothelium (Bottos et al., 2009), suggesting that they play a role in the formation of vascular layers.

The second point of view relates to the possibility that neurexins and neuroligins mediate part of the physical cross talk between neurons and blood vessels. These events could take place in very different settings and timings. One is the recognition/adhesion between the axon terminals (varicosities) and the target region of the blood vessels that takes place in the final phase of the autonomic innervation of the vascular system (Glebova and Ginty, 2005). These cell-to-cell adhesive events would be temporary, since, normally, the varicosities are located at a certain distance from the smooth muscle layer (Burnstock, 2008). An additional fascinating possibility is that neurexin and neuroligin mediate the interactions between the nervous and vascular system during angiogenesis/synaptogenesis/neurogenesis phenomena that take place in the cerebellum and hippocampus upon repetitive physical activities and/or motor skill learning (Isaacs et al., 1992; Van der Borght et al., 2009). Neurexin-neuroligin mediated cross talk between blood vessels and neuronal cells could be at play also in the so called “vascular niche” of the subgranular zone (SGZ) of the hippocampus where neuronal precursors are in intimate contact with the blood vessels (reviewed in (Doetsch, 2003)). As a final point, the widespread expression of neurexin and neuroligin in the vessels

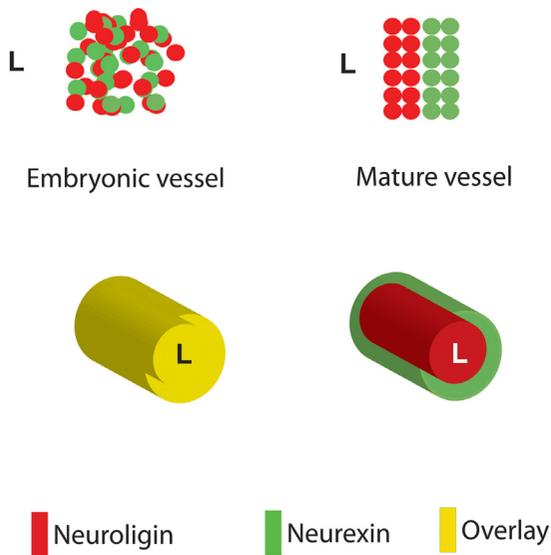


Fig. 3. Expression of neurexin and neuroligin during blood vessel assembly (for details see text). *L* stands for Luminal side of blood vessel.

of the brain parenchyma (Bottos *et al.*, 2009), indicates that they could mediate a pervasive interaction between the vasculature and the surrounding neural tissue. They could indeed be part of the so called “neurovascular unit” that matches the cerebral blood flow to the metabolic demands of neurons (Drake and Iadecola, 2007). In this last circumstance, neurexin and neuroligin would have a double relations with autism spectrum disorders. In fact, cerebral temporal regions that are implicated in social perception, language, and theory-of-mind (abilities that are impaired in autism) can undergo hypoperfusion in autistic children (Ohnishi *et al.*, 2000; Zilbovicius *et al.*, 2000). In the same way, significant negative correlation has been observed between rCBF (Regional Cerebral Blood Flow) in the left superior temporal gyrus and the diagnostic score for autism ADI-R. The more severe the autistic syndrome, the more rCBF is low in this region, suggesting that left superior temporal hypoperfusion is related to autistic behavior severity (Gendry Meresse *et al.*, 2005). These last observations are particularly intriguing when related to the involvement of β neurexin in vessel tone maintenance (Bottos *et al.*, 2009).

In conclusion, can we predict a general significance of the synaptic/vascular parallels? Obviously, the capacity to form billions of specific and plastic cell-to-cell contacts through the synapses represents the most fascinating anatomical and biochemical features of the nervous system, and the enormous heterogeneity of neurexins and neuroligins matches this perspective perfectly. While the task of forming and operating such a network is enormously complex, blood vessels do not appear to have the same needs. However, as in many other instances, we should consider that nature saves its resources by using the same molecules or molecular arrangements with different purposes, while complex and specific functions can be built upon molecular “mixing”. For example, it is an established fact that the proteins positioned at the proximal intracellular side of tight junctions (typical of endothelial and epithelial linings) and those at synapses display a similar domain organization and build up comparable scaffolding structures (Bottos *et al.*, 2009; Butz *et al.*, 1998; Dejana, 2004).

Alternatively, we should not exclude that the vascular system

embraces processes unknown at this time or underestimated in their complexity. As a matter of fact blood vessels are highly heterogeneous (Aird, 2006) and perform a multitude of tasks, some of which require fine cell-to-cell recognition events (e.g. leukocyte extravasation (Vestweber, 2002)). In conclusion, it is very likely that the discovery of neurexin and neuroligin in the vascular system will fuel new concepts into the field.

Acknowledgment

This work was supported by grant of Associazione Italiana per la Ricerca sul Cancro (IG #10133) (to F.B.), (IG # 9211) (to G.S.); Telethon Italy (GGP04127 and GGP09175) (to G.S.); Compagnia di San Paolo - Neuroscience Program Multicentre Projects (to G.S.); Fondazione Piemonte per la Ricerca sul Cancro - ONLUS – Intramural Grant 2010 (to G.S.); Ministero della Salute - Programma Ricerca Oncologica 2006 and Ricerca Finalizzata 2006 (to G.S. and F.B.), Associazione Augusto per la Vita (G.S.); Regione Piemonte (Finalized Health Research 2006, 2008 and 2009; Industrial Research and Precompetitive Development 2006: grants PRESTO and SPLASERBA; Technological Platforms for Biotechnology: grant DRUIDI; Converging Technologies: grant PHOENICS; Industrial Research 2009: grant BANP) (to F.B. and G.S.); CRT Foundation (to F.B.).

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