Genome Biology

Protein family review

The netrin protein family

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Summary

The name netrin is derived from the Sanskrit *Netr*, meaning 'guide'. Netrins are a family of extracellular proteins that direct cell and axon migration during embryogenesis. Three secreted netrins (netrins 1, 3 and 4), and two glycosylphosphatidylinositol (GPI) -anchored membrane proteins, netrins G1 and G2, have been identified in mammals. The secreted netrins are bifunctional, acting as attractants for some cell types and repellents for others. Receptors for the secreted netrins include the Deleted in Colorectal Cancer (DCC) family, the Down's syndrome cell adhesion molecule (DSCAM), and the UNC-5 homolog family: Unc5A, B, C and D in mammals. Netrin Gs do not appear to interact with these receptors, but regulate synaptic interactions between neurons by binding to the transmembrane netrin G ligands NGL1 and 2. The chemotropic function of secreted netrins has been best characterized with regard to axon guidance during the development of the nervous system. Extending axons are tipped by a flattened, membranous structure called the growth cone. Multiple extracellular guidance cues direct axonal growth cones to their ultimate targets where synapses form. Such cues can be locally derived (short-range), or can be secreted diffusible cues that allow target cells to signal axons from a distance (long-range). The secreted netrins function as short-range and long-range guidance cues in different circumstances. In addition to directing cell migration, functional roles for netrins have been identified in the regulation of cell adhesion, the maturation of cell morphology, cell survival and tumorigenesis.

Gene organization and evolutionary history

UNC-6, the first reported netrin, was identified in the nematode worm *Caenorhabditis elegans* in a screen for proteins that regulate neural development [1]. It is now clear that netrins have a highly conserved function in bilaterally symmetric animals, directing axons toward the ventral midline of the developing nervous system during embryogenesis. Orthologs of the secreted netrin UNC-6 have since been identified in invertebrates other than *C. elegans*, such as *Drosophila melanogaster* (Netrin A and Netrin B) [2], and the leech (Netrin) [3] (Figure 1a). Notably, a secreted netrin (NvNetrin) has been identified in the sea anemone *Nematostella vectensis*, an animal that exhibits some of the earliest hallmarks of bilateral symmetry [4]. Vertebrates express multiple netrin genes. In mammals, expression of five netrins has been identified (netrins 1, 3, 4, G1 and G2). Netrins 1 to 4 are secreted, whereas netrins G1 and G2 are tethered to the plasma membrane by carboxy-terminal glycosylphosphatidylinositol (GPI) tails [5,6]. Netrin Gs are thought to have evolved independently of netrins 1, 2 and 3 [5]. Orthologs of the netrin Gs have been identified only in vertebrate species. All netrins characterized so far in invertebrates, including *C. elegans* UNC-6, are secreted proteins.

All netrins are members of the laminin superfamily (Figure 1a). The sequence of the amino-terminal two-thirds of netrin 1, 2 and 3 is most similar to amino-terminal sequences found in the laminin-γ1 chain [7,8], whereas the amino-terminal domains of netrins G1, G2 and 4 are most similar to the amino terminus of the laminin-β1 chain (Figure 1b,c) [5,6,9]. In heterotrimers of laminin-α, -γ, and -β chains these amino-terminal domains mediate self-assembly, leading to the formation of complex laminin networks [10]. Netrin 4 integrates into basement membranes through interaction of its amino-terminal domains with laminins [11]; however, other secreted netrins do not seem to bind laminins in extracellular matrix (ECM).

Characteristic structural features

On the basis of their homology with laminin amino-terminal domains, the amino-terminal netrin domains were named VI and V. Domain VI, at the amino terminus, is globular. It is followed by domain V, which is composed of three epidermal growth factor (EGF) repeats (Figure 1b,c). Domains VI and V bind to the Deleted in Colorectal Cancer (DCC) and UNC-5 families of netrin 1 receptors; however, the precise molecular details of the interaction have not been determined. The remaining carboxy-terminal sequence of netrins 1 to 4 (the C-domain) is enriched in basic amino acids [7-9]. This sequence binds heparin with high affinity, and may contribute to presenting secreted netrins on cell surfaces or retaining them in ECM through interactions with heparin sulfate proteoglycans (HSPGs) [12].

