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Neuropilin ligands in vascular and neuronal patterning

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Abstract

Blood vessels and neurons share guidance cues and cell surface receptors to control their behaviour during embryogenesis. The transmembrane protein neuropilin 1 (NRP1) is present on both blood vessels and nerves and binds two structurally diverse ligands, the class 3 semaphorin SEMA3A and the VEGF164 isoform of the vascular endothelial growth factor VEGF (VEGF-A). *In vitro*, SEMA3A competes with VEGF164 for binding to NRP1 to modulate the migration of endothelial cells and neuronal progenitors. It was therefore hypothesised that NRP1 signalling controls neurovascular co-patterning by integrating competing VEGF164 and SEMA3A signals. However, SEMA3A, but not VEGF164, is required for axon patterning of motor and sensory nerves, and, vice versa, VEGF164 rather than SEMA3A is required for blood vessel development. Ligand competition for NRP1 therefore does not explain neurovascular congruence. Instead, these ligands control different aspects of neurovascular patterning that impact on cardiovascular function. Thus, SEMA3A/NRP1 signalling guides the neural crest cell (NCC) precursors of sympathetic neurons as well as their axonal projections. In addition, VEGF164 and a second class 3 semaphorin termed SEMA3C contribute to the remodelling of the embryonic pharyngeal arch arteries and primitive heart outflow tract by acting on endothelium and NCCs, respectively. Consequently, loss of either of these NRP1 ligands disrupts blood flow into and out of the heart. Multiple NRP1 ligands therefore cooperate to orchestrate cardiovascular morphogenesis.

Keywords

neuropilin; VEGF; semaphorin; neuron; blood vessel; neural crest

Introduction

Blood vessels and neurons share guidance cues and cell surface receptors to control their behaviour during embryogenesis (reviewed in [1]). For example, the transmembrane protein neuropilin 1 (NRP1) is present on both blood vessels and nerves. Neuropilin 1 is a single pass transmembrane protein that is specific to vertebrates. It has a large extracellular N-terminal domain that contains two complement-binding homology domains, known as a1 and a2, two coagulation factor V/VIII homology domains, known as b1 and b2, and a domain separating the b2 from the transmembrane domain, termed c [2](Fig. 1A). The a- and b-domains mediate ligand binding, the c domain promotes dimerisation and the interaction with other cell surface receptors, and the intracellular domain binds a PDZ-domain containing protein termed synectin or GIPC (Fig. 1A). Even though NRP1 was originally identified as an adhesion molecule in the nervous system, it is more commonly studied as the ligand binding subunit of the receptor for the semaphorin SEMA3A and as a receptor for the VEGF165 isoform of the vascular endothelial growth factor VEGF-A in endothelial and tumour cells (VEGF164 in mice)[3-6]. The closely related neuropilin 2 (NRP2) protein was originally identified as a SEMA3F receptor, but has also been

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implicated in VEGF isoform binding and intercellular adhesion [7-9]. NRP1 recruits a member of the A-type plexin family to transduce semaphorin signals [10, 11]. In contrast, it is not yet clear, if NRP1 requires a co-receptor such as the VEGF receptors VEGFR1 or VEGFR2 to transduce VEGF164 signals, or if signalling through its cytoplasmic domain is more important to promote VEGF signalling in vivo (see below).

Role of NRP1 ligands in neuronal development

SEMA3A was the first ligand reported to bind NRP1 and is well known for its ability to collapse the growth cones of NRP1-expressing axons in vitro (e. g. [4, 5, 12]). In vivo, SEMA3A is essential to control fasciculation and branching of various types of nerves in the peripheral nervous system (PNS) and central nervous system (CNS) of the mouse embryo (Fig. 1A; for example [13], but also axon guidance of sympathetic neurons (Fig. 1B,C; see below). The defects of *Sema3a*-null mutants are phenocopied in mouse embryos lacking NRP1 or carrying a mutation in the $\alpha 1$ domain of NRP1 that impairs binding to class 3 semaphorins [14, 15]. VEGF164 also binds NRP1-expressing axon tracts, at least in the CNS [16]. However, loss of VEGF164 does not compromise axon patterning outside the CNS, as the cranial nerves and limb nerves appear normal in mice expressing only the VEGF120 isoform of VEGF-A, which does not signal through NRP1 (*Vegfa*^{120/120} mice) [16, 17]. In contrast, NRP1 can act as a VEGF-A receptor to control neuronal cell body migration. Thus, the cell body migration of facial branchiomotor (FBM) neurons from their birthplace in the hindbrain to the site where they assemble into the paired facial motor nuclei requires VEGF164/NRP1 signalling, rather than semaphorins (Fig. 1A)[17]. Because *Vegfa* is expressed in cells along the migration path of FBM neurons, and VEGF164-coated beads attract them in hindbrain explants, VEGF164 appears to provide a chemoattractive signal for these neurons.

