Development 138, 2153-2169 (2011) doi:10.1242/dev.044529


# Netrins: versatile extracellular cues with diverse functions

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## Summary

Netrins are secreted proteins that were first identified as guidance cues, directing cell and axon migration during neural development. Subsequent findings have demonstrated that netrins can influence the formation of multiple tissues, including the vasculature, lung, pancreas, muscle and mammary gland, by mediating cell migration, cell-cell interactions and cell-extracellular matrix adhesion. Recent evidence also implicates the ongoing expression of netrins and netrin receptors in the maintenance of cell-cell organisation in mature tissues. Here, we review the mechanisms involved in netrin signalling in vertebrate and invertebrate systems and discuss the functions of netrin signalling during the development of neural and non-neural tissues.

**Key words: DCC, Adhesion, Axon, Neogenin, Netrin, UNC5**

## Introduction

Netrins are a family of extracellular, laminin-related (see Glossary, Box 1) proteins that function as chemotropic guidance cues for migrating cells and axons during neural development. They act as chemoattractants for some cell types and chemorepellents for others. Loss-of-function mutations in netrin 1 or in certain netrin receptors are lethal in mice, highlighting the importance of netrin signalling during development. Insights into the functions of netrins have arisen from studies across a wide range of animal species, including invertebrates such as the nematode worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, non-mammalian vertebrates such as the frog *Xenopus laevis*, and mammals including rats, mice and humans.

Since its discovery in the early 1990s, it is now becoming clear that the netrin gene family exhibits a rich biology, with significance beyond neural development, and contributes to the organisation of multiple tissues. Along with a number of other identified axon guidance cues (Hinck, 2004), secreted netrins influence organogenesis outside the central nervous system (CNS), directing cell migration and mediating cell-cell adhesion in the lung, pancreas, mammary gland, vasculature and muscle (Kang et al., 2004; Lejmi et al., 2008; Liu et al., 2004; Lu et al., 2004; Srinivasan et al., 2003; Yebra et al., 2003). Here, we discuss the cell biology of netrin and netrin receptor functions and review the downstream signal transduction mechanisms that they activate. We also provide an overview of netrin function during development, both within the nervous system and within other developing organs and tissues.

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## Netrin family members

The first reported member of the netrin family, uncoordinated-6 (UNC-6), was identified in a search for gene products that regulate neural development in *C. elegans* (Ishii et al., 1992). Netrins have since been identified and studied in multiple vertebrate and invertebrate species (Table 1), including *X. laevis* (de la Torre et al., 1997), *D. melanogaster* (Harris et al., 1996; Mitchell et al., 1996) and the sea anemone *Nematostella vectensis* (Matus et al., 2006), an animal that exhibits early hallmarks of the origins of bilateral symmetry. In mammals, three secreted netrins, netrin 1, 3 and 4, and two membrane-tethered glycophosphatidylinositol (GPI)-linked (see Glossary, Box 1) netrins, netrin G1 and G2, have been identified (Table 1). Orthologues of netrin 1, which have been identified in all bilaterally symmetrical animals studied so far, play a highly conserved role directing cell and axon migration in the embryonic nervous system. Among the secreted netrins, netrin 1 expression and function have been best characterised. Netrin 1 is expressed in regions of both the developing and adult nervous systems, including the optic disc, forebrain, cerebellum and spinal cord (Deiner et al., 1997; Hamasaki et al., 2001; Kennedy et al., 1994; Livesey and Hunt, 1997). Netrin 1 is also highly expressed in various tissues outside of the nervous system, including the developing heart, lung, pancreas, intestine and mammary gland (Liu et al., 2004; Shin et al., 2007; Srinivasan et al., 2003; Yebra et al., 2003; Zhang and Cai, 2010).

All netrins are composed of ~600 amino acids and belong to the superfamily of laminin-related proteins (Yurchenco and Wadsworth, 2004). N-terminal netrin sequences are homologous to domains VI and V found at the N-termini of laminins; the N-terminal domains of netrin 1 and netrin 3 show most similarity to the laminin γ1 chain (Serafini et al., 1994; Wang et al., 1999), and those of netrins 4, G1 and G2 are most similar to the laminin β1 chain (Nakashiba et al., 2000; Nakashiba et al., 2002; Yin et al., 2000) (Fig. 1A). In secreted netrins, these domains (VI and V) are linked to a C-terminal domain termed 'domain C' or the netrin-like (NTR) module. This module is not homologous to any laminin domain (Serafini et al., 1994) but exhibits sequence similarity to the tissue inhibitor of metalloproteinases (TIMPs) (Banyai and Patthy, 1999), is rich in basic amino acid residues and can bind heparin (see Glossary, Box 1) (Kappler et al., 2000). Although all netrins include laminin-like domains, a clear functional distinction can be made between the secreted netrins and the GPI-linked netrin G proteins due to the engagement of different sets of receptor proteins.

## Netrin receptors

In mammals, receptors for the secreted netrins include deleted in colorectal cancer (DCC), the DCC paralogue neogenin, the UNC-5 homologues UNC5A-D, and Down syndrome cell adhesion molecule (DSCAM) (Table 1). The GPI-linked netrins, by contrast, function by binding to the netrin G ligands (NGLs) NGL-1 and NGL-2 (also known as LRRC4C and LRRC4, respectively), which belong to a family of transmembrane proteins that are structurally

and functionally distinct from the secreted netrin receptors. All netrin receptors identified thus far are single-pass type I transmembrane proteins and are members of the immunoglobulin (Ig) superfamily (see Glossary, Box 1) (Fig. 1B).

### DCC receptor family

DCC was originally identified in humans as a candidate tumour suppressor associated with an allelic deletion of chromosome 18q21 in colon cancer (Fearon et al., 1990; Vogelstein et al., 1988). The most commonly studied members of the DCC family include DCC and neogenin in mammals (Cho et al., 1994; Vielmetter et al., 1994), UNC-40 in *C. elegans* (Chan et al., 1996) and Frazzled in *D. melanogaster* (Kolodziej et al., 1996) (Table 1). Extracellularly, all members of the DCC family are composed of four Ig domains (see Glossary, Box 1) and six fibronectin type III domains (FNIII, see Glossary, Box 1; Fig. 1B), with evidence to suggest that netrin 1 binds to the fourth and fifth FNIII repeats (Geisbrecht et al., 2003; Kruger et al., 2004). Intracellularly, DCC does not encode any obvious catalytic domain but contains three highly conserved sequences termed the P1-3 motifs (Keino-Masu et al., 1996). DCC mediates chemoattractant responses to netrin 1-4, and also contributes to chemorepellent signalling (Colavita and Culotti, 1998; Hong et al., 1999; Jarjour et al., 2003; Kennedy et al., 1994; Qin et al., 2007; Wang et al., 1999). Neogenin, another member of the DCC family, shares ~50% amino acid identity with DCC (Vielmetter et al., 1994). Although less well studied than DCC, recent reports have provided insight into neogenin function and signalling (De Vries and Cooper, 2008). Interestingly, neogenin appears to act as an attractive axon guidance receptor in response to netrin 1, but also as a repellent receptor when bound to repulsive guidance molecule (RGMa), an alternative ligand that does not belong to the netrin family (Rajagopalan et al., 2004; Wilson and Key, 2006). In addition to their roles in axon guidance, both DCC and neogenin regulate cell-cell adhesion and tissue organisation through interactions with the secreted netrins (Jarjour et al., 2008; Kang et al., 2004; Krauss, 2010; Lejmi et al., 2008; Park et al., 2004; Srinivasan et al., 2003).

### The UNC5 receptor family

Four orthologues of *C. elegans* UNC-5, UNC5A-D, have been characterised in vertebrates (Table 1). Extracellularly, they are composed of two Ig domains and two thrombospondin type I (TSP-1) domains (see Glossary, Box 1; Fig. 1B), with the Ig repeats required for netrin 1 binding (Geisbrecht et al., 2003; Krauss, 2010; Leonardo et al., 1997). The UNC5 intracellular domain encodes a ZU-5 domain (see Glossary, Box 1) (Leonardo et al., 1997), a DCC-binding (DB) motif, and a death domain (DD, see Glossary, Box 1) (Hofmann and Tschopp, 1995).

Netrin 1 and 3 are chemorepellents for axons of *Xenopus* spinal neurons and rodent trochlear motoneurons (see Glossary, Box 1), which express UNC5 homologues (Colamarino and Tessier-Lavigne, 1995; Hong et al., 1999; Wang et al., 1999). Although these chemorepellent responses require expression of an UNC5 protein, in some cases this response also depends upon the co-expression of DCC with UNC5 (Colavita and Culotti, 1998; Hong et al., 1999). In fact, many neurons in vertebrates and invertebrates express both UNC5 homologues and DCC. However, in contrast to DCC-dependent chemorepulsion, genetic analyses in *C. elegans* and *D. melanogaster* have provided examples of UNC-5-dependent repellent responses that occur in the absence of the DCC homologues UNC-40 and Frazzled (Keleman and Dickson, 2001; Merz et al., 2001).