The C-domain of netrin 1, also known as the netrin-like (NTR) module, is homologous to similar domains found in a disparate group of proteins that have diverse biological functions. So far, the NTR module has been identified in complement proteins C3, C4 and C5, which are components

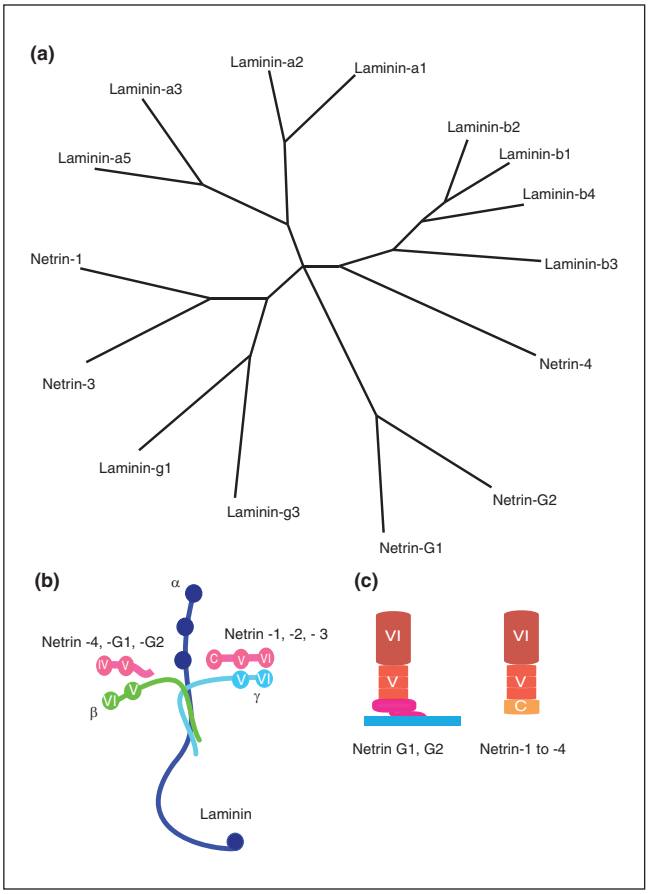

Figure 1

The netrin family of proteins. **(a)** Netrins are members of the laminin superfamily. A phylogenetic tree based on human protein sequences illustrates the relationship between netrin and laminin family members. **(b)** Laminin 1 is a heterotrimer composed of α (blue), β (green), and γ (turquoise) chains. The amino-terminal VI and V domains of netrins 1 to 3 (red) are homologous with the γ chain of laminin 1. These domains in netrins 4, G1 and G2 are more similar to the β chain of laminin 1. **(c)** Domain organization of the netrin family members. Netrins 1 to 4 are secreted proteins and contain a carboxy-terminal C-domain (C), whereas netrins G1 and G2 are linked to the plasma membrane by a GPI linker (magenta).

of the innate immune system, in secreted Frizzled-related proteins that are involved in axon guidance, in the type I procollagen C-proteinase enhancer proteins (PCOLCE), which are metalloproteinase inhibitors, and in tissue inhibitors of metalloproteinases [13]. The functional significance of the NTR module is unknown in netrin and poorly understood in other proteins, but structural and functional data suggest an inhibitory activity towards proteinases [14-16]. The C-domain of secreted netrins (netrins 1 to 4) is not required for binding to DCC or the UNC-5 homologs [17-19]. Deletion of the UNC-6 C-domain generates axon-guidance defects in *C. elegans*, but these are relatively mild compared to the effects of complete loss of UNC-6 function [20].

Localization and function

Netrin-1 expression in the central nervous system

Multiple extracellular guidance cues direct axonal growth cones to their ultimate targets, where synapses are formed [21]. Studies of the distribution and function of secreted netrins, netrin 1 in particular, provide evidence for roles as both short-range and long-range cues. 'Short-range' refers to a role when netrin is in the immediate vicinity of its cellular source, either close to the secreting cell or attached to its surface. By contrast, 'long-range' secreted cues function at a distance from the cell secreting the factor. There is substantial evidence that netrin 1 functions as a long-range cue in the embryonic nervous system. The localization and function of netrin 1 has been particularly well studied in the developing chick, mouse and rat spinal cord. Netrin 1 is secreted by the floor plate, a specialized group of secretory cells at the ventral midline of the embryonic neural tube [22]. A gradient of netrin 1 protein emanating from the floor plate orients cell and axon migration with respect to the ventral midline [23]. In both the developing and mature central nervous system (CNS), most of the netrin 1 protein is tightly associated with cell membranes and ECM [7,24], probably through interactions with HSPGs or other proteins that bind netrin 1. In contrast to the long-range function in the embryonic nervous system, in the adult mammalian CNS netrin 1 is expressed by oligodendrocytes, the myelinating cells of the CNS. It is enriched in non-compact oligodendrocyte membranes [24] at axo-oligodendroglial paranodal junctions [25], where it regulates the organization of cell-cell contact.

