Netrin-1 signaling through DCC enhances synapse number and function

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ABSTRACT

Actin-based changes in filopodia dynamics and spine morphology play an important role in initiating synapse formation and mediating synaptic plasticity. During embryonic development, the guidance cue netrin-1 signals through Deleted in Colorectal Cancer (DCC) receptors to modulate actin cytoskeleton dynamics, enhancing the formation and motility of filopodia. We recently reported that netrin-1 and DCC continue to be expressed in the adult spinal cord, but their function remains elusive. Here, we report that netrin-1 and DCC are expressed in adult cortical and hippocampal neurons, where they distribute to synaptic sites. In primary cortical neuron cultures, while netrin-1 is restricted to dendrites, DCC localizes to both axonal and dendritic compartments. We find that netrin-1 increases the density of axonal and dendritic filopodia and branches in a DCC-dependent manner. We demonstrate that, although dispensable for synapse formation and maintenance, netrin-1-DCC signaling enhances the number and efficacy of synaptic sites *in vitro*. These observations suggest a novel role for netrin-1 and DCC in synaptogenesis and synaptic plasticity.

RÉSUMÉ

Les changements de la dynamique des filopodes et de la morphologie des épines dendritiques dépendent de la régulation du cytosquelette d'actine et jouent un rôle important lors de l'initiation de la formation de synapses et de la médiation de la plasticité synaptique. Durant le développement embryonnaire, la molécule de guidance nétrine-1 signale via son récepteur DCC la modulation des dynamiques du cytosquelette d'actine, favorisant la formation et la motilité des filopodes. Nous avons récemment démontré que nétrine-1 et DCC continuent d'être exprimé dans la moelle épinière chez l'adulte, mais leur fonction reste élusive. Dans cet article, nous demontrons que nétrine-1 et DCC sont exprimés dans les neurones adultes du cortex et de l'hippocampe, où ils se distribuent à des sites synaptiques. Dans des cultures primaires de neurones corticaux, alors que nétrine-1 est restreinte dans le compartiment dendritique, DCC se localise tant au niveau des axones que des dendrites. Nous avons découvert que nétrine-1 augmente la densité des filopodes et des branches axonales et dendritiques, par un mécanisme requérant DCC. Bien que non essentiel à la formation et maintenance de synapses, nous rapportons que le signal engendré par nétrine-1 et DCC augmente le nombre et l'efficacité des sites synaptiques *in vitro*. Ces observations suggèrent un nouveau rôle pour nétrine-1 et DCC dans la synaptogenèse et la plasticité synaptique.

CONTRIBUTIONS OF AUTHORS

Chapter 2. Netrin-1 signaling through DCC enhances synapse number and function

Figure 1 — *In situ* hybridization for *netrin-1* and *dcc* were performed by Dr. Cecilia Flores using riboprobes created by Colleen Manitt.

Figure 2-9 — I performed all of the experiments required for the assembly of these figures.

LIST OF ABBREVIATIONS

A	Adult
A2b	Adenosine receptor 2b
ADF	Actin-Depolymerizing Factor
AMPA	α-Amino-3-hydroxy-5-Methyl-isoxasole Propionic Acid
Arp2/3	Actin-Related Protein complex 2/3
AZ	Active Zone
BDNF	Brain-Derived Neurotrophic Factor
Ca^{2+}	Calcium
CAM	Cell Adhesion Molecule
CaMK	Calcium/calmodulin-dependent Kinase
cAMP	Adenosine 3',5'-monophosphate
CapZ	Capping Protein
CASK	CaMK-, SH3- and guanylate-Kinase-domain containing protein
cc	corpus callosum
Cdc42	Cell Division Cycle 42
cGMP	Guanosine 3',5'-monophosphate
CNS	Central Nervous system
CNR	Cadherin-related Neuronal Receptor
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
DB	DCC-Binding domain
DCC	Deleted in Colorectal Cancer
DCCfb	Deleted in Colorectal Cancer function-blocking antibody
DD	Death Domain
DIV	Days *In Vitro*
E	Embryonic
ECM	Extracellular Matrix
F-actin	Filamentous actin
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FNIII	Fibronectin-type III
GAP-43	Growth Associated Protein of 43 kDa
GFP	Green Fluorescent Protein
GluR1	Glutamate Receptor 1
GRIP	Glutamate Receptor Interacting Protein
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HB-GAM	Heparin-Binding Growth-Associated Molecule
HEK	Human Embryonic Kidney cell
ICAM	Intercellular Adhesion Molecule
Ig	Immunoglobulin
LTD	Long-Term Depression
LTM	Long-Term Memory
LTP	Long-Term Potentiation
MAG	Myelin-Associated Glycoprotein