**Box 1. Glossary**

**Amphetamine.** Stimulant drugs that modulate neurotransmitter levels in the brain and are used to treat narcolepsy and attention deficit disorders.
**Autaptic synapse.** A synapse made by a neuron onto itself.
**Axon turning assay.** Typically, an in vitro assay to examine the capacity of a factor to turn an extending axon.
**Death domain.** A protein-protein interaction domain consisting of six alpha-helices that is associated with apoptotic signalling.
**Dopaminergic (DA) neurons.** Neurons that use dopamine as a neurotransmitter and function in controlling voluntary movements, mood and behaviour.
**Fibronectin type III domain (FNIII).** A protein module that is similar to a domain in the ECM protein fibronectin.
**Filopodia.** Dynamic finger-like actin-rich protrusions that extend from the motile edges of cells.
**Floor plate.** A cluster of highly secretory cells located at the ventral midline of the embryonic spinal cord.
**Glycophosphatidylinositol (GPI).** Glycolipid that anchors proteins to the plasma membrane.
**Growth cone.** The enlarged motile tip of an extending axon.
**Heparan sulphate proteoglycans (HSPGs).** Complex high-molecular-weight transmembrane and secreted extracellular glycoproteins with attached glycosaminoglycan (GAG) chains.
**Heparin.** Highly glycosylated carbohydrate that functions as an anti-coagulant.
**Ig domain.** A protein structural domain first identified in immunoglobulins, characterised by its β-sheet folds.
**Immunoglobulin (Ig) superfamily.** A large family of secreted and transmembrane proteins that contain regions homologous to immunoglobulins.
**Lamellipodia.** Ruffling sheet-like extensions of the plasma membrane that contain filamentous actin networks extending from the leading edge.
**Laminins.** A family of ECM glycoproteins found in basal lamina.
**Leucine-rich repeat (LRR).** A protein structural domain that contains a high frequency of leucine residues.
**Nodes of Ranvier.** The uninsulated portions of an axon between myelin internodes where action potentials are regenerated.
**Nucleokinesis.** The movement of a nucleus within a cell during cell migration.
**Oligodendrocyte.** Glial cell in the CNS that produces the myelin wrap that insulates axons.
**Optic tectum.** A structure in the dorsal part of the midbrain of non-mammalian vertebrates that is innervated by retinal ganglion cells. Homologous to the superior colliculus in mammals.
**Paranode.** Specialised adhesive junction between an oligodendrocyte and an axon that flanks the node of Ranvier.
**Pontine nuclei.** A region of the pons that relays information between the cerebral cortex and cerebellum.
**Retinal ganglion cells (RGCs).** The output neurons of the retina that project an axon along the optic nerve.
**Rho GTPase.** A family of GTP-hydrolyzing proteins that regulate actin cytoskeletal dynamics and cell adhesion.
**Rhombic lip.** Proliferative region of the dorsal hindbrain.
**Spinal commissural neurons.** Neurons that project their axons to the contralateral side of the spinal cord to innervate targets on the other side of the nervous system.
**Thrombospondin type I (TSP-1) domain.** A protein domain homologous to a sequence first identified in thrombospondin 1 (THBS1).
**Trochlear motoneurons.** Motoneurons that project an axon dorsally away from the ventral midline to innervate the extraocular muscles of the eye.
**ZU-5 domain.** A protein structural domain with homology to sequence of the tight junction protein zona occludens 1 (also known as TJP1).

**Table 1. Netrin and netrin receptor homologues**

| Species | Latin name | Netrin | Netrin receptor | |
|---|---|---|---|---|
| | | | DCC family | UNC5 family |
| Human | *Homo sapiens* | Netrin 1 | Neogenin | UNC5A |
| | | Netrin 3 (netrin 2-like) | DCC | UNC5B |
| | | Netrin 4 | | UNC5C |
| | | Netrin G1 | | UNC5D |
| | | Netrin G2 | | |
| Mouse | *Mus musculus* | Netrin 1 | Neogenin | UNC5A |
| | | Netrin 3 | DCC | UNC5B |
| | | Netrin 4 | | UNC5C |
| | | Netrin G1 | | UNC5D |
| | | Netrin G2 | | |
| Rat | *Rattus norvegicus* | Netrin 1 | Neogenin | UNC5A |
| | | Netrin 3 | DCC | UNC5B |
| | | Netrin 4 | | UNC5C |
| | | Netrin G1* | | UNC5D |
| | | Netrin G2 | | |
| Chicken | *Gallus gallus* | Netrin 1 | Neogenin | UNC5A* |
| | | Netrin 2 | DCC | UNC5B |
| | | Netrin 4* | | UNC5C |
| | | Netrin G1* | | UNC5D* |
| | | Netrin G2* | | |
| Zebrafish | *Danio rerio* | Netrin 1a | Neogenin | Unc5a* |
| | | Netrin 1b | Dcc | Unc5b |
| | | Netrin 2 | | Unc5c* |
| | | Netrin 4 | | Unc5d* |
| | | Netrin G1* | | |
| Clawed frog | *Xenopus laevis* | Netrin 1 | DCC | UNC5 |
| Fruit fly | *Drosophila melanogaster* | Netrin-A | Frazzled | UNC-5 |
| | | Netrin-B | | |
| Nematode | *Caenorhabditis elegans* | UNC-6 | UNC-40 | UNC-5 |
| Lamprey | *Petromyzon marinus* | Netrin | Neogenin | UNC-5 |
| Medicinal leech | *Hirudo medicinalis* | Netrin | – | – |
| Amphioxus | *Branchiostoma floridae* | AmphiNetrin | – | – |
| Sea squirt | *Ciona intestinalis* | Ci-netrin | – | – |
| Sea urchin | *Hemicentrotus pulcherrimus* | HpNetrin | – | – |
| Sea anemone | *Nematostella vectensis* | Netrin | – | – |

The four vertebrate UNC-5 homologues UNC5A-D are sometimes described as UNC5H1-4. To date, receptors have not been identified for the medicinal leech, amphioxus, sea squirt, sea urchin or sea anemone.
*Sequences identified in GenBank but not published in the literature.
Human: netrin 1 (Meyerhardt et al., 1999), netrin 3 (netrin 2-like) (Van Raay et al., 1997), netrin 4 (Koch et al., 2000), netrin G1 (Nakashiba et al., 2000), netrin G2 (Nakashiba et al., 2002), neogenin (Meyerhardt et al., 1997), DCC (Fearon et al., 1990), UNC5A (Tanikawa et al., 2003; Thiebault et al., 2003), UNC5B (Komatsuzaki et al., 2002; Tanikawa et al., 2003), UNC5C (Ackerman and Knowles, 1998), UNC5D (Wang, H. et al., 2008).
Mouse: netrin 1 (Serafini et al., 1996), netrin 3 (Wang et al., 1999), netrin 4 (Koch et al., 2000; Yin et al., 2000), netrin G1 (Nakashiba et al., 2000), netrin G2 (Nakashiba et al., 2002), neogenin (Keeling et al., 1997), DCC (Cooper et al., 1995), UNC5A (Engelkamp, 2002; Leonardo et al., 1997), UNC5B (Engelkamp, 2002; Leonardo et al., 1997), UNC5C (Ackerman et al., 1997), UNC5D (Engelkamp, 2002).
Rat: netrin 1 (Manitt et al., 2001), netrin 3 (Manitt et al., 2001), netrin 4 (Zhang et al., 2004), netrin G2 (Pan et al., 2010), neogenin (Keino-Masu et al., 1996), DCC (Fearon et al., 1990; Keino-Masu et al., 1996), UNC5A (Leonardo et al., 1997), UNC5B (Leonardo et al., 1997), UNC5C (Kuramoto et al., 2004), UNC5D (Zhong et al., 2004).
Chicken: netrin 1 (Serafini et al., 1994), netrin 2 (Serafini et al., 1994), neogenin (Vielmetter et al., 1994), DCC (Chuong et al., 1994), UNC5B (Bouvree et al., 2008), UNC5C (Guan and Condic, 2003).
Zebrafish: Netrin 1a (Lauderdale et al., 1997), Netrin 1b (Strahle et al., 1997), Netrin 2 (Park et al., 2005), Netrin 4 (Park et al., 2005), Neogenin (Shen et al., 2002), Dcc (Hjorth et al., 2001), Unc5b (Kaur et al., 2007; Lu et al., 2004).
Frog: netrin 1 (de la Torre et al., 1997), DCC (Pierceall et al., 1994), UNC5b (Anderson and Holt, 2002; Karaulanov et al., 2009).
Fruit fly: Netrin-A (Harris et al., 1996; Mitchell et al., 1996), Netrin-B (Harris et al., 1996; Mitchell et al., 1996), Frazzled (Kolodziej et al., 1996), UNC-5 (Keleman and Dickson, 2001).
Nematode: UNC-6 (Ishii et al., 1992), UNC-40 (Chan et al., 1996), UNC-5 (Leung-Hagesteijn et al., 1992).
Lamprey: netrin (Shifman and Selzer, 2000b), neogenin (Shifman et al., 2009), UNC-5 (Shifman and Selzer, 2000a).
Leech: netrin (Gan et al., 1999).
Amphioxus: AmphiNetrin (Shimeld, 2000).
Sea squirt: Ci-netrin (Hotta et al., 2000).
Sea urchin: HpNetrin (Katow, 2008).
Sea anemone: netrin (Matus et al., 2006).

The extracellular and intracellular domains of DCC and UNC5 exhibit a remarkable modularity of function. For example, expression in cultured *Xenopus* spinal neurons of a chimeric receptor composed of the extracellular domain of rat DCC fused to the intracellular domain of an UNC5 homologue is sufficient to elicit an axonal chemorepellent response to netrin 1 that is similar to that of full-length UNC5 (Hong et al., 1999). Conversely, a chimera composed of the intracellular domain of DCC with an UNC5 extracellular domain signals chemoattraction (Hong et al., 1999; Keleman and Dickson, 2001). An important generalisation