Role of NRP1 ligands in blood vessel development

NRP1 is not only involved in neuronal patterning, but is also essential for the formation of a functional vasculature during vertebrate development. Thus, overexpression of NRP1 in the mouse embryo leads to increased vessel growth, and vessels are leaky and haemorrhagic, causing lethality by embryonic day (E) 17.5 [18]. Vice versa, the targeted disruption of NRP1 reduces vessel growth, in particular in the central nervous system (CNS) [19](Fig. 1D,E).

Since NRP1 was cloned as a VEGF165 receptor in human endothelial and tumour cells [6], much effort has been directed at identifying its precise role in vascular growth. Its contribution to VEGF-A signalling was first studied in cells derived from porcine aortic endothelium (PAE), because they lack expression of the main endothelial VEGF receptor, VEGFR2, as well as NRP1, and could therefore be engineered to selectively express either receptor alone or in combination. In this model, NRP1 increases the affinity of VEGF165 for VEGFR2 to stimulate cell migration [6]. However, others have contested the idea that NRP1 increases the affinity of VEGF165 for VEGFR2 and suggested that NRP1 instead increases VEGFR2 clustering and stability [20]. Consistent with the latter idea, more recent work suggests that synectin/GIPC binds to the NRP1 cytoplasmic tail to promote ligand-dependent endocytosis of VEGFR2/NRP1 complexes [21]. Alternatively, or additionally, NRP1 may act on endothelium from a non-endothelial cell type *in trans*, as has been described for tumour cells [22] and haematopoietic cells [23].

Based on the cell culture studies described above, and because the phenotype of tissue-specific knockout mice lacking NRP1 in vascular endothelium is similar to that of complete NRP1 knockouts, the essential role of NRP1 in vascular development is commonly attributed to its ability to act as a VEGF164 receptor in endothelial cells [14]. However, the

vascular defects caused by loss of NRP1 are surprisingly different to those caused by loss of VEGF164. Thus, NRP1 deficiency impairs neural tube vascularisation more severely than loss of VEGF164, but it affects perisomitic vessel growth less severely [24, 25]. The loss of VEGF isoform signalling cannot therefore be entirely responsible for the vascular deficiency of *Nrp1*-null mutants, and it is likely that NRP1 ligands other than VEGF164 contribute to vessel patterning.

SEMA3A has also been proposed to act as an alternative NRP1 ligand in vascular growth. Thus, VEGF164 and SEMA3A were reported to compete for binding to NRP1 in PAE cells to modulate VEGF/VEGFR2-mediated cell migration [26]. However, recent biochemical data demonstrated that SEMA3A and VEGF165 bind to distinct domains of NRP1 and can therefore bind simultaneously, rather than competitively [27]. SEMA3A has also been proposed to affect the migration of primary human umbilical cord endothelial cells by modulating integrin signalling [28]. Consistent with a role for SEMA3A in vessel patterning, it is able to bind blood vessels in the brain, the organ most severely affected by vascular defects in *Nrp1*-null mutants [16]. However, neither SEMA3A nor any form of semaphorin signalling through NRP1 appear to be essential for vascular development [16], and the combined loss of SEMA3A and VEGF164 does not impair brain vascularisation as severely as the loss of NRP1 [16]. Together, these observations suggest that NRP1 has a function in vascular development above and beyond its role as a VEGF164 receptor that is not due to SEMA3A binding. Recently, NRP1 was shown to interact with a host of additional proteins, including several growth factors and adhesion molecules [29-33]. It will present a formidable challenge to vascular biologists to identify which of these interactions are physiologically relevant. We are particularly intrigued by the idea that neuropilins interact with two types of adhesion molecules, L1-CAM and integrins, as it reminds us of the original discovery of NRP1 as an adhesion molecule.

Role of neuropilin ligands in the sympathetic nervous system

Whilst semaphorin signalling through NRP1 is not essential for blood vessel growth, it does play an essential role in the cardiovascular system by patterning the sympathetic nervous system (SNS). Thus, loss of SEMA3A leads to an insufficient supply of sympathetic innervation to the heart, causing heart arrhythmia and sudden death in adult mice [34]. These innervation defects can be attributed to an abnormal SNS development in the embryo [35]. The SNS consists of two chains of ganglia that lie adjacent to the vertebral column and project axons to target organs, such as the heart and resistance arteries. Loss of function for either NRP1 or SEMA3A leads to ectopic sympathetic neuronal progenitors in the limb and abnormally small sympathetic chains [35] (Fig. 1B,C). These defects were originally attributed to a role for SEMA3A in guiding sympathetic neuronal progenitors [35]. However, our own investigations have revealed that SEMA3A and NRP1 are required at an even earlier stage of development, i.e. for the guidance of neural crest cells, which are the precursors of sympathetic neuronal progenitors [36].