Netrin 1 is expressed in many other regions of the developing and mature mammalian CNS, including the visual system [26-29], the developing and postnatal olfactory system [27,30], the developing forebrain [31], the cerebellum [27] and the adult forebrain [32]. Netrin 1 regulates cell and axon migration in each of these brain regions during development.

Netrin receptors and signal transduction

Netrins engage a number of different receptors to activate chemotropic responses and adhesive mechanisms. Several netrin receptors belong to the immunoglobulin superfamily, which encodes a large group of proteins involved in processes such as signal transduction and cell adhesion [33]. Netrin 1 binding to its receptors alters the architecture of the cytoskeleton through reorganization of actin and microtubule networks [34].

The first netrin receptors to be identified belong to the DCC subfamily of proteins, which in vertebrates includes DCC and neogenin, a protein with approximately 50% amino-acid identity to DCC [17]. Orthologs of DCC are present in *C. elegans* and *Drosophila*, named UNC-40 and Frazzled, respectively [35,36]. DCC, neogenin, and their orthologs are transmembrane proteins with an extracellular

domain composed of four immunoglobulin domains and six fibronectin type III (FNIII) repeats [37,38] (Figure 2a), with the fourth and fifth FNIII repeats in DCC being required to bind netrin 1 [39,40]. Following a single-pass transmembrane domain are three highly conserved intracellular domains, named P1, P2 and P3 [36,41], that are thought to have important roles in the recruitment of cytoplasmic signal transduction molecules; however, the specific functional contribution of each of these domains is not known.

Vertebrates have four netrin 1 receptors homologous to *C. elegans* UNC-5: Unc5A-D [42]. The Unc5 proteins are transmembrane proteins with an extracellular domain consisting of two immunoglobulin repeats followed by two thrombospondin type-I modules [18,43] (Figure 2a). The intracellular domain of Unc5 proteins includes a ZU-5 domain of undetermined function, named for its homology to a portion of Zona Occludens-1 [18], and a death domain associated with apoptotic signaling [44]. The immunoglobulin repeats of Unc5 proteins are required for netrin binding [39].

Down's syndrome cell adhesion molecule (DSCAM) is a type I transmembrane receptor that contains ten immunoglobulin domains and six FNIII repeats in its extracellular domain [45] (Figure 2a). For a long time an orphan receptor without an identified ligand, DSCAM has recently been shown to function as a receptor for netrin 1 that contributes to netrin-dependent axon guidance during development [46-48]. Netrin 1 binds to the immunoglobulin domains of DSCAM [48]. Signaling mechanisms activated by netrin 1 downstream of DSCAM have not been identified.

The signaling pathways leading from the DCC and Unc5 receptors are shown in Figure 2b. Essential roles for downstream signal-transducing proteins that regulate cytoskeletal reorganization, such as focal adhesion kinase (FAK), the protein tyrosine kinase Fyn, the Rho family of small GTPases, and microtubule-associated proteins, have been identified.

The membrane-linked netrin G proteins bind the netrin G ligands NGL-1 and NGL-2, which are type 1 transmembrane proteins with extracellular domains composed of leucine-rich repeats (LRRs) and immunoglobulin domains [6,49,50]. Netrin G proteins are expressed by neurons, and have little expression outside the nervous system. Mutation of the gene for netrin G1 in humans produces an atypical form of Rett syndrome, a neurodevelopmental disorder with symptoms that include spasmodic hand and facial movements (chorea), lack of social interest, and seizure [51-53]. Although gross neuroanatomical structure and neural circuitry are intact in netrin-G1-null mice, the mice exhibit altered synaptic responses and defects in sensorimotor gating behavior [54]. Consistent with a role for netrin Gs in synapse formation and function, glutamatergic synaptogenesis has been found to be regulated by interactions between NGL-2 and the postsynaptic scaffold protein PSD-95 [49]. Interesting, NGL-3, a third NGL family member found in mammals, does not appear to bind either netrin G1 or G2, but also regulates glutamatergic synaptogenesis through an interaction with the protein tyrosine phosphatase LAR [55].