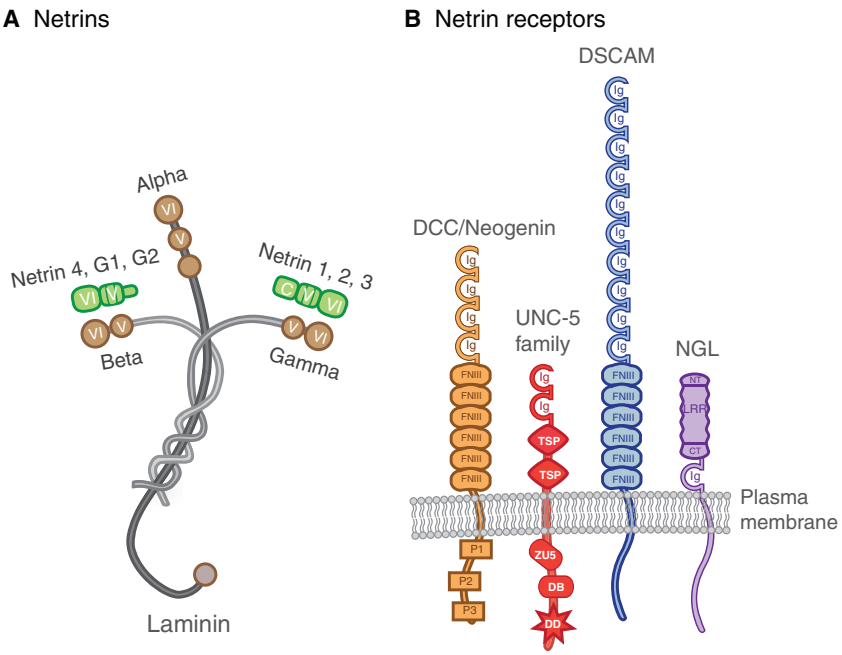


**Fig. 1. Netrin proteins and their receptors.** (**A**) Netrins are members of the laminin superfamily. N-terminal netrin sequences encode domains VI and V (green), which are homologous to the N-terminal domains VI and V of laminins (brown). These domains in netrin 1, 2 and 3 are most similar to the laminin γ chain, whereas those in netrin 4, G1 and G2 are most similar to the laminin β chain. The C-terminal C domains (C) of netrins 1-4, G1 and G2 are not homologous to laminin, nor are the C domains of netrins 1-4 homologous to the C domains of the netrin G proteins. (**B**) The netrin receptors illustrated are all single-pass transmembrane proteins and members of the Ig superfamily. They include deleted in colorectal cancer (DCC), the DCC paralogue neogenin found in vertebrates, members of the UNC5 homologue family, DSCAM and the netrin G ligands (NGLs). CT, C-terminal cysteine-rich capping structure; DB, DCC-binding domain; DD, death domain; FNIII, fibronectin type III domain; Ig, immunoglobulin domain; LRR, leucine-rich repeat; NT, N-terminal cysteine-rich capping structure; P1, P2 and P3, conserved regions in the cytoplasmic domain of DCC; TSP, thrombospondin type 1 (TSP-1) domain; ZU5, zona occludens 5 (ZU-5) domain, with homology to zona occludens 1.

drawn from these studies is that the intracellular domain of netrin receptors is crucial for their ability to mediate attractant or repellent responses to netrin.

## DSCAM

*DSCAM* was originally identified as a gene that is duplicated in Down syndrome (Yamakawa et al., 1998) and was recently reported to function as a netrin receptor (Andrews et al., 2008; Liu et al., 2009; Ly et al., 2008). DSCAM is expressed by embryonic spinal commissural neurons (see Glossary, Box 1) in mammals and contributes to guiding these axons to the floor plate (see Glossary, Box 1) of the developing spinal cord (Liu et al., 2009; Ly et al., 2008). In *Drosophila*, DSCAM and DSCAM3 similarly promote midline crossing by axons in response to Netrin-A and -B (Andrews et al., 2008). The DSCAM extracellular domain is composed of ten Ig domains and six FNIII repeats (Yamakawa et al., 1998) (Fig. 1B), with netrin 1 proposed to bind to the Ig loops (Ly et al., 2008). Current findings suggest that DSCAM evokes chemoattractant responses to netrin 1 independently of DCC (Ly et al., 2008).

## Netrin G ligands

NGL-1 and NGL-2 bind to netrin G1 and netrin G2, respectively, and are thus considered to be receptors for the netrin G proteins (Kim et al., 2006; Lin et al., 2003; Nakashiba et al., 2002). The NGL transmembrane proteins are composed of leucine-rich repeats (LRRs, see Glossary, Box 1) and Ig domains (Kim et al., 2006; Lin et al., 2003) (Fig. 1B). NGL receptors and the netrin G proteins are enriched at synapses and regulate glutamatergic synaptogenesis (Woo et al., 2009b). Notably, NGL-2 also interacts with the post-synaptic intracellular scaffolding protein PSD95 (DLG4) (Kim et al., 2006). A third member of the NGL family, NGL-3 (LRRC4B), does not bind to netrin G1 or netrin G2, but contributes to the regulation of glutamatergic synaptogenesis through interactions with the transmembrane receptor tyrosine phosphatases LAR (PTPRF), protein-tyrosine phosphatase δ (PTPδ) and PTPσ (Kwon et al., 2010; Woo et al., 2009a).

## Other netrin receptors and binding proteins

Secreted netrins and DCC also interact with heparin (Bennett et al., 1997; Serafini et al., 1994; Shipp and Hsieh-Wilson, 2007), suggesting that they bind heparan sulphate proteoglycans (HSPGs, see Glossary, Box 1). The positively charged C domain of secreted netrins interacts tightly with heparan sulphate, perhaps localising and multimerising netrin in the extracellular matrix (ECM) (Geisbrecht et al., 2003; Kappler et al., 2000; Shipp and Hsieh-Wilson, 2007). The conditional ablation of the mouse exostosin 1 (*Ext1*) gene, which encodes an enzyme required for heparan sulphate synthesis, has revealed a cell-autonomous function for heparan sulphate in embryonic spinal commissural neurons (Matsumoto et al., 2007). EXT1 is required for axonal chemoattraction to netrin 1, providing evidence for a functional interaction between HSPGs and at least one netrin receptor.

Netrins can also bind to integrins, a large family of transmembrane receptors that link the actin cytoskeleton to ECM proteins (Nikolopoulos and Giancotti, 2005). Netrin 1 binds to α6β4 and α3β1 integrins, and this is suggested to regulate epithelial cell adhesion and migration (Yebra et al., 2003). Although netrin domains VI and V are homologous to laminins, and certain integrins function as laminin receptors, α6β4 and α3β1 integrins do not bind to these domains in netrins. Instead, they bind a sequence of positively charged amino acids found at the C-terminus of netrin 1 (Yebra et al., 2003). Interestingly, however, the C-terminal domain does not appear to be required for axon chemoattraction, as a VI-V-Fc chimeric protein that lacks domain C is sufficient to promote outgrowth from rat embryonic spinal commissural axons in vitro (Keino-Masu et al., 1996). Although this suggests that α6β4 and α3β1 integrins are not essential for chemotropic responses to netrins, it does not rule out the possibility that integrins might functionally interact with netrins in other contexts.

Laminins are known to multimerise through their VI domains (Yurchenco and Wadsworth, 2004). Netrin 4, but not netrin 1, 3, G1 or G2, can be incorporated into basement membranes of various tissues through the interaction of its domain VI with

domain VI of laminin (Schneiders et al., 2007). Netrin 4 thereby inhibits basement membrane assembly by interfering with laminin multimerisation, and also inhibits branching morphogenesis in the developing lung and salivary gland (Koch et al., 2000; Liu et al., 2004; Schneiders et al., 2007). As such, netrin 4 may directly influence organogenesis by signalling to cells from the basement membrane or by modifying the structure of the basement membrane itself.

## Netrin function in the nervous system

The development of a functional nervous system depends on the establishment of precise connections between neurons. This requires the migration of neural precursors to appropriately position cell bodies, and the projection of axons to synaptic targets. Studies of knockout mice have provided substantial insight into netrin and netrin receptor function in the nervous system (see Box 2). These studies, together with studies of netrin function in other model species, have revealed that netrins direct cell and axon migration and subsequently influence axon arborisation and synapse formation during neural development. In the mature CNS, recent findings provide evidence that netrins also regulate cell-cell interactions, including maintaining the organisation of oligodendroglial paranodal junctions (Jarjour et al., 2008). Furthermore, recent studies have suggested that netrin-related changes can influence human neural circuitry and the progression of neurodegenerative diseases (see Box 3).

### Neuronal precursor cell migration

Netrin function has been extensively studied during cerebellar development. Netrin 1 attracts migrating progenitor cells that originate from the lower rhombic lip (see Glossary, Box 1) towards the ventral midline to form the pontine nuclei (see Glossary, Box 1) in the hindbrain (Alcantara et al., 2000). This process depends on the expression of netrin 1 by midline cells and on DCC expression by migrating progenitors. Netrin 1 promotes precerebellar neuron migration in mice, which is disrupted by inhibiting RHOA-dependent nucleokinesis (see Glossary, Box 1) (Causeret et al., 2004). Netrin 1-directed migration and subsequent axon outgrowth by precerebellar neurons require phosphorylation of the microtubule-associated protein MAP1B (MTAP1B) through the activation of the serine/threonine kinases cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3) (Del Rio et al., 2004). Consistent with this, MAP1B-deficient mice exhibit defects in the pontine nuclei and in several forebrain axon tracts, similar to the phenotypes of netrin 1 or *Dcc* mutants (Bloch-Gallego et al., 1999; Del Rio et al., 2004). Interestingly, during post-natal maturation, netrin 1 repels migrating cerebellar granule cell precursors, which upregulate UNC5 expression (Alcantara et al., 2000). Netrin 1 has also been implicated as a chemorepellent for migrating adult neural stem cells at sites of injury in the mature nervous system (Petit et al., 2007), highlighting similar functions for netrin in directing cell migration during development and in adulthood.

### Axon guidance

Although netrins are widely expressed in a range of tissues, they have largely been studied for their role as axon guidance cues during neural development. Substantial evidence supports the notion that netrins function as long-range chemotropic guidance cues in the embryonic vertebrate CNS. Floor plate cells express the netrin 1 gene, and a gradient of netrin 1 protein is present in the embryonic spinal cord as commissural axons extend to the ventral midline (Kennedy et al., 1994; Kennedy et al., 2006; Placzek et al., 1990; Serafini et al., 1996; Tessier-Lavigne et al., 1988) (Fig. 2A). In vitro axon turning assays (see Glossary, Box 1) have demonstrated that recombinant netrin 1 mimics the capacity of the floor plate to promote commissural axon outgrowth from explants of dorsal neural epithelium (Serafini et al., 1994). Similarly, a cellular source of netrin 1 attracts extending commissural axons, deflecting them from their dorsoventral trajectory in the embryonic neural tube (Kennedy et al., 1994). In this axon turning assay, growth cones (see Glossary, Box 1) turned up to 250 μm away from the floor plate, revealing the capacity of netrin 1 protein to diffuse at least this distance through the embryonic neural epithelium (Kennedy et al., 1994; Placzek et al., 1990). In a further reduced axon turning assay that utilised *Xenopus* retinal ganglion cells (RGCs, see Glossary, Box 1) in dispersed culture, axonal growth cones could be attracted up a gradient of netrin 1 ejected from a pipette (de la Torre et al., 1997). In addition to chemoattraction, netrin 1 functions as a repellent for other cell types, such as the trochlear motoneurons and oligodendrocyte precursor cells (OPCs) (Colamarino and Tessier-Lavigne, 1995; Jarjour et al., 2003) (Fig. 2A). Subsequent studies have demonstrated that secreted netrins direct axon extension in many different parts of the developing nervous system.