Neural crest cells (NCCs) are a population of highly migratory cells that exists transiently in the vertebrate body and gives rise to many different tissues (reviewed in [37]). Within the nervous system, they form the entire SNS and peripheral nervous system of the trunk, and, together with placodal neurons, they form the cranial ganglia. Within the cardiovascular system, they provide a smooth muscle coat for head vessels and the pharyngeal arch arteries. Early in development, NCCs delaminate from the neural folds as they invaginate from the ectoderm to form the neural tube. NCCs then disseminate along distinct pathways, depending on their fate. Whilst NCCs destined for the heart delaminate at the level of the hindbrain, NCCs destined to become sympathetic neurons delaminate at trunk level. Sympathetic NCCs then travel towards the dorsal aorta, where they aggregate and

differentiate into postmitotic sympathetic precursors. Subsequently, these precursors undergo a secondary migration step, which culminates in the formation of the definitive sympathetic ganglia. To reach the dorsal aorta, the sympathetic NCCs initially migrate ventrally between the somites, transient epithelial structures that are arranged segmentally along the anterior-posterior axis of the vertebrate trunk and later give rise to skin, muscle and bones. Following this route, the sympathetic NCCs migrate alongside intersomitic blood vessels, which extend between the dorsal aorta and the perineural vascular plexus. In contrast, later born sympathetic NCCs migrate through the somites, concomitant with the segregation of the somite into a dermomyotome and sclerotome layer.

At first, it was thought that trunk NCC migration was unaffected by loss of NRP1 signalling in mice [35]. However, NRP1 has been implicated in NCC guidance in chick embryos [38], and a subset of cranial NCCs in the mouse requires SEMA3A/NRP1 signalling to identify their appropriate migratory route [39]. We therefore re-investigated the possibility that the defective sympathetic chain assembly in *Nrp1*-null mutants is due to a defect in trunk NCC guidance, using novel markers that are more specific to NCCs than those that had been previously employed [36]. We observed that an excess of NCCs migrated alongside intersomitic blood vessels in *Sema3a*-null and NCC-specific *Nrp1*-null mutant embryos, even though this route was taken by only a small subset of very early migrating NCCs in wild type littermates. These displaced NCCs then aggregated distal to the dorsal aorta, explaining the position of the previously observed ectopic sympathetic ganglia. These findings provided the first evidence that SEMA3A/NRP1 signalling contributes to cardiovascular development by controlling the migration of NCCs. It is not yet known if the alternative NRP1 ligand VEGF164 also affects NCC migration, but there is evidence that VEGF-A affects the SNS to promote nerve regeneration [40].

Role of NRP1 ligands in heart development

The finding that NRP1 signalling is important for CNS vascularisation and the sympathetic innervation of heart and arteries suggests that it is a key player in cardiovascular morphogenesis and function. However, the midgestation lethality of *Nrp1*-null mutants is more readily explained by defective remodelling of the heart outflow tract (OFT) and the pharyngeal arch arteries into the great vessels that leave the heart [19]. Importantly, these remodelling processes rely on the interaction of endothelial cells and a subset of NCCs, which is derived from the lower hindbrain and is commonly referred to as the cardiac NCC. The role of cardiac NCCs in vessel remodelling is explained in Box 1 and is reviewed in [41].

Genetic studies have implicated both semaphorin and VEGF ligands for NRP1 in the remodelling of the great vessels. Thus, loss of VEGF164 inhibits OFT remodelling in mice. Moreover, in zebrafish, VEGF164 interacts genetically with the gene encoding TBX1 [42], and TBX1 gene function is compromised in many patients with DiGeorge syndrome, a birth defect that affects many tissues containing NCC-derivatives, for example the OFT (reviewed in [41]). Finally, a specific *VEGFA* promoter haplotype is associated with an increased risk for cardiovascular complications in patients with DiGeorge syndrome [42]. The deletion of NRP1 in vascular endothelium also leads to an OFT defect [14], suggesting that VEGF164/NRP1 signalling is more important in endothelial cells than cardiac NCCs during OFT septation.

In contrast, loss of SEMA3A has not been reported to cause OFT defects. Rather, an alternative neuropilin ligand termed SEMA3C is essential for OFT septation [43]. SEMA3C is likely to signal through NRP1/PLXND1 complexes in endothelial cells of the OFT [44]. Accordingly, loss of PLXND1 results in similar OFT defects to loss of SEMA3C [44]. In

contrast, ablating semaphorin signalling through both neuropilins is necessary to reproduce the OFT defect of *Sema3c*-null mutants [14], presumably because SEMA3C can bind NRP2 in addition to NRP1 [45].