MAP2	Microtubule Associated Protein 2
MAPK	Mitogen Activated Protein Kinase
mEPSC	miniature Excitatory Post-Synaptic Current
Mint1	Munc18-1 Interacting protein 1
mRNA	messenger Ribonucleic Acid
Munc	Mouse Unc homologs
N-Cadherin	Neural Cadherin
NCAM	Neural Cell Adhesion Molecule
NFM	Medium-sized Neurofilament
NGF	Nerve Growth Factor
N-GFP	Netrin-1::Green Fluorescent Protein chimera
NMDA	N-Methyl-D-Aspartate
NMJ	Neuromuscular Junction
NT	Neurotrophin
N-WASP	Neuronal Wiskott-Aldrich Syndrome Protein
P	Postnatal
PBS	Phosphate Buffered Saline
PDZ	PSD-95, Dlg and ZO-1
PFA	Paraformaldehyde
PI_3K	Phosphatidylinositol 3-Kinase
PKA	cAMP-dependent Protein Kinase A
PKC	Calcium-dependent Protein Kinase C
PLC	Phospholipase C
PSD	Post-Synaptic Density
Rac	Ras-related C3 botulinum toxin substrate
RCM	Repulsive Guidance Molecule
RFP	Red Fluorescent Protein
RIM	Rab3-Interacting Molecule
Rho	Ras Homolog
rNet	Recombinant Netrin-1 protein
SNAP-25	Synaptosomal-Associated Protein 25
STM	Short-Term Memory
STP	Short-Term Potentiation
SV	Synaptic Vesicle
SV2	Synaptic Vesicle protein 2
SVP-38	Synaptic Vesicle Protein of 38 kDa
SynCAM	Synaptic Cell-Adhesion Molecule
TIMP	Tissue Inhibitor of Metalloproteinase
Trk	Tropomyosin-Receptor Kinase
Tsp-1	Thrombospondin type 1
UNC	Uncoordinated
VAMP2	Vesicle-Associated Membrane Protein 2
ZO-1	Zona Occludens protein 1
ZU5	ZO-1 and Unc5-like domain

INTRODUCTION

The generation of a functional neuronal network requires the elaboration of precise synaptic connections between neurons and their targets. This connectivity is established during the development of the nervous system in a series of continuous events that can be divided into three stages: (1) neurite guidance, (2) target recognition, and (3) synapse formation. Fine-tuning of neural circuitry is not limited to neural development as experience can alter synapse number, structure and function; a process referred to as synaptic plasticity.

Despite substantial progress in the identification of molecules responsible for the targeting of axons and dendrites (Dickson, 2002; Huber et al., 2003), as well as the characterization of the molecular components of synapses (Scannevin and Huganir, 2000; Sheng, 2001), the molecular events that trigger synaptogenesis remain unclear. There is evidence that the initial contact between axons and dendrites is sufficient to establish a functional synaptic site (Fiala et al., 1998; Ziv and Garner, 2001; Ziv and Smith, 1996), and that dynamic actin-based filopodial extensions from both axonal and dendritic compartments are important in initiating this contact (Chang and De Camilli, 2001; Jontes and Smith, 2000). Moreover, activity-dependent actin reorganization was shown to contribute to synaptic plasticity (Fukazawa et al., 2003; Krucker et al., 2000; Lendvai et al., 2000; Trachtenberg et al., 2002). The identification of signals that influence actin dynamics and the formation of axonal and dendritic filopodia is therefore essential to understand the molecular mechanisms of synapse formation and synaptic plasticity.