Several axon-guidance cues interact with components of the ECM [56]. The initial purification of netrins from embryonic chick brain homogenate involved heparin sepharose affinity chromatography [7], suggesting that netrin might interact with HSPGs. Subcellular fractionation of embryonic brain and adult rat spinal cord indicated that the vast majority of netrin 1 protein is associated with cell membranes and ECM, consistent with an interaction with HSPGs or other extracellular netrin-binding proteins [7,24]. Subsequent studies demonstrated that netrin 1 binds heparin through its C-domain [57], but a function for this interaction between netrin 1 and proteoglycans remains to be demonstrated.

Roles for secreted netrin family members have been identified in distinct cellular processes, including axon guidance (netrin 1, 2 and 3) [22], tissue morphogenesis and cell-cell adhesion (netrin 1) [25,58], synaptogenesis (netrin-Gs, UNC-6) [49,59], and angiogenesis (netrin-4) [60-62]. The diverse functional roles of secreted netrins have been best characterized in studies of netrin 1 [63].

Roles of netrin 1 in axon guidance, neuron migration and glial development

Floor-plate cells in the ventral embryonic spinal cord secrete netrin 1 [22], which is distributed in a decreasing ventral-dorsal gradient in the cord [23]. This gradient of netrin 1 attracts the axons of embryonic spinal commissural neurons, directing them towards the dorsal midline of the spinal cord [22]. DCC is required for this attractive effect of netrin 1, and loss of either netrin 1 or DCC function results in loss of several commissures, which are locations where axons coalesce to cross from one side of the CNS to the other. Deficiencies of netrin or DCC function cause loss of the ventral commissure in the embryonic spinal cord, the corpus callosum that connects the left and right cerebral hemispheres, and the hippocampal and anterior commissures in the brain [64,65].

Netrin 1 secreted by floor-plate cells also acts as a chemorepellent for migrating cells and axons. This was first demonstrated for the axons of trochlear motor neurons, whose cell bodies are located in the ventral neural tube and innervate the extra-occular muscles of the eye. The gradient of netrin 1 emanating from the ventral midline directs these axons away from the floor plate towards the