**Box 2. Netrins and netrin receptors: insights from knockout mice**

Mice lacking netrin 1 exhibit severe neurodevelopmental defects and die within a few hours of birth (Serafini et al., 1996), highlighting the importance of netrin signalling during development. Deficits in these mice include the disruption of multiple CNS commissures, including the ventral spinal commissure, the corpus callosum and the anterior and hippocampal commissures (Serafini et al., 1996). Mice lacking DCC phenocopy the netrin 1 null mice remarkably closely (Fazeli et al., 1997), highlighting the role of DCC as a key netrin 1 receptor. *Unc5a* null mice are viable and live to adulthood, but exhibit reduced neuronal apoptosis (Williams et al., 2006). *Unc5b* knockout mice die during embryogenesis due to heart failure and substantial disruption of their vasculature (Lu et al., 2004). *Unc5c* null mice survive to adulthood, but are ataxic and exhibit cell migration defects in the cerebellum (Ackerman et al., 1997; Goldowitz et al., 2000). The ventral-dorsal trajectories of axons that normally project away from netrin 1 expressed at the ventral midline are also disrupted in *Unc5* nulls, including the axons of trochlear motoneurons (Burgess et al., 2006) and of hindbrain cerebellar, inferior olivary and pontine axons (Kim and Ackerman, 2011). These findings provide evidence that UNC5 homologues direct axon extension in the mammalian CNS.

### Axon branching, innervation and synaptogenesis

Once an axon has reached its target, appropriate innervation often involves axon branching. Secreted netrins regulate branching and, similar to their roles in axon guidance, this contribution of netrins to neural development is evolutionarily conserved. For example, increased expression of the DCC homologue UNC-40 (Gitai et al., 2003), or the misexpression of the N-terminal domain of the netrin homologue UNC-6 (Lim et al., 1999), increases axon branching by motoneurons in *C. elegans* (Wang and Wadsworth, 2002). UNC-40 promotion of axon branching in *C. elegans* requires MADD-2, a tripartite motif protein that recruits the actin regulatory protein MIG-10, the homologue of lamellipodin (RAPH1) in vertebrates (Hao et al., 2010). Studies using mammalian neocortical neurons in vitro have demonstrated that local application of netrin 1

**Box 3. Neural circuitry and neurodegenerative diseases**

A recent study has identified functional *DCC* heterozygosity in humans as an underlying cause of congenital mirror movements (Srour et al., 2010), which are involuntary contralateral movements that mirror unilateral voluntary movements. These findings suggest that a reduction in *DCC* gene dosage during human development sufficiently disrupts neural circuitry to result in mirror movements. Another series of human genetic studies reported that single nucleotide polymorphisms found in the human genes encoding netrin 1 and DCC correlate with the susceptibility to develop Parkinson's disease and amyotrophic lateral sclerosis (ALS) (Lesnick et al., 2007; Lesnick et al., 2008; Lin et al., 2009). These findings are particularly intriguing in light of studies that implicate the secreted netrins and DCC in synapse formation, and lead to the hypothesis that netrin-related changes in synapse function might influence the development and progression of certain forms of human neurodegenerative diseases. The recent identification of netrin 1 as a ligand for DSCAM, a gene implicated in Down syndrome, suggests that some of the deficits associated with this disorder might result from altered netrin signalling (Liu et al., 2009; Ly et al., 2008). Netrin G proteins have also emerged as important regulators of glutamatergic synaptogenesis, and mutations in the human gene for netrin G1 are associated with an atypical form of Rett syndrome, a neurological disorder (Archer et al., 2006; Borg et al., 2005; Nectoux et al., 2007).

promotes de novo axon branch formation by rapidly inducing $Ca^{2+}$ transients, polymerisation of F-actin, and the formation of filopodial protrusions that may become a branch point (Dent et al., 2004). The induced increase in intracellular $Ca^{2+}$ appears to be crucial because inhibiting the netrin 1-mediated $Ca^{2+}$ signalling pathway disrupts axon branch formation induced by netrin 1 (Tang and Kalil, 2005).

Initial evidence in support of a role for netrins in synaptogenesis came from genetic analyses of *Drosophila* motoneurons, which form glutamatergic synapses on body wall muscles. Upregulating the expression of Netrin by muscle cells results in the increased formation of synaptic connections, whereas fewer synapses are established in the absence of Frazzled expression by the motoneuron (Kolodziej et al., 1996; Mitchell et al., 1996; Winberg et al., 1998). Interestingly, the axon guidance cue Semaphorin was found to have an opposite effect to Netrin at this synapse (Winberg et al., 1998). When the expression of Netrin and Semaphorin was either simultaneously upregulated or absent, synaptic innervation was normal, suggesting that these factors are not required for the axon to find the muscle but rather that they modulate the number of connections that are made between motoneurons and muscles. In *C. elegans*, UNC-6 regulates synaptogenesis by organising the subcellular distribution of presynaptic proteins (Colon-Ramos et al., 2007; Poon et al., 2008). These findings have also been extended to vertebrates; perfusion of netrin 1 into the *Xenopus* optic tectum (see Glossary, Box 1) during development results in a DCC-dependent increase in RGC axon branching and the formation of additional presynaptic puncta (Manitt et al., 2009).

These data support a role for netrin during the early stages of synaptogenesis, but also raise the possibility that netrin 1 and DCC influence synapse structure and function in the mature nervous system. DCC is highly expressed, particularly by dopaminergic (DA) neurons (see Glossary, Box 1), during development and in adulthood (Livesey and Hunt, 1997; Volenec et al., 1998). Mice heterozygous for loss of *Dcc* function are viable but express reduced levels of DCC protein (Fazeli et al., 1997). Intriguingly, adult *Dcc* heterozygous mice exhibit a blunted response to amphetamine (see Glossary, Box 1) and do not develop behavioural sensitisation to repeated doses of this drug (Flores et al., 2005). A recent examination of newborn *Dcc* heterozygous and null mice revealed defects in DA precursor cell migration, axon guidance and terminal arborisation (Xu et al., 2010). In particular, increased DA innervation was present in the medial prefrontal cortex of *Dcc* heterozygotes (Xu et al., 2010), a brain region that is associated with drug addiction (Steketee, 2003). Although increased innervation was detected in adult *Dcc* heterozygotes, newborns were indistinguishable from wild-type littermates (Xu et al., 2010). This indicates that the increase in DA axon arborisation occurs during post-natal development and is consistent with findings demonstrating that the response of *Dcc* heterozygotes to amphetamine changes during maturation (Grant et al., 2009). Further in vitro studies demonstrated that loss of DCC function inhibits netrin 1-induced DA axon branching and results in fewer autaptic synaptic (see Glossary, Box 1) connections per cell (Xu et al., 2010). These findings support the conclusion that DCC expression regulates the extent of axonal and terminal arborisations in the mammalian brain.

## Oligodendroglial development and maturation

Netrin 1 makes key contributions to several stages of the maturation of oligodendrocytes (see Glossary, Box 1). In the embryonic spinal cord, OPCs, which express DCC and UNC5A, are repelled by the gradient of netrin 1 that emanates from the floor plate (Jarjour et al., 2003; Tsai et al., 2006; Tsai et al., 2009; Tsai et al., 2003) (Fig. 2A). This directs OPCs away from the ventricular zone where they were born and towards axons at the edge of the neural tube (Jarjour et al., 2003; Tsai et al., 2006). Upon reaching the nascent white matter, post-mitotic oligodendrocytes elaborate highly branched processes that then extend in search of axons (Haber et al., 2009; Kirby et al., 2006) (Fig. 2B). At this point in their differentiation, oligodendrocytes express netrin 1, and both autocrine and paracrine sources promote process branching and the elaboration of myelin-like membrane sheets (Rajasekharan et al., 2009). Through DCC, netrin 1 activates the Src family kinase (SFK) member FYN in differentiating oligodendrocytes, an event confirmed by the fact that netrin 1 does not induce process branching in oligodendrocytes derived from *Fyn* knockout mice (Rajasekharan et al., 2009). In migrating OPCs, netrin 1 activates the Rho GTPase RHOA and requires DCC and the RHOA effector ROCK to mediate chemorepulsion (Rajasekharan et al., 2010). By contrast, netrin 1 inhibits RHOA in differentiating post-mitotic oligodendrocytes and this is required for netrin 1-dependent oligodendroglial process branching (Rajasekharan et al., 2010). These findings indicate that differential regulation of RHOA contributes to the distinct responses made by OPCs and post-mitotic oligodendrocytes to netrin 1.