Netrins are a family of secreted molecules involved in cellular, axonal and dendritic guidance during the development of the central nervous system (CNS) (Kim and Chiba, 2004; Manitt and Kennedy, 2002). Netrin-1 signals through Deleted in

Colorectal Cancer (DCC) receptors (KeinoMasu et al., 1996) to modulate actin cytoskeleton dynamics, enhancing the formation and motility of filopodia (Dent et al., 2004; Li et al., 2002b; Shekarabi and Kennedy, 2002). Although both netrin-1 and DCC continue to be expressed in the adult CNS (Manitt et al., 2001; 2004), their function remains unclear. We sought to elucidate the role of netrin-1 and DCC in the mature nervous system, specifically their involvement in the formation of axonal and dendritic filopodia, and their possible role in initiating synaptic contacts and modulating synaptic transmission.

Here, we report that netrin-1 enhances axonal and dendritic branching and filopodia formation in a DCC-dependent manner *in vitro*, suggesting that netrin-1 functions to augment the chances of axo-dendritic contact. Accordingly, we report that netrin-1 increases the number of synapses in primary cortical cultures. In addition, netrin-1 improved synaptic transmission *in vitro*. Our data suggest novel functions for netrin-1 and DCC in synapse formation and synaptic plasticity.

Chapter 1

Literature Review

1. Axonal Guidance

1.1 Guidance Mechanisms

The generation of a functional neuronal network requires that neurites – axons and dendrites – navigate precisely over long distances to establish contact with their appropriate targets. To ensure accurate targeting, extending axons and dendrites are guided by growth cones at their leading edge, which sense and respond to molecular cues in the environment (Huber et al., 2003; Kim and Chiba, 2004). During the past decade, several proteins have been functionally characterized as guidance cues based on their ability to specify guidance decisions, including semaphorins, netrins, ephrins and Slits (reviewed in Dickson, 2002; Huber et al., 2003). While these factors were originally characterized as either permissive or inhibitory cues, it is now clear that individual guidance molecules can function as both attractants and repellents. The response of a growth cone to a given cue depends on the intracellular state of the cell, the differential expression of receptor complexes, and cross-talk between intracellular signaling cascades (Guan and Rao, 2003). In particular, intracellular calcium and cyclic nucleotide levels were shown to modulate attractive or repulsive axonal turning responses (reviewed in Song and Poo, 1999). For example, the attractive effects of brain-derived neurotrophic factor (BDNF) and netrin-1 on *Xenopus* spinal neurites *in vitro* can be converted to repulsion in the presence of agents that lower adenosine 3',5'-monophosphate (cAMP) levels, or inhibit of cAMP-dependent protein kinase A (PKA) activity (Ming et al., 1997; Song et al., 1997). Conversely, the repellent actions of semaphorin III and myelin-associated glycoprotein (MAG) were converted to attraction upon pharmacological elevation of guanosine 3',5' monophosphate (cGMP) and cAMP, respectively (Song et al., 1998). Furthermore, elevated cytoplasmic calcium levels were demonstrated to induce

growth cone turning, to enhance netrin-1 attraction, and to convert MAG/myelin-mediated repulsion into attraction (Ming et al., 2001; Zheng, 2000). Although it remains unclear how these intracellular second messenger pathways ultimately influence axonal pathfinding, we and others have recently shown that PKA (Bouchard et al., 2004) and protein kinase C (PKC) activities (Williams et al., 2003) differentially influence the cell surface presentation of guidance cue receptors, providing one mechanism to explain these observations.

