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Netrin Ligands and Receptors: Lessons from neurons to the endothelium

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Abstract

Netrins were initially identified as secreted ligands regulating axon guidance and migration through interaction with canonical receptors.

Netrins were then shown necessary for development of a range of tissues, including lung, mammary gland and the vasculature.

While new netrin receptors, as well as alternative ligands for classical netrin receptors, were described in the neuronal and epithelial fields, there was a singular focus on canonical netrin receptors in the vascular system, leading to controversy on netrin function and the nature of receptor-mediated netrin signaling in the endothelium.

Herein, we summarize the current state of knowledge on netrin ligands and receptors and discuss questions, controversies and perspectives surrounding netrin functions and receptor identity in the vasculature.

Keywords

netrin; netrin receptor; endothelium; integrin

Over the past 30 years, loss- and gain-of function experiments of netrin ligands and receptors proved their importance as axon guidance cues and as critical players in embryonic physiology and adult pathology (Corset et al. 2000, Srinivasan et al. 2003, Yebra et al.

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Conflict-of-interest disclosure.

The authors are or were previously employed by the University of Utah, which has filed intellectual property surrounding the therapeutic uses of vascular guidance cues and with the intent to license this body of intellectual property for commercialization.

2003,Liu et al. 2004,Lu et al. 2004,Ly et al. 2005,Wilson et al. 2006,Navankasattusas et al. 2008,Castets et al. 2009,Larrieu-Lahargue et al. 2010,Primo et al. 2010,Larrieu-Lahargue et al. 2011,Lambert et al. 2012). However, contradictory data has led to confusion regarding their function during vascular development and the identity of their endothelial receptors (Lu et al. 2004,Wilson et al. 2006,Navankasattusas et al. 2008,Castets et al. 2009,Lambert et al. 2012). We herein review the current knowledge of netrins and their receptors, discuss their implication in the vascular field, and suggest potential new netrin ligands and receptors.

1. Multiple Receptors for Netrins

Accurate patterning of the nervous system requires the correct guidance of axonal growth cones. Among all the neural guidance cues attracting or repulsing axons through the direct activation of neuronal cell surface receptors, netrin-1 was the first soluble factor identified based on its ability to induce commissural cone growth (Kennedy et al. 1994,Serafini et al. 1994).

In vertebrates, three secreted netrins, netrin-1, -3 and -4, and two glycosylphosphatidylinositol (GPI)-anchored membrane proteins, netrin G1 and G2, have been reported (Figure 1). They are laminin-related proteins with a N-terminal laminin VI domain, a central laminin V domain and a C-terminal domain (C or netrin-like domain (NTR)) (Cirulli et al. 2007). Netrin-1's axonal functions have been linked to two classes of receptors, the Deleted in Colon Cancer (DCC) family, including DCC and neogenin, and the Unc5s (Unc5A to D, Figure 1). DCC and neogenin are implicated in netrin-1-induced axon chemoattraction and repulsion (Cirulli et al. 2007), while all four Unc5 family members have been shown to mediate netrin-1-induced axon chemorepulsion in the absence or the presence of DCC receptors (Cirulli et al. 2007).

Because the nervous and vascular systems share similarities at the anatomical and cellular level (Tam et al. 2010), it was asked if neural guidance cues, such as netrins, pattern vasculature growth (angiogenesis), by direct interaction with endothelial cell surface receptors. Even though an abundant literature fairly demonstrated their importance, the precise angiogenic roles of netrins and netrin canonical receptors, DCC and Unc5s family, remains unclear (Lu et al. 2004,Wilson et al. 2006,Navankasattusas et al. 2008,Castets et al. 2009,Lambert et al. 2012).

Controversy emerged with the generation in mice of a deleted allele for Unc5B, the unique netrin-1 canonical receptor detected in murine endothelial cells (Lu et al. 2004). Lu et al. reported that embryos homozygous for the unc5B mutation developed normally until E12.5, in CD1 genetic background, and died from heart failure (Lu et al. 2004). An increased branching in the internal carotid artery, intersomitic and hindbrain vessels were also observed in E10.5 and E12 mutant embryos. Conversely, Navankasattusas and colleagues reported that the deletion of the unc5b locus, was also embryonic lethal, between E12 and E13.5 (Navankasattusas et al. 2008). A decrease in placental arteriole density, leading to a defective umbilical blood flow and an increased embryonic hypoxia, was the only vascular impairment observed in E12 Unc5b-deleted homozygote embryos (Navankasattusas et al. 2008). The opposing phenotypes of Unc5B-deficient mice might be partially explained by different gene deletion strategies and mouse genetic background (Lu et al. 2004,Navankasattusas et al. 2008) as well as a mechanism recently described by Mehlen's group, Unc5B functioning as a "dependence" receptor (Castets et al. 2009). In the absence of netrin-1, the receptor induces an intracellular signal promoting apoptosis, while, in the presence of ligand, the same receptors stimulate cell survival, proliferation, and migration (Castets et al. 2009).