In the mature CNS, myelinating oligodendrocytes continue to express netrin 1, DCC and UNC5 homologues (Manitt et al., 2001; Manitt et al., 2004). Netrin 1 and DCC are particularly enriched at oligodendroglial paranodal junctions (Jarjour et al., 2008) (Fig. 2B). Paranodes (see Glossary, Box 1) become disorganised in the absence of DCC or netrin 1, which results in the disruption of the nodes of Ranvier (see Glossary, Box 1) (Jarjour et al., 2008). DCC localised to the oligodendroglial membrane loops is thought to bind netrin 1 on the axon surface to mediate oligo-axonal adhesion and organise the cytoskeleton within the oligodendrocyte (Jarjour et al., 2008). Together, these

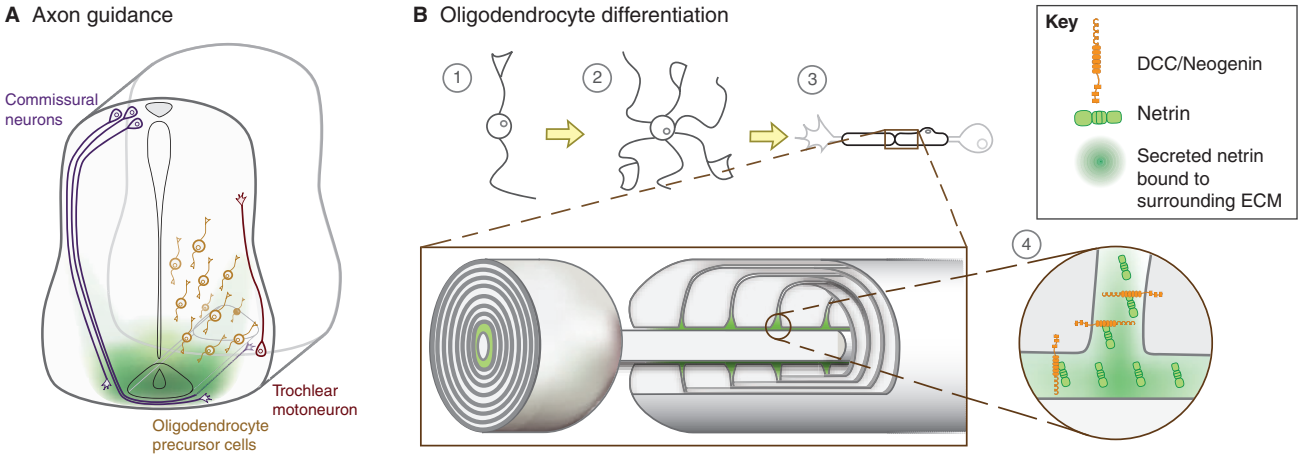


**Fig. 2. Netrin function in the nervous system.** (**A**) Within the developing spinal cord, netrin 1 (green) secreted by floor plate cells forms a gradient emanating from the ventral midline. The netrin 1 gradient is bifunctional, attracting the migration of some cells, such as spinal commissural axons (purple), and repelling others, such as migrating oligodendrocyte precursor cells (OPCs) in the spinal cord (orange) and the axons of trochlear motoneurons in the brainstem (red). (**B**) Netrin 1 influences oligodendrocytes at several stages of their differentiation: bipolar migrating OPCs (1) express DCC and UNC5A and are repelled by a gradient of netrin 1. Multipolar post-mitotic differentiating oligodendrocytes (2) express netrin 1, DCC and UNC5 homologues. Netrin 1 protein, from autocrine and paracrine sources, promotes process branching and myelin-like membrane sheet formation (3). Netrin 1 and DCC expressed by mature myelinating oligodendrocytes (4) are enriched at paranodal junctions, which are specialised junctions formed between non-compacted oligodendroglial membranes and the axon. The paranode flanks the node of Ranvier. Netrin 1 and DCC are required to maintain the organisation of paranodal junctions.

findings identify three distinct roles for netrin 1 at various points during development of the oligodendrocyte lineage: repelling precursor cell migration, promoting process elaboration during differentiation, and maintaining specialised cell-cell junctions in the mature cell. Although implicated at each stage of differentiation, how netrin 1 and DCC fulfil these different roles, and the unique signalling mechanisms involved in these events, are not well understood.

## Netrin function outside the nervous system

Netrins and netrin receptors are also expressed in a number of tissues outside of the nervous system and play key roles during development by regulating cell adhesion and tissue morphogenesis (Fig. 3). In the developing mammary gland, terminal end buds are the growing tips of the ductal network and consist of two layers: the luminal epithelial cells and the cap cells. Netrin 1 secreted by the luminal cells binds to the DCC homologue neogenin, which is expressed by the adjacent cap cells. This mediates adhesion between the two cell layers, an event required for proper terminal end bud formation (Srinivasan et al., 2003) (Fig. 3A). Another example of a non-neuronal role for netrins occurs during branching morphogenesis of the embryonic lung, where netrin 1 and 4 are expressed by epithelial stalk cells and inserted into the basement membrane surrounding the developing endoderm buds. This sheath of netrin around the developing bud functions to constrain DCC- and UNC5B-expressing distal tip cells, thereby preventing excessive branching and ectopic bud formation (Liu et al., 2004) (Fig. 3B). Pancreatic development also requires netrin 1, which is produced by epithelial ductal cells and associates with collagen IV and fibronectin in the local ECM (Yebra et al., 2003). In this context, interactions between netrin 1 and the α6β4 and α3β1 integrins are thought to contribute to epithelial cell-matrix adhesion (Yebra et al., 2003).

Netrins also contribute to the elaboration of vascular networks (Fig. 3C). Vascular endothelial tip cells exhibit highly motile protrusions that are reminiscent of axonal growth cones. These cells express UNC5B and their motility is inhibited by netrin 1, thereby limiting endothelial cell migration and blood vessel branching (Larrivee et al., 2007; Lejmi et al., 2008; Lu et al., 2004). Controversy exists, however, with regard to the precise role of netrins during vascular development, as other studies have reported that netrin promotes angiogenesis (Epting et al., 2010; Park et al., 2004; Wilson et al., 2006), perhaps reflecting differences in the populations of endothelial cells examined or experimental conditions employed. A recent study describes a role for netrin 4 in the development of the lymphatic vascular system (lymphangiogenesis) and implicates netrin 4 activation of ERK, AKT and S6 kinase in vessel formation (Larrieu-Lahargue et al., 2010). Notably, these findings provide multiple examples of netrins directing the formation of branched networks by promoting or constraining elongation and branching in different contexts. Netrin 1 can also inhibit leukocyte migration (Ly et al., 2005), and recent findings provide intriguing evidence that upregulation of netrin expression provides protection against the deleterious effects of inflammation in several tissues (Mirakaj et al., 2010; Rosenberger et al., 2009; Tadagavadi et al., 2010; Wang, W. et al., 2008).

## Netrin signalling mechanisms

Studies investigating the signal transduction mechanisms engaged by secreted netrins have focused largely on netrin 1, and relatively little is known about the specific signalling mechanisms activated by other netrin family members. Functions described for netrin 1 include the regulation of cell migration, axon extension and guidance, cell-cell and cell-substrate adhesion, cell survival and cellular differentiation. As we discuss below, recent studies have identified a number of molecular signalling components that

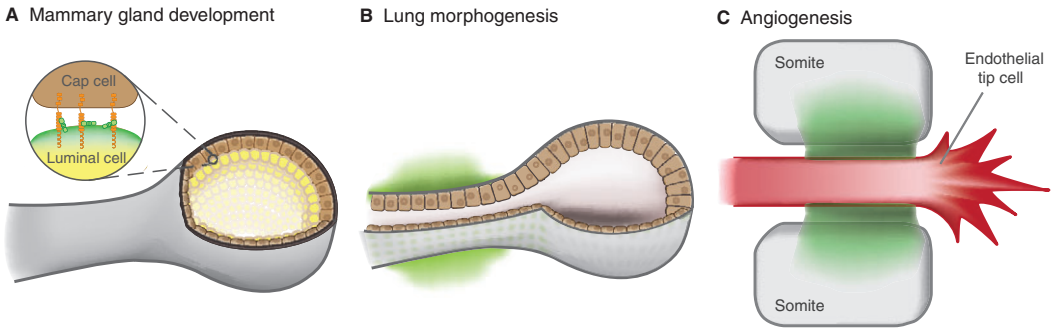


**Fig. 3. Netrin function in other developing organs and tissues.** (**A**) In mammary gland morphogenesis, the terminal end buds of ductal branches consist of two layers of cells – cap cells and luminal cells. The luminal cells express netrin 1 (green), which binds neogenin (orange) expressed by the cap cells and provides a stable adhesive interaction between the two layers. (**B**) During lung morphogenesis and the development of the bronchial tree, epithelial stalk cells secrete netrin 1 and netrin 4 into the surrounding basal lamina to inhibit inappropriate proximal branching and bud formation. (**C**) Endothelial tip cells that pioneer vascular formation are highly motile protrusive cells, similar to axonal growth cones. During angiogenesis, somites secrete netrin (green) that inhibits vascular branching via a mechanism that is dependent on UNC5B expression by endothelial tip cells.

function downstream of netrin 1, although the molecular details of how these elements interact to generate specific cellular responses are not well understood.

### Chemoattractant signal transduction cascades

In vertebrate species, studies of the axonal projections made by embryonic spinal commissural neurons and RGCs have been particularly useful for investigating the mechanisms that underlie netrin 1-mediated axon chemoattraction. The growth cone, which is found at the tip of an extending axon, projects filopodia (see Glossary, Box 1) and lamellipodia (see Glossary, Box 1) that probe the extracellular environment for guidance cues. Cytoplasmic signal transduction molecules in the growth cone link the activation of axon guidance receptors to the reorganisation of the actin cytoskeleton (Huber et al., 2003). For example, growth-promoting extracellular guidance cues induce the formation of adhesive complexes that then enhance membrane extension on one side of the growth cone by locally restricting the retrograde flow of F-actin, resulting in directional extension (Dickson, 2002; Huber et al., 2003). Recent studies have shown that netrin 1 can activate multiple downstream signal transduction molecules that regulate cytoskeletal dynamics and process extension, including SFKs and members of the Rho GTPase family (see Glossary, Box 1) (Huber et al., 2003).

In neurons that respond to netrin 1 as a chemoattractant, the intracellular domain of DCC is constitutively bound to the adaptor protein NCK1 and to focal adhesion kinase (FAK; PTK2) (Li et al., 2004; Li et al., 2002a; Ren et al., 2004). The binding of netrin 1 to DCC triggers the dimerisation of DCC via its P3 intracellular domain (Stein et al., 2001), as well as FAK autophosphorylation and tyrosine phosphorylation of the DCC intracellular domain (Ren et al., 2004). This initiates the recruitment of several intracellular signalling components to the DCC-NCK1-FAK complex (Fig. 4A), which subsequently act to regulate SFK signalling, Rho GTPase activation, the release of $Ca^{2+}$ stores, protein translation and rearrangements of the cytoskeleton.