1.2 Growth Cone Cytoskeletal Dynamics

It is well established that changes in growth cone shape and direction are dictated by the reorganization of the cytoskeleton, especially actin filaments (Gallo and Letourneau, 2004). Neuronal growth cones typically consist of a central microtubule-rich core and a flattened peripheral domain composed of two actin-rich membrane structures at its leading edge called filopodia and lamellipodia. Filopodia are dynamic finger-like protrusions composed of bundles of filamentous actin (F-actin) that extend away from the growth cone and sample the extracellular environment. Filopodia contain receptors on their surface that respond to guidance cues, the contact of a single filopodium with extracellular guidance molecules being sufficient to modulate the path of an extending axon (Chien et al., 1993; O'Connor et al., 1990). Lamellipodia contain cross-linked networks of actin filaments and provide adhesion with the substrate (Jay, 2000). Both structures are highly motile, constantly expanding and retracting as they sample the environment and guide growth cones along their paths (Kater and Rehder, 1995; Zheng et al., 1996). Growth cone advancement is controlled by the properties of actin filament dynamics within filopodia and lamellipodia, namely (1) the assembly of actin monomers

into F-actin filaments at the growth cone leading edge (Mallavarapu and Mitchison, 1999), (2) the continuous retrograde flow of F-actin filaments towards the central domain (Lin et al., 1996; Lin and Forscher, 1995), and (3) the disassembly and recycling of actin filaments in the central domain (Suter and Forscher, 2000). Consequently, attractive guidance cues were proposed to promote actin polymerization and/or prevent retrograde flow, whereas repulsive cues were suggested to mediate opposite effects on these processes (reviewed in Mueller, 1999).

1.3 Guidance Cue Signaling Mechanisms

How do guidance molecules regulate cytoskeletal dynamics within growth cones? One model, referred to as the "substrate cytoskeletal coupling" model, relies on the ability of substrate-bound cues to create the pulling forces necessary for growth cone extension and guidance. In this model, forward movement is generated by the linkage of motile actin filaments to a fixed substrate via cell surface receptors. This functional 'coupling' attenuates retrograde actin flow and produces the tensile forces required to pull the central domain of the growth cone forward. Simultaneous polymerization of actin filaments at the leading edge further ensures growth cone extension (Lin et al., 1994; Mitchison and Kirschner, 1988; Suter and Forscher, 2000).

This model however, cannot account for the ability of soluble cues to guide axonal and dendritic growth cones. It has become evident during the past few years that guidance factors regulate cytoskeletal dynamics by signaling through proteins belonging to the Rho family of small GTPases. Members of the Rho family of GTP-binding proteins are key regulators of actin polymerization, branching and depolymerization (see Dickson, 2002; Hall, 1998; Luo, 2002 for review). The best studied Rho GTPases are

Rac, Cdc42 and RhoA. Whereas RhoA is primarily responsible for the formation of stress fibers and actin-myosin-dependent contractility, Rac and Cdc42 regulate the formation of protrusive lamellipodia and filopodia, respectively (Hall, 1998). Thus, guidance cues causing growth cone repulsion and collapse were proposed to signal through RhoA, and attractive cues through Rac and Cdc42 (Mueller, 1999; Yuan et al., 2003). Consistent with this, the activity of Rho GTPases has been implicated in the signaling pathways of several guidance cues including netrins (Gitai et al., 2003; Li et al., 2002b; Shekarabi and Kennedy, 2002), semaphorins (Liu and Strittmatter, 2001), ephrins (Wahl et al., 2000) and Slits (Wong et al., 2001). However, some evidence suggests that growth cone guidance signals can also be transduced independently of Rho GTPases (Luo, 2002). Other modulators of actin assembly such as barbed end cappers (CapZ), polymerization factors (ADF), actin monomer sequestering agents (profilin, thymosin β4), actin nucleation factors (Arp2/3), actin stabilizer (filamin), as well as modulators of myosin motor function are possible candidates for the regulation of the cytoskeletal architecture in growth cones by guidance cues and adhesion molecules (Bridgman et al., 2001; Mallavarapu and Mitchison, 1999; Wylie and Chantler, 2001).