The opposing vascular phenotypes of Unc5B-deficient mice were further accentuated by conflicting netrins-mediated *in vitro* endothelial functions. We and others reported that netrins induced human umbilical vein, artery and dermal microcapillary endothelial cell proliferation, migration, tube formation and survival (Wilson et al. 2006, Lambert et al. 2012), independent of canonical netrin receptor expression; Lu et al. demonstrated that netrins inhibited artery, but not vein, endothelial cell migration and filopodia extension in an Unc5B-dependent manner (Lu et al. 2004). Ligand concentration and subsequent receptor activation might explain part of the controversy observed *in vitro*. We and others reported that netrins-induced effects were biphasic, following a bell-shaped curve, with both induction and inhibition of biological functions at low and high doses of recombinant netrins, respectively (Wilson et al. 2006, Castets et al. 2009, Larrieu-Lahargue et al. 2010, Lambert et al. 2012). Sequential activation of high and low affinity receptors might be responsible of this biphasic response to netrins doses, as demonstrated by Yang et al (Yang et al. 2007).

We have recently shown that netrin-4 binds with high affinity both lymphatic endothelial $\alpha 6 \beta 1$ integrin and laminin-1, leading to activation of $\alpha 6 \beta 1$ and integrin-dependent downstream effectors, and subsequent formation of focal adhesions. Netrin-4,-stimulated endothelial cell adhesion, migration and integrin dependent signaling cascades were inhibited with $\alpha 6$ or $\beta 1$ function-blocking antibodies or siRNAs, while Unc5B or neogenin siRNA had no effect (Larrieu-Lahargue et al. 2010, Larrieu-Lahargue et al. 2011). Finally, we reported that netrin-4 and endothelial $\alpha 6 \beta 1$ integrin colocalize in specific vascular beds. The *in vivo* relevance of such netrin/integrin interaction thus deserves serious investigation. Interestingly, no major vascular defect was detected in ocular vasculature of netrin-4 knockout mice (Li et al. 2012), indicating either a compensatory effect from other netrin/growth factor or a restriction of its role to pathological adult angiogenesis. There is strong evidence for a role of $\alpha 6 \beta 1$ integrin in pathological angiogenesis, though again, the data is contradictory. Short hairpin RNAs or function-blocking antibodies against $\alpha 6 \beta 1$ integrin abrogated tumor angiogenesis in both breast cancer MDA-MB231 and pancreatic RIP-Tag2 tumor models (Lee et al. 2006, Primo et al. 2010), while tumor vasculature was increased in B16/F0 melanoma and LL-2 lung carcinoma engrafted in mice deleted for $\alpha 6$ integrin in the endothelium (Germain et al. 2010). It is not known if these differences are due to differences in tumor type or to target gene silencing in non-endothelial cells. The observation that $\alpha 6 \beta 1$ blockage modulates angiogenic growth factors, such as VEGF-A, VEGF-C and FGF-2 (Lee et al. 2006, Germain et al. 2010, Primo et al. 2010, Larrieu-Lahargue et al. 2011) suggests additional experimentation is merited. Therefore, a combination of tissue specific netrin overexpression (Larrieu-Lahargue et al. 2010), with an endothelial-specific integrin-deletion might indeed prove informative.

Together these data emphasize that the relationship between netrins and their receptors, in the endothelium, is more complex than the simple idea of a single ligand signaling through the unique endothelium receptor, Unc5B. Identification of new binding partners as well as new receptors for netrin receptors and ligands, have reinforced this hypothesis.

2. New “Non-canonical” Ligands for canonical Netrin-1 Receptors

Genetic evidence indicates clearly that endothelial ligands of netrin-1 canonical receptors remain to be identified. Beyond the controversy, it is clear that inactivation of murine Unc5B induces a major vascular defect (Lu et al. 2004, Navankasattusas et al. 2008), while none has been reported in either netrin-1 or netrin-4 *knockout* mice (Serafini et al. 1996, Park et al. 2004, Li et al. 2012). Similarly, netrins-deficient mice are viable or die postnatally (Serafini et al. 1996, Park et al. 2004, Li et al. 2012), while Unc5B deletion causes mouse embryonic lethality (Lu et al. 2004, Navankasattusas et al. 2008). Finally, netrins-induced *in*

vitro functions were retained in Unc5B siRNA-transfected endothelial cells (Wilson et al. 2006, Larrieu-Lahargue et al. 2010, Lambert et al. 2012).