Netrin 1 induces the recruitment and activation of FYN, which binds to DCC between P2 and P3 (Li et al., 2004; Meriane et al., 2004). FYN is thought to then regulate the activity of Rho GTPases: RAC1 and CDC42 become activated (Li et al., 2002b; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005), whereas RHOA is inhibited (Moore et al., 2008) (Fig. 4A). Consistent with these findings, which were obtained using mammalian neurons, the Rac-like GTPase in *C. elegans*, CED-10, was shown to be required for netrin-dependent axon guidance in this organism (Gitai et al., 2003). Two guanine nucleotide exchange factors (GEFs) for RAC1, TRIO and DOCK180 (DOCK1), have since been reported to function downstream of DCC in vertebrate neurons (Briancon-Marjollet et al., 2008; Li et al., 2008). These GEFs regulate the activation of Rho GTPases by promoting the exchange of GDP for GTP. Genetic analysis in *Drosophila* has also identified a role for TRIO in netrin signalling (Forsthoefel et al., 2005), whereas in *C. elegans* the Trio homologue UNC-73 is required for appropriate localisation of UNC-40 to the cell surface (Watari-Goshima et al., 2007). However, the mechanisms responsible for regulating CDC42 and RHOA downstream of DCC in neurons remain unclear. Neither *Trio* nor *Dock180* knockout mice (Briancon-Marjollet et al., 2008; Laurin et al., 2008) phenocopy the severity of the neural developmental defects found in *Dcc* or netrin 1 knockouts (Fazeli et al., 1997; Serafini et al., 1996), indicating that additional mechanisms must contribute to DCC signalling during chemotropic axon guidance.

Netrin 1 also activates the serine/threonine kinase PAK1 and, in embryonic rat spinal commissural neurons, promotes its recruitment into a complex with DCC (Shekarabi et al., 2005) (Fig. 4A). PAK1 is a downstream effector of CDC42 and RAC1 and functions as an adaptor that links NCK1 to CDC42 or RAC1 (Bagrodia and Cerione, 1999). Disruption of PAK1 binding to NCK1 blocks netrin 1-induced recruitment of PAK1 to DCC and inhibits netrin 1-induced growth cone expansion (Shekarabi et al., 2005). Additional downstream effectors of CDC42 that are activated by DCC include the actin-binding proteins Enabled/vasodilator-stimulated phosphoprotein (ENA/VASP) and neuronal Wiskott-Aldrich syndrome protein (N-WASP), which are both modulators of actin polymerisation (Lebrand et al., 2004; Shekarabi et al., 2005).

DCC-dependent commissural axon chemoattraction also involves the activation of the mitogen-activated protein kinase (MAPK) cascade (Campbell and Holt, 2003; Forcet et al., 2002; Ma et al., 2010). The extracellular signal-regulated kinases 1 and 2 [ERK1

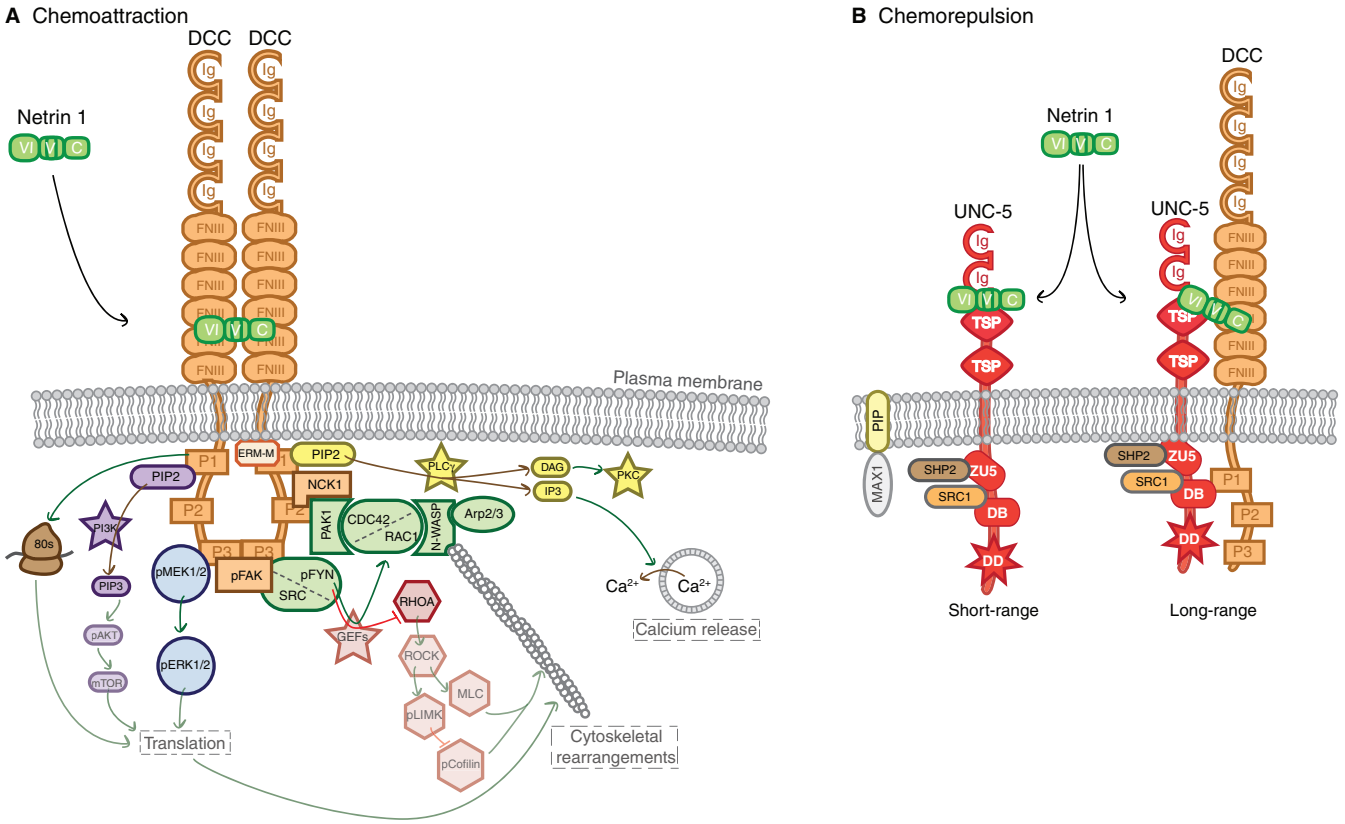


**Fig. 4. Netrin signalling.** As a guidance cue, netrin 1 dictates either an attractive or repellent response depending on the receptors expressed by the migrating cell and the signal transduction mechanisms activated. (**A**) During chemoattraction, netrin 1 binding triggers DCC homodimerisation (mediated by DCC intracellular P3 domains), and activation of constitutively bound NCK1 and FAK. This initiates the recruitment of a number of intracellular signalling components that activate Src family kinases, Rho GTPases, the release of $Ca^{2+}$ stores, protein translation and, ultimately, the rearrangement of the actin cytoskeleton. (**B**) During the generation of a chemorepellent response, netrin 1 signals through UNC5/DCC heterodimers, which are thought to facilitate long-range responses by increasing the sensitivity to relatively low netrin concentrations, or through UNC5 in the absence of DCC to mediate relatively short-range repellent responses. Although multiple proteins are known to be required for netrin 1-mediated chemoattraction and chemorepulsion, how these proteins work together to regulate chemotropic turning by a growth cone remains poorly understood. Signal transduction components illustrated as 'faded' are speculative and direct evidence for their involvement has not been obtained. 80s, eukaryotic ribosomes; Arp2/3, complex of the actin-related proteins ARP2 (ACTR2) and ARP3 (ACTR3); CDC42, cell division cycle 42; DAG, diacylglycerol; ERM-M, ezrin/radixin/moesin and merlin protein family; GEFs, guanine exchange factors; IP3, inositol 1,4,5-triphosphate; MAX1, motor axon guidance PH/MyTH4/FERM domain cytoplasmic protein; MLC, myosin light chain; mTOR, mammalian target of rapamycin; N-WASP, neuronal Wiskott-Aldrich syndrome protein; NCK1, non-catalytic region of tyrosine kinase adaptor protein 1; pAKT, phosphorylated RAC-alpha serine/threonine protein kinase; pCofilin, phosphorylated cofilin; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; pFAK, phosphorylated focal adhesion kinase; pFYN, phosphorylated Src family kinase FYN; pLIMK, phosphorylated LIM domain kinase 1; pMEK1/2, phosphorylated mitogen-activated protein kinase kinase 1/2; PAK1, p21-activating kinase 1; PI3K, phosphatidylinositol-3 kinase; PIP, phosphatidylinositol phosphate; PIP2, phosphatidylinositol (4,5) bisphosphate; PIP3, phosphatidylinositol (3,4,5) trisphosphate; PKC, protein kinase C; PLCγ, phospholipase Cγ; RAC1, ras-related C3 botulinum toxin substrate 1; RHOA, Ras homologue gene family member A; ROCK, RhoA kinase; SHP2, Src homology region 2 domain-containing phosphatase 2; SRC, tyrosine kinase sarcoma.

(MAPK3) and ERK2 (MAPK1)] are phosphorylated following netrin receptor activation, which results in the activation of specific transcription factors such as ELK1 (Forcet et al., 2002), implicating a role for netrin 1 upstream of transcriptional activation. The binding of netrin 1 to DCC also promotes the synthesis of the phosphoinositide phosphatidylinositol (4,5) bisphosphate ($PIP_2$) (Xie et al., 2005), which is phosphorylated by phosphatidylinositol-3 kinase (PI3K) and results in phosphatidylinositol (3,4,5) trisphosphate ($PIP_3$) production. Notably, $PIP_3$ facilitates the binding of GTPases to their effectors, thereby enhancing signalling (Di Paolo and De Camilli, 2006). Netrin 1 also induces $PIP_2$ hydrolysis by phospholipase Cγ (PLCγ) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$), which in turn activate protein kinase C (PKC) and stimulate the release of $Ca^{2+}$ from intracellular stores, respectively (Ming et al., 1999; Xie et al., 2006). PKC additionally mediates cytoskeletal rearrangements and translational control (Larsson, 2006), whereas increased levels of intracellular $Ca^{2+}$ are required for the axons of *Xenopus* spinal neurons to turn towards a source of netrin 1 (Hong et al., 2000). In addition to the release of $Ca^{2+}$ from intracellular stores, netrin 1 also activates transient receptor potential (TRP) channels to trigger a $Ca^{2+}$ influx across the plasma membrane of *Xenopus* spinal neurons, which is required for axon chemoattraction to netrin 1 (Wang and Poo, 2005).