2. CNS Synaptogenesis

Central synapses are asymmetric sites of cell-cell contact designed to mediate fast and efficient transmission of chemical signals between nerve cells. The presynaptic plasma membrane contains an electron-dense region, known as the active zone (AZ), where neurotransmitter-filled synaptic vesicles (SVs) dock and fuse to release their content into the synaptic cleft (Dresbach et al., 2001; Ziv and Garner, 2004). In close juxtaposition to the AZ is the postsynaptic density (PSD), a specialized electron-dense

membrane domain which serves to cluster neurotransmitter receptors and ion channels (Scannevin and Huganir, 2000; Sheng, 2001).

The processes underlying central synapse formation have fascinated neuroscientists for decades but, until recently, attempts made at understanding CNS synaptogenesis were limited to ultrastructural analysis (Bartlett and Banker, 1984; Vaughn, 1989). It is well established that most CNS synapses are formed during early postnatal development up until young adulthood (Blue and Parnavelas, 1983; Cragg, 1975; Vaughn, 1989; Zecevic and Rakic, 1991). In rodents, it is estimated that half the total number of synapses in the adult is generated during the first two postnatal weeks, reaching 80% of adult numbers by the end of the third week (Hinds and Hinds, 1976; Smolen and Raisman, 1980). Recent molecular and imaging techniques provided significant insight in the cellular and molecular dynamics involved in the formation of synaptic contact. Synapse assembly is considered to be a multi-step process. Following initial contact, a stable synaptic adhesion site is established and both the axonal and dendritic compartments differentiate into pre- and postsynaptic specializations, respectively (Garner et al., 2002; Ziv and Garner, 2001). Furthermore, a precise understanding of the mechanisms by which initial synaptic contacts are established during the development of the CNS is thought to provide insight into the processes of structural plasticity in the adult brain (Lamprecht and LeDoux, 2004).

2.1 Axo-Dendritic Contact

There is evidence that the initial contact between axons and dendrites is sufficient to establish a functional synaptic site (Ahmari et al., 2000; Alsina et al., 2001; Fiala et al., 1998; Friedman et al., 2000; Ziv and Garner, 2001; Ziv and Smith, 1996). Factors

enhancing the chances of axo-dendritic contact therefore play an important role in the initiation of synaptogenesis. Because of the motility, responsiveness and exquisite sensitivity of axonal growth cones, extending axons have typically been considered the determining factor in initiating specific contact with the dendritic arbor or soma of appropriate target cells. This model, which is mainly based on the well-characterized development of the neuromuscular junction (NMJ) (Burden, 2002; Sanes and Lichtman, 1999), where target muscles are rather stationary, attributes a relatively passive function to dendrites. While it is well accepted that axonal growth cones play a central role in wiring the nervous system and initiating axo-dendritic contact, numerous studies have recently suggested a role for actin-based dendrite extensions, namely dendritic filopodia and spines, in synaptogenesis and the remodeling of connectivity (Jontes et al., 2000; Luscher et al., 2000; Ziv and Smith, 1996). The observation that the majority of excitatory synapses in the CNS are formed between spines and varicosities along en passant axons (Gray, 1959; Harris and Kater, 1994; Vaughn, 1989), suggested that dendritic extensions function to efficiently connect axon fibers with a multitude of dendritic arbors, without the need for axons to run convoluted paths (Anderson and Martin, 2001). In the past few years, dendritic filopodia have been extensively studied both *in vitro* and *in vivo* (Yuste and Bonhoeffer, 2004). Because their presence often precedes the formation of synaptic contacts and spines, and because their number and motility decreases as neurons mature, dendritic filopodia have been proposed to initiate axo-dendritic synaptogenesis and to be the precursors for dendritic spines and branches (Dailey and Smith, 1996; Smith, 1999; Ziv and Smith, 1996). Thus, one emerging principle is that dendrites as well as axons are active in seeking out nearby synaptic partners.