Recent reports have shown that endothelial Unc5B interacts with ligands other than netrin-1. Robo receptors (Robo1-4) and slit ligands (slit1-3) were initially identified as negative regulators of commissural axon crossing (Kidd et al. 1999, Nguyen Ba-Charvet et al. 1999). Even though Robo4 expression was reported specifically in the vasculature, mice lacking this gene are viable and do not display any vascular phenotype mice (Jones et al. 2008). More recently, investigators have demonstrated that in retinal and choroidal vascular pathologies, slit2-inhibited vascular permeability and neovascularization were abrogated in Robo4 deficient mice (Jones et al. 2008, London et al. 2010). However, Robo4 and slit2 binding is controversial (Park et al. 2003, Suchting et al. 2005, Koch et al. 2011). One recent finding suggested that VEGF-A-induced permeability and corneal neovascularization were inhibited by Unc5B/Robo4 direct trans-interaction (Koch et al. 2011). The ligand, Robo4 extracellular domain was sufficient to activate the endothelial receptor Unc5B, demonstrating that canonical netrin receptors were able to bind non canonical ligands, thus, adding another intricacy level to our understanding of netrin signaling. It is intriguing that Robo ligands, slits, and netrins combined activity has been already reported in axonal wiring (Bielle et al. 2011). Relevance of such Unc5B/Robo4 interaction, in the presence of their canonical ligands netrin-1 or slit2, remains to be investigated to clarify ligand/receptor spatial and chronological interaction, in the endothelium, as well as in the nervous system, as all these axon guidance cues might also directly interfere with the nervous system development, which might subsequently block endothelial growth.

3. Netrins effects outside the endothelium

Development of the overlapping vascular and nervous network might be completed either by their simultaneous growth or by neurons and endothelial cells guiding each other subsequently. Therefore, direct or indirect effects of axon guidance cues, on the endothelium, must be investigated thoroughly. Netrins are known to be secreted and act on various cell types (Kennedy et al. 1994, Park et al. 2004, Ly et al. 2005, Lim et al. 2011, van Gils et al. 2012).

For example, Lim et al. showed both netrin-1a and DCC deficient morpholinos induced vascular deficiencies in zebrafish even though their expression was not detected in the endothelium (Lim et al. 2011). Netrin-1a-expressed muscle pioneer cells attract DCC-positive rostral primary motoneuron (RoP) axons along the horizontal myoseptum, adjacent to the future trajectory of the parachordal endothelial chain, which secreted in turn, an unidentified factor attracting EC from the posterior cardinal vein. Thus, silencing of either netrin-1a or DCC abrogated PAC formation while netrin-1-expressing muscle pioneer cell transplant and DCC overexpression was sufficient to rescue this vascular defect phenotype.

Park et al and others reported that netrin-1 stimulated *in vitro* smooth muscle cell migration in a neogenin dependent manner (Park et al. 2004). The *in vivo* relevance of such ligand/receptor signaling has been demonstrated in an atherosclerotic context. Van Gils et al. have reported that atherosclerotic plaques-infiltrating macrophages expressed netrin-1, and its specific deletion in myeloid lineage resulted in a decrease in alpha-smooth muscle actin-positive cells in atherosclerotic lesions (van Gils et al. 2012).

Additionally, the pro-inflammatory state modifies vascular integrity (Chavez et al. 2011). Endothelium-secreted netrin-1 has been shown to be an inflammation suppressor by inhibiting leukocyte infiltration in an Unc5B dependent process (Ly et al. 2005) while Van Gils and colleagues have demonstrated that atherosclerotic plaques-infiltrating macrophages

were retained in the inflammation sites, through their autocrine netrin-1/Unc5b signaling (van Gils et al. 2012). Additional work will be required to investigate the indirect role of netrin-1/neogenin on vascular maturation as well as netrin-1/Unc5B inflammation in vascular integrity.

Together, these data highlight the need for careful investigation into the side effects of wide range-axon guidance cues, such as netrins, and their subsequent consequences on the endothelium.

4. Perspectives

Recent evidence implicating new non canonical receptors for netrin signaling, new ligands for canonical netrin receptors as well as indirect netrins effect on non-vascular cells suggest that netrin function, on the endothelium, might be more complex than considered a decade ago. These observations may partially explain the lack of concordance of phenotypes in mice lacking canonical netrins or netrin receptors. The discovery of other non-canonical classes of netrin receptors and ligands, such as draxin, RGM, FLRT, DSCAM or A2b (Figure 1), in different research fields (Corset et al. 2000, Ly et al. 2008, Mirakaj et al. 2011, Yamagishi et al. 2011), encourages thorough investigations of their direct/indirect effects on the endothelium.

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Figure 1. Netrins and their receptors

Schematic representations of netrins receptors and the described ligand-receptor interactions, identified in different biological systems. Canonical and non-canonical netrin receptors are squared in red and blue respectively while typical and atypical ligands for canonical netrin receptors are reported just above, in red and blue, respectively. DCC; Deleted in colorectal cancer, DSCAM; Down syndrome cell adhesion molecule, Draxin; Dorsal repulsive axon guidance protein, FLRT-2 and 3; Fibronectin and leucine-rich transmembrane protein-2 and 3, RGM-A; Repulsive Guidance Molecule-A, Robo4; Magic Roundabout.

Ig; immunoglobulin domain, FNIII; fibronectin type III domain, P1-3; conserved region of the DCC cytoplasmic domain, TSP; thrombospondin type-1 domain, ZU-5; zona occludens 5 domain, UPA; Unc5, PIDD and ankyrin domain, DD; death domain.