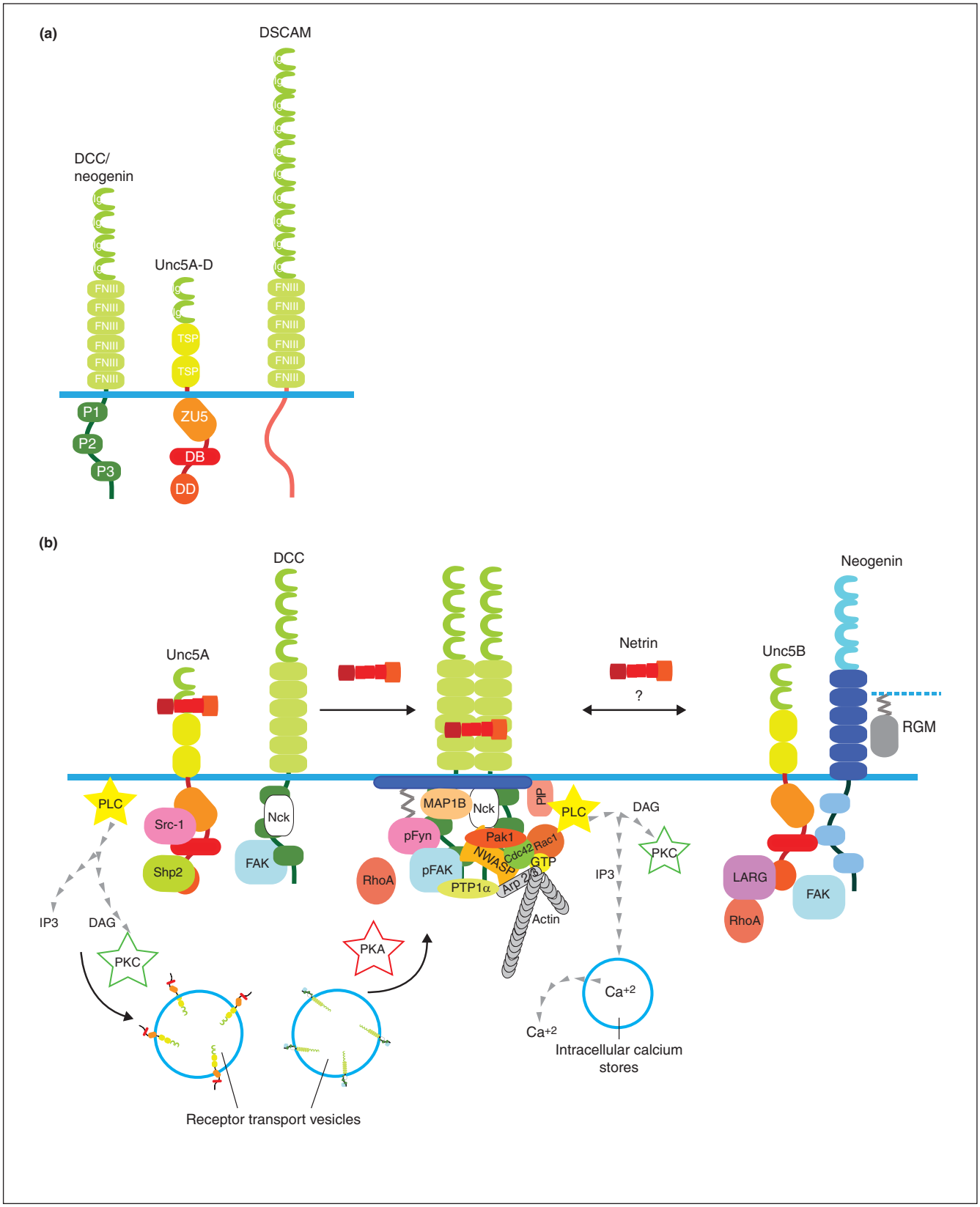

Figure 2

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Figure 2 continued
Canonical netrin receptors and their mechanism of action. **(a)** The DCC/neogenin family, the Unc5 homologs (Unc5A-Unc5D), and DSCAM are receptors for netrin 1. DB, DCC-binding domain; DD, death domain; Ig, immunoglobulin domain; FNIII, fibronectin type III repeat; TSP, thrombospondin type-I module; ZU5, domain homologous to part of Zona Occludens-1. **(b)** Signal transduction components that act downstream of DCC and Unc5 homologs. Netrin 1 binding to DCC results in the recruitment of intracellular signaling molecules associated with reorganization of the actin cytoskeleton. Netrin binding to DCC induces homodimer formation, which is the active form of the receptor. These proteins include members of the Rho family of GTPases (RhoA, Rac1, Cdc42), Src-family kinases (Src-1, pFyn), the serine/threonine kinase Pak1, the Wiskott-Aldrich syndrome related protein family (NWASP), and the Arp2/3 actin-binding complex. Proteins are shape and color-coded according to family. The tyrosine phosphatase Shp2 and the tyrosine kinase Src-1 have been implicated in Unc5 function, but signaling downstream of the Unc5 receptors is relatively poorly understood. Within neuronal growth cones, activation of protein kinase A (PKA, red star) recruits DCC to the plasma membrane from an intracellular pool of vesicles. Endocytosis of Unc5A is triggered by activation of protein kinase C (PKC, green star). Activation of PLC by DCC leads to release of calcium from intracellular calcium stores. DAG, diacylglycerol; FAK, pFAK, focal adhesion kinase; IP3, inositol trisphosphate; LARG, leukemia associated Rho guanine nucleotide exchange factor; MAP1B, microtubule associated protein 1B; Nck, an adaptor protein; PIP, phosphatidylinositol phosphate; PTP1α, protein phosphatase 1α; RGM, repulsive guidance molecule. RGM is a GPI-linked cell-membrane-associated ligand for neogenin that is not related to netrins. RGM is thought to signal growth-cone collapse through a complex of neogenin and Unc5B. The double-headed arrow with the question mark indicates that it is not clear how netrin-1 may interact with the Unc5B, neogenin, and RGM complex. For further discussion of the mechanisms regulating netrin signaling and receptor trafficking, see [34,79].

dorsal midline [66]. Unc5C is required for this netrin-1-dependent chemorepellent effect [67]. These findings first indicated that netrin 1 is a bifunctional axon-guidance cue in vertebrates, an observation consistent with the function of UNC-6, the netrin ortholog in *C. elegans* [1]. Following these studies, netrin 1 has been found to function as an attractive or repellent guidance cue for a number of neuronal cell types in the vertebrate CNS, including dopaminergic neurons (attraction) [68] and cerebellar granule neurons (repulsion) [69].