In contrast to the signal transduction cascades activated downstream of DCC, little is known regarding signal transduction downstream of DSCAM in vertebrate species. It is known from in

vitro studies that the intracellular domain of human DSCAM interacts with PAK1 (Li and Guan, 2004), and netrin 1 binding triggers the activation of PAK1 and FYN (Liu et al., 2009), which are downstream signalling molecules shared with DCC (Meriane et al., 2004; Shekarabi et al., 2005). In *Drosophila*, DSCAM binds DOCK, a homologue of NCK1 in mammals, and activates PAK1 (Schmucker et al., 2000), which is reminiscent of DCC signalling in mammalian neurons (Li et al., 2002a; Shekarabi et al., 2005). Curiously, the extracellular domains of mammalian and *Drosophila* DSCAM are well conserved, but their intracellular domains are not (Schmucker et al., 2000).

### Chemorepellent signal transduction cascades

The signalling mechanisms that underlie netrin 1-induced chemorepulsion are considerably less well understood than those underlying chemoattraction, although they appear to be mediated primarily by UNC5 and UNC5-DCC signalling. Studies carried out in *Drosophila* in vivo and in mammalian cell lines in vitro support the conclusion that expression of an UNC5 homologue in the absence of DCC mediates short-range repulsion in response to netrin, whereas long-range netrin-induced repulsion requires multimerisation of UNC5 with DCC, mediated by interaction between the DB domain of UNC5 and the P1 domain of DCC (Hong et al., 1999; Keleman and Dickson, 2001) (Fig. 4B). Interestingly, studies in *Drosophila* indicate that the DB domain of UNC-5 is also required for short-range repulsion, which does not require the DCC orthologue Frazzled (Keleman and Dickson, 2001), perhaps revealing the contribution of an alternative UNC5 co-receptor. In *C. elegans*, neurons that express both UNC-5 and UNC-40 are repelled by the netrin homologue UNC-6 (Hedgecock et al., 1990) (Table 1). Initial evidence for UNC-40-dependent and -independent functions of UNC-5 in *C. elegans* came from studies demonstrating that, in *unc-5* null worms, defects in neuronal migration away from an UNC-6 source are almost as severe as those observed in *unc-6* nulls, whereas the deficits in chemorepulsion that are present in *unc-40* nulls are not as severe (Hedgecock et al., 1990). This indicates that UNC-40 is not essential for UNC-5 function in all cells. Subsequent studies in *C. elegans* demonstrated that ectopic expression of UNC-5 in neurons that normally project ventrally is sufficient to direct their axons dorsally, away from the UNC-6 source in vivo, and that this response requires UNC-40 function, supporting a role for both UNC-5 and UNC-40 in chemorepulsion in response to UNC-6 (Colavita and Culotti, 1998; Hamelin et al., 1993). This conclusion is also consistent with studies of cell and axon migration in vertebrates, which have demonstrated that the genetic ablation of *Dcc* or disruption of DCC function compromises the repellent responses of neurons to netrin 1 (Hong et al., 1999; Jarjour et al., 2003).

UNC5 function is absolutely dependent on its cytoplasmic domain (Hong et al., 1999; Keleman and Dickson, 2001; Killeen et al., 2002). Remarkably, expression of the UNC5B cytoplasmic domain alone is sufficient to trigger repulsion in *Xenopus* spinal neurons through its association with the intracellular domain of DCC (Hong et al., 1999). Deletion analyses of UNC5 revealed a functional contribution of the cytoplasmic juxtamembrane domain to axon guidance in *C. elegans*, whereas deletion of the cytoplasmic ZU-5 domain disrupted function in both *Drosophila* and *C. elegans* (Keleman and Dickson, 2001; Killeen et al., 2002), confirming that the cytoplasmic domain of UNC5 is crucial for its function. Both long- and short-range repellent responses of axons to netrin secreted from the *Drosophila* embryo midline require an intact DD (Keleman and Dickson, 2001). By contrast, the DD was found to be dispensable in *Xenopus* for chemorepellent axon turning to netrin 1 (Hong et al., 1999), perhaps owing to a species difference or to the reduced complexity of the in vitro assay employed.

Studies of the signal transduction pathway downstream of UNC5 have identified a limited number of components. Netrin 1 induces phosphorylation of UNC5 (on Y482) in a DCC-dependent manner, through the actions of SRC and FAK (Killeen et al., 2002; Li et al., 2006). This leads to the binding of the tyrosine phosphatase SHP2 (PTPN11) to UNC5 (Fig. 4B) (Tong et al., 2001). Studies in *C. elegans* have also identified roles for the PAK family member MAX-2 and the adaptor protein MAX-1 as modulators of UNC-5-mediated axon repulsion (Huang et al., 2002; Lucanic et al., 2006).

### Cell adhesion pathway signalling

When DCC binds to immobilised netrin 1 in vitro, it mediates cell-substrate adhesion (Moore et al., 2008; Shekarabi et al., 2005). The importance of this has been highlighted in a recent study showing that axon chemoattraction requires that DCC adheres to immobilised netrin 1 so as to transduce force across the plasma membrane (Moore et al., 2009). These findings support the idea that, during netrin-induced chemoattraction, DCC has two simultaneous functions: as a transmembrane bridge that links extracellular netrin 1 to the actin cytoskeleton, and as the core of a protein complex that directs the reorganisation of F-actin. In further support of this mechanism of action, it has been shown that netrin 1 and DCC, when expressed by mature myelinating rodent oligodendrocytes, are both required to maintain axo-oligodendroglial paranodal junctions (Jarjour et al., 2008).

Outside of the nervous system, netrin 1, netrin 3, netrin 4, DCC and neogenin have been shown to regulate epithelial morphogenesis in the mammary gland, pancreas, lung and lymphatic vasculature, in part by influencing cell-cell adhesion (Hebrok and Reichardt, 2004; Larrieu-Lahargue et al., 2010; Liu et al., 2004; Slorach and Werb, 2003; Srinivasan et al., 2003; Yebra et al., 2003). Furthermore, during muscle development, myoblasts express neogenin and netrin 3, and myoblast fusion to produce myotubes requires neogenin and is enhanced by the addition of netrin (Kang et al., 2004). The intracellular domain of DCC also contains a proposed ezrin/radixin/moesin and merlin (ERM-M)-binding domain to which the ERM proteins ezrin and merlin (neurofibromin 2) can bind (Martin et al., 2006). ERM proteins are ubiquitous cytoplasmic adaptors that function as links between transmembrane adhesion proteins and the actin cytoskeleton, and influence protein trafficking and signal transduction to regulate tissue organisation (Tepass, 2009). Interestingly, ectopic expression of DCC in a colon cancer cell line increased cell-cell adhesion while reducing cell-matrix adhesion, increasing the number of desmosomes between cells and reducing focal adhesions that link cells to the substrate (Martin et al., 2006). Overall, these findings suggest a role for netrins and their receptors in modulating cell-cell and cell-matrix adhesion; however, the details of these interactions and their full functional significance remain to be investigated.

### Modulation of netrin signalling by cAMP, receptor trafficking and calcium

Cyclic adenosine monophosphate (cAMP) is a well-characterised second messenger that exerts a profound influence on axon guidance and axon regeneration. Increasing cAMP activates protein kinase A (PKA), which in turn regulates Rho GTPase activation (Lang et al., 1996) and ENA/VASP function (Gertler et al., 1996; Krause et al.,