NRP1 is also expressed in cardiac NCCs as they migrate within the pharyngeal arches [46]. Consistent with the idea that NRP1 plays a role in aortic arch smooth muscle development, loss of NRP1 in mice leads to agenesis or transposition of the remodelling aortic arch arteries [19]. It is not yet known if these defects are due to endothelial cells failing to recruit cardiac NCC-derived cells to induce smooth muscle cell formation, or if cardiac NCCs are recruited, but fail to differentiate into proper smooth muscle cells on the arch arteries. PLXND1 is required for smooth muscle cell differentiation on the 4th and 6th pharyngeal arch arteries, most likely because it forms a heterodimer with NRP1 to mediate SEMA3C signals, as in OFT development [44]. However, the absence of VEGF164 also perturbs aortic arch remodelling [42], raising the possibility that NRP1 plays a dual role in pharyngeal arch development as a VEGF and semaphorin receptor.

Conclusion

NRP1 plays multiple roles in neuronal and vascular development by interacting with a variety of different ligands and co-receptors, and several of these pathways are critical for cardiovascular morphogenesis and function. Even though the combination of in vitro assays, expression studies and genetic models performed by a large number of different laboratories has helped to elucidate the significance of semaphorin and VEGF binding to NRP1, much remains to be learnt about the physiological role of the adhesion ligands and GIPC/synectin in neurovascular development.

Box 1

After delamination from the lower hindbrain, cardiac NCCs migrate into the pharyngeal arches, where some come into close contact with the pharyngeal arch arteries and differentiate into vascular smooth muscle cells. The interaction between NCC-derived vascular smooth muscle and endothelial cells promotes the remodelling of the pharyngeal arch arteries. Defective remodelling of the pharyngeal arch arteries can lead to an overriding aorta and a hypoplastic pulmonary trunk in a condition known as Tetralogy of Fallot. Other cardiac NCCs continue to migrate to invade the primitive cardiac outflow tract (OFT), where they induce the formation of a septum that separates the OFT into aorta and pulmonary artery. The absence of OFT separation leads to a lethal condition known as persistent truncus arteriosus, in which arterial and venous blood mixes as it leaves the heart. Both defects can occur together with craniofacial, thymic and parathyroid abnormalities in a relatively common congenital disease known as DiGeorge syndrome. Because cardiac NCCs interact with multiple cell types during heart development, including pharyngeal arch epithelium and mesenchyme and the vascular endothelium that lines the aortic arch arteries and the outflow tract, the functional analysis of genetic mutations that affect OFT and aortic arch remodelling is complex and not yet complete. For a more detailed description of the role of NCCs in cardiac development, see [41].

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Fig. 1. NRP1 in neurovascular patterning

(A) Schematic representation of neuropilin organisation, ligand binding and signalling roles in neuronal versus vascular patterning. The extracellular part of NRP1 contains two domains termed a1 and a2, two domains termed b1 and b2, one domain termed c and a transmembrane domain (tm). Class 3 semaphorin (SEMA3)-binding requires the a1/a2 domain and VEGF165-binding the b1/b2 domain; the b1/b2 domain also contains the adhesion domain, whose physiological function is not yet understood. The three last amino acid residues of the cytoplasmic domain mediate binding to the PDZ domain-containing protein GIPC1/synectin, which is important for vascular patterning in zebrafish. (B,C) NRP1 is essential for sympathetic chain assembly and sympathetic axon guidance. The sympathetic nervous system of wild type (B) and *Nrp1*-null (C) mouse embryos at E12.5 was visualised by wholemount immunohistochemistry for tyrosine hydroxylase. In wild types, sympathetic neurons form a tight chain on each side of the dorsal aorta. In *Nrp1*-null mutants, most cell bodies are only loosely packed into chains, and there are many ectopic neurons, especially at limb level (the boxed area is shown in higher magnification in the inset of panel C). In addition, axons extend between the two chains, rather than running up and down each chain (arrowhead in panel C). (D,E) NRP1 is essential for proper brain vascularisation. The subventricular vascular plexus of wild type (D) and *Nrp1*-null (E) hindbrains at E11.5 was visualised by wholemount fluorescent staining for isolectin B4; only the right side of each flatmounted hindbrain is shown. Note that the vessels in mutants are larger in diameter and form a sparse vascular network. Isolectin B4 also labels brain macrophages, which appear as scattered single cells and are clearly visible in *Nrp1*-null mutants due to the paucity of blood vessels. Scale bars: (B,C) 500 μ m; (D,E) 200 μ m.