In addition to directing axon guidance, netrin 1 also acts as a bifunctional regulator of neuron migration. During cerebellar development, netrin 1 acts as an attractant for precerebellar neurons, specifically the neurons that initially migrate away from the lower rhombic lip to ultimately populate the pontine nuclei [69]. This attractive effect of netrin 1 requires DCC. By contrast, netrin 1 does not appear to influence migrating precursors of cerebellar granule neurons (CGNs), which originate in the upper rhombic lip. Subsequently, during postnatal cerebellar development, netrin 1 acts as a repellent for the CGN precursors, which upregulate Unc5 expression as they exit the external granule layer [69]. Netrin 1 also acts as an attractant for DCC-expressing lateral olfactory tract neuronal cells, which migrate to specific regions of the lateral olfactory tract where they act as guidepost cells for olfactory bulb axons [70].

Netrin 1 released from the ventricular zone of the third ventricle repels migrating glial precursor cells, which have the capacity to differentiate into oligodendrocytes or astrocytes [29]; however, a direct role in the development of astrocytes has not been identified. In the embryonic spinal cord, netrin 1 repels migrating oligodendrocyte precursor cells [71,72]. Later in development, netrin 1 regulates the morphological maturation of developing oligodendrocytes, and is required for the maintenance of axo-oligodendroglial paranodal junctions in mature myelin [25,73]. Looking beyond neural development, netrin 1 has been implicated in directing the migration of adult neural stem cells at sites of injury in the mature CNS [74].

A role for netrin 1 in non-neural cells

Recent work has identified roles for netrin 1 in non-neural cell types, including tissue morphogenesis, vascular development and dysregulation leading to cancer [63]. Netrin 1 is required for development of the mammary gland terminal end buds, which form the growing tips of the network of ducts in the gland. In this context, netrin 1 expressed by a layer of luminal epithelial cells binds to the DCC homolog neogenin, expressed by an adjacent layer of cap cells, to stabilize the organization of these cell layers in the developing bud [58]. During development of the vasculature, netrin 1 acts through Unc5B to inhibit vessel branching [75] and also promotes DCC-dependent migration and proliferation of vascular endothelial cells [76].

A role for netrin 1 as an anti-apoptotic survival factor has been identified in tumorigenesis [77]. The 'dependence-receptor' hypothesis proposes that DCC and Unc5 homologs mediate cell death in the absence of netrin 1, and binding of the ligand to these receptors switches between a pro-apoptotic signal and the promotion of survival and motility [77]. While it appears that netrin 1 can influence cell survival, the dependence-receptor hypothesis remains controversial, not least because an analysis of the nervous system of mice lacking netrin 1 function did not reveal an increase in the number of apoptotic cells, arguing against an essential role for netrin 1 as dependence ligand during neural development [78].

Frontiers

Members of the netrin family are essential chemotropic guidance cues that direct cell and axon migration in the developing nervous system during embryogenesis. The significance of this function is particularly evident with regard to axon migration toward and away from the floor

plate, a major source of netrin 1 located at the ventral midline of the neural tube. While directing commissure formation has been a major focus of the study of netrin function, it is now clear that netrin family members also play key roles in directing the formation of neural circuits other than guiding axons relative to the midline in the developing CNS. The concept that gradients of netrin protein direct migration during development is now well established, and netrin receptors and intracellular signaling mechanisms mediating the chemotropic response have been identified. Current studies are addressing the functional significance of the recently identified netrin 1 receptor DSCAM. Determining how DSCAM interacts with and influences the function of the canonical families of netrin receptors, the DCC and UNC-5 family members, is a major goal. Ongoing studies aim to develop a complete understanding of the biochemical mechanisms that convert a gradient of extracellular netrin protein into directed cell movement.

Studies of netrin function in the nervous system and in non-neural tissues have revealed important contributions of netrins in regulating cell-cell adhesion and tissue organization. In some cases, cells that initially migrated in response to a source of netrin subsequently require netrin to regulate appropriate cell-cell interactions. It remains to be determined how the signaling mechanisms that direct the motility of these cells switch during maturation to subsequently regulate the organization of cell-cell adhesive contacts. Leaving embryogenesis and entering the realm of tissue repair, exciting findings have implicated netrin 1 in directing adult neural stem-cell migration, suggesting that netrin 1 may influence recovery following injury.

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