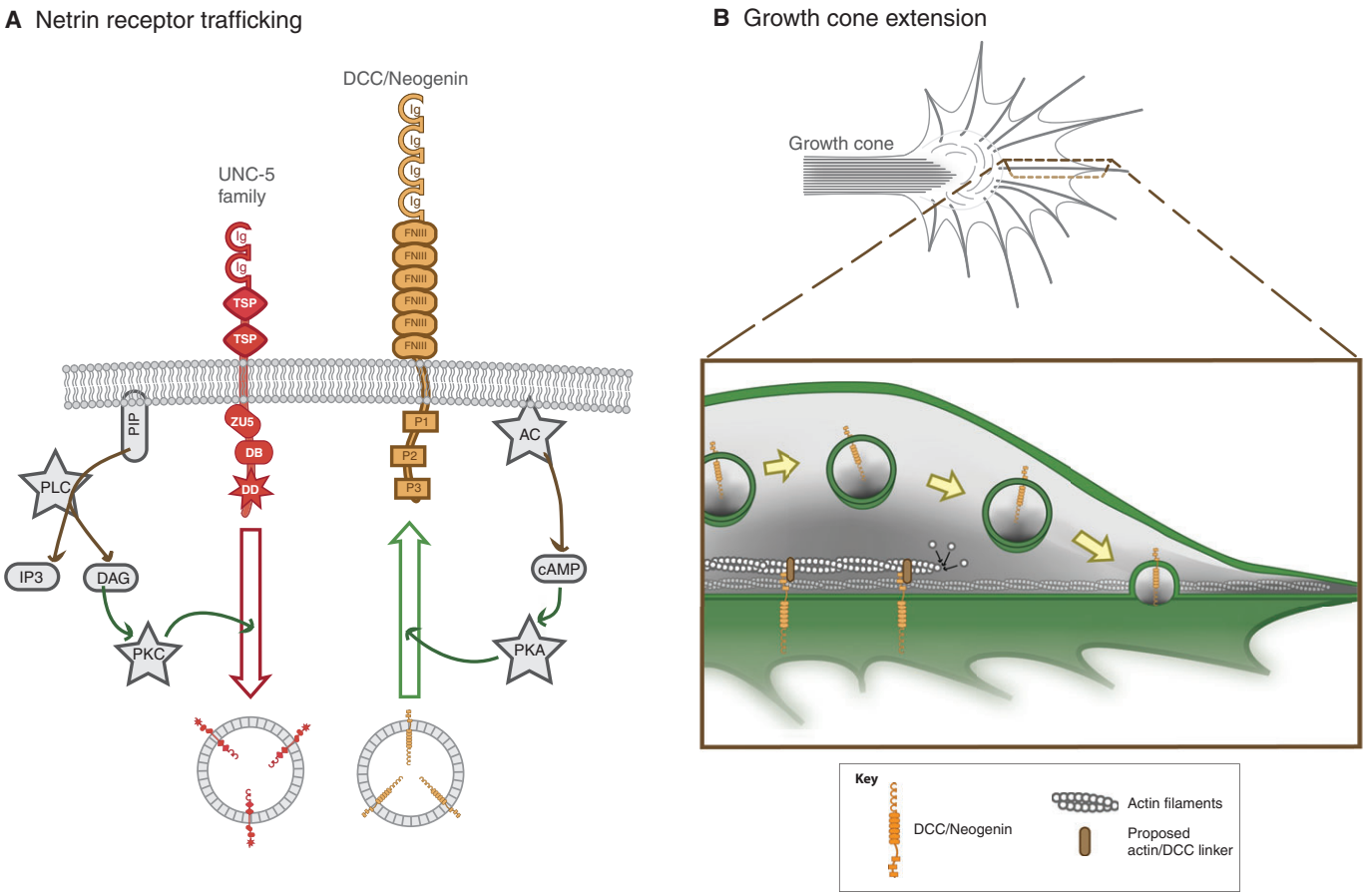


**Fig. 5. Regulation of netrin receptor trafficking and membrane recruitment.** (**A**) In axonal growth cones, the activation of protein kinase A (PKA) recruits DCC from an intracellular pool of vesicles to the plasma membrane, which enhances the axon outgrowth evoked by netrin 1. Activation of protein kinase C (PKC) activates endocytosis of UNC5A, causing neurons to switch from chemorepellent to chemoattractant responses to netrin 1. (**B**) DCC is proposed to function in axonal growth cones simultaneously as a transmembrane bridge that links extracellular netrin to the F-actin cytoskeleton and as the core of a protein complex that directs the reorganisation of F-actin. Membrane extension is hypothesised to be driven by the insertion of DCC at the leading edge of the growth cone, DCC stabilisation in the plasma membrane through binding to immobilised matrix-associated netrin, and linkage of DCC to polymerising filaments of F-actin. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate.

2003), both of which signal downstream of netrin 1 to direct cytoskeletal rearrangements (Gitai et al., 2003; Lebrand et al., 2004; Moore et al., 2008; Shekarabi and Kennedy, 2002). Importantly, it has been shown that, in response to PKA inhibition, the axons of *Xenopus* spinal neurons can shift their response to a netrin 1 gradient from attraction to repulsion (Ming et al., 1997). These findings led to the hypothesis that PKA activation regulates the direction of axon turning by altering signal transduction pathways downstream of netrin 1. More recently, it was demonstrated that PKA activation in embryonic rat spinal commissural neurons or neocortical neurons causes the relocation of DCC from an intracellular vesicular pool to the plasma membrane of the growth cone (Bouchard et al., 2008; Bouchard et al., 2004). This increased DCC presented by growth cones enhances axon outgrowth and the turning responses of these axons to netrin 1 (Bouchard et al., 2008; Bouchard et al., 2004; Moore and Kennedy, 2006). The inhibition of RHOA, which is a downstream consequence of PKA activation, also causes DCC to be recruited to the plasma membrane and promotes commissural axon outgrowth in response to netrin 1 (Moore et al., 2008). Interestingly, PKA inhibition did not result in embryonic rat spinal commissural axons switching their response to repulsion, but instead reduced the extent of their attraction to a gradient of netrin 1 (Moore and Kennedy, 2006). These findings indicate that PKA regulates the sensitivity of embryonic spinal commissural neurons to a gradient of netrin 1 by modulating the trafficking of DCC (Fig. 5A). Membrane extension is thus hypothesised to be driven by the insertion of DCC at the leading edge of growth cones, the stabilisation of DCC in the plasma membrane, the linking of DCC to actin filaments, and by signalling mechanisms that are activated by DCC to promote actin polymerisation (Fig. 5B). This mechanism combines the substrate-cytoskeletal coupling model that describes the action of adhesion molecules, such as L1CAM (Suter and Forscher, 2000), with the localised activation of signalling that directs cytoskeletal reorganisation (Hall and Lalli, 2010).

Conversely, the activation of PKC triggers the endocytosis of UNC5 homologues (Fig. 5A), resulting in cultured cerebellar granule cell neurons switching from repellent to attractant responses to netrin 1 (Bartoe et al., 2006). In rat hippocampal neurons, activating PKCα recruits the adaptor protein interacting with C kinase 1 (PICK1) to the UNC5A intracellular domain, triggering internalisation of UNC5A but not DCC, consequently switching the response of these cells to netrin 1-mediated attraction

(Williams et al., 2003). Together, these findings identify the regulation of netrin receptor trafficking as a key determinant of the migratory response made by axonal growth cones.

Decreasing cytoplasmic $Ca^{2+}$ levels, by blocking $Ca^{2+}$ release from intracellular stores or by inhibiting its influx through $Ca^{2+}$ channels, can also convert *Xenopus* spinal neuron responses to netrin 1 from attraction to repulsion (Hong et al., 2000). This requires calcium-calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN) phosphatase, with high local $Ca^{2+}$ concentrations favouring CaMKII-induced attraction and moderate levels of $Ca^{2+}$ activating CaN to mediate repulsion (Wen et al., 2004). Cyclic guanosine monophosphate (cGMP) signalling also influences the response to netrin 1: a high intracellular ratio of cAMP to cGMP promotes the attraction of *Xenopus* spinal neuron growth cones to netrin 1 by activating $Ca^{2+}$ entry through L-type calcium channels (LCCs), whereas a low ratio results in decreased $Ca^{2+}$ influx and netrin 1-mediated repulsion (Nishiyama et al., 2003).

### Effects of netrin signalling on localised protein translation

Extending axons contain a subpopulation of transported mRNAs and the machinery for local protein translation, providing the growth cone with a substantial level of functional autonomy from the cell body during embryogenesis (Lin and Holt, 2008). Recent studies suggest that localised binding of netrin to its receptors can activate translation and can function to restrict new protein synthesis to specific subdomains of a cell or growth cone, thereby influencing axon growth. In *Xenopus* RGCs, for example, the application of netrin 1 rapidly activates translation initiation factors and increases the local synthesis of proteins such as β-actin (Leung et al., 2006). Importantly, activation of translation is required for the netrin-mediated growth cone turning of *Xenopus* RGCs (Campbell and Holt, 2001) and is regulated by the ERK and p38 MAPK (MAPK14) pathways (Campbell and Holt, 2003). Finally, a recent study provides evidence that DCC binds directly to large and small ribosomal subunits, eukaryotic initiation factors and monosomes (Tcherkezian et al., 2010), suggesting that DCC can act to anchor the translation machinery to the plasma membrane and spatially restrict protein synthesis.

**Box 4. Netrins and cancer: the 'dependence-receptor' hypothesis**

Although evidence suggests that DCC has an anti-oncogenic role, how the disruption of netrin signalling contributes to malignancy remains controversial. In colorectal cancer, allelic deletion involving chromosome 18q21, which encodes DCC, occurs in over 70% of tumours (Vogelstein et al., 1988; Fearon et al., 1990). *DCC* expression is reduced in many cancers (Ekstrand et al., 1995; Reyes-Mugica et al., 1997) and DCC loss correlates with the development of highly invasive glioblastoma multiformae (Reyes-Mugica et al., 1997). Ectopic expression of *DCC* in transformed epithelial cells reduces tumourigenicity (Klingelhutz et al., 1995; Rodrigues et al., 2007) and expression of *DCC* antisense RNA in transformed fibroblasts results in faster cell growth, anchorage independence and tumourigenicity when cells are transplanted into nude mice (Narayanan et al., 1992). Such findings suggest that DCC loss dysregulates a mechanism that normally restrains cell motility. However, increased tumour formation is not detected in *Dcc* knockout mice (Fazeli et al., 1997), although tumours might not have had time to develop owing to the early post-natal lethality of *Dcc* nulls.

Substantial evidence supports a role for netrin 1 as an anti-apoptotic survival factor (Mehlen and Furne, 2005). A dependence mechanism has been proposed to underlie this function, whereby DCC, neogenin or an UNC5 homologue trigger apoptosis in the absence of netrin, and loss of receptor expression or upregulation of netrin expression is predicted to provide a selective advantage to tumour cell growth. Although netrin 1 can influence cell survival, the dependence-receptor hypothesis remains controversial. Mice lacking netrin 1 do not exhibit increased apoptosis in the CNS, arguing that netrin 1 is not an essential dependence ligand in the developing CNS (Williams et al., 2006). Additionally, quantitative analyses of apoptosis by specific neural cell types in *Dcc* knockouts have not supported a pro-apoptotic role for DCC signalling in vivo (Jarjour et al., 2003; Tsai et al., 2003; Shi et al., 2010; Xu et al., 2010).

## Conclusions

Netrins are essential chemotropic cues for migrating cells and axons during neural development. Although the majority of studies thus far have focused on this guidance role in the embryonic nervous system, it is now apparent that netrin family members and their receptors participate in a range of functions in several tissues, both throughout development and in adulthood. Tremendous advances have been made in identifying the signal transduction components required for netrin function. Determining how netrin receptors and signal transduction proteins function as an ensemble in the axonal growth cone to regulate motility remains a major challenge for current studies. Netrins and netrin receptors have now also been demonstrated to regulate adhesion in several cellular contexts; however, how the signalling mechanisms that direct motility during development subsequently switch during maturation to regulate cell-cell interactions and adhesion remains to be determined. In mature tissues, exciting recent findings implicate netrins in the regulation of adult stem cell migration, in tumour cell survival (see Box 4) and as modulators of inflammation, suggesting potentially novel means of promoting recovery from injury or disease. In this regard, netrins, netrin receptors and the downstream signalling mechanisms involved are promising targets for the development of treatments for neurodegenerative disease, vascular disease and cancer.

**Acknowledgements**

We thank Katherine E. Horn and Sathyanath Rajasekharan for comments on the manuscript. Supported by a grant to T.E.K. from the Canadian Institutes of Health Research (CIHR). K.L.W.S. was supported by a Jeanne-Timmins Costello Graduate Fellowship. T.E.K. is a Killam Foundation Scholar and holds a Chercheur National award from the Fonds de la Recherche en Santé du Québec.

**Competing interests statement**

The authors declare no competing financial interests.

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