2.2 Time Course of Synapse Assembly

Live imaging and retrospective immunolabeling of cultured hippocampal neurons revealed that many components of glutamatergic synaptic junctions are assembled with surprising speed following initial contact between axons and dendrites. Indeed, synaptic transmission can be recorded within an hour of axo-dendritic contact and individual synaptic proteins accumulate to new synaptic sites within minutes (Antonova et al., 2001; Friedman et al., 2000; Okabe et al., 2001; Washbourne et al., 2002). The presynaptic AZ scaffold protein Bassoon and the SV membrane protein synaptophysin were detected at new synaptic sites before postsynaptic NMDA receptors and the postsynaptic scaffold protein PSD-95 (Friedman et al., 2000; Okabe et al., 2001), indicating that presynaptic differentiation precedes postsynaptic specialization. This being said, relatively few synaptic molecules have been studied in detail, and a broader spectrum of synaptic components need to be analyzed before conclusively determining whether one synaptic side differentiates before the other. Nevertheless, it is clear that synaptogenesis takes place within hours *in vitro*, as opposed to days as previously inferred from ultrastructural analyses (Lee and Sheng, 2000; Rao et al., 1998).

2.3 Presynaptic Assembly

Because of the speed at which synapses assemble, it has been proposed that synapses form by rapid recruitment of pre-assembled synaptic components to the sites of cell contact, thereby bypassing the need to built synapses 'from scratch'. This hypothesis was recently supported by two independent groups that reported the existence of two types of precursor complexes. Using a GFP-tagged SV molecule (synaptobrevin II/VAMP2), Ahmari and colleagues (2000) reported rapid accumulation of mobile

vesicular packets at new axo-dendritic contact sites. These VAMP2-positive puncta colocalize with presynaptic molecules such as voltage-gated calcium channels, synaptic vesicle protein 2 (SV2), amphiphysin and synapsin I. In another study, a previously unknown 80 nm dense-core vesicle was shown to contain multiple presynaptic AZ components, including the presynaptic scaffold proteins Piccolo, Bassoon and RIM, and the cell adhesion molecule N-Cadherin (Zhai et al., 2001). This vesicle also contains AZ proteins implicated in SV exocytosis such as Munc13, Munc18, syntaxin, SNAP-25 and N-type calcium channels (Shapira et al., 2003). Dense-core vesicles differ from the previously identified VAMP2 vesicles in that they are devoid of SV molecules. Instead, they are thought to constitute 'active zone precursor vesicles'. It has been proposed that the formation of a functional presynaptic site results from the fusion with the presynaptic plasma membrane of 2-5 dense-core vesicles and 1-4 VAMP2 packets (Ahmari et al., 2000; Friedman et al., 2000). However, it remains unclear whether all excitatory active zones are assembled in such a manner, and what the molecular signals for the exocytosis of such vesicles are.

2.4 Postsynaptic Receptor Complex Assembly

The mechanisms of assembly of postsynaptic receptor complex are less well characterized than on the presynaptic side. The postsynaptic scaffold protein PSD-95 and NMDA receptors are first detected on the postsynaptic side within 20-60 min following contact establishment (Bresler et al., 2001; Okabe et al., 2001). AMPA receptor accumulation follows that of PSD-95 (Friedman et al., 2000). Unlike active zones, there is no compelling evidence suggesting that prefabricated packets of PSD components are recruited to postsynaptic sites. Whereas discrete clusters of non-synaptic PSD-95 (Marrs

et al., 2001; Prange and Murphy, 2001; Rao et al., 1998) and NMDA receptors (Washbourne et al., 2002) have been reported, live imaging of GFP-tagged PSD-95 suggests that it is recruited to nascent synaptic sites by diffusion from a cytoplasmic pool (Bresler et al., 2001; Marrs et al., 2001). PSD-95, NMDA receptors and AMPA receptors exist largely in non-overlapping clusters and their rate of appearance at postsynaptic sites differs (Friedman et al., 2000; Rao et al., 1998; Washbourne et al., 2002), indicating that the major postsynaptic components do not exist in preassembled PSD precursor vesicles. Moreover, AMPA receptors can be recruited to synaptic sites by lateral migration in the plasma membrane (Borgdorff and Choquet, 2002). Finally, the cytoplasmic domains of AMPA and NMDA receptor subunits bind to different sets of proteins (Sheng and Sala, 2001), suggesting distinct regulatory mechanisms of recruitment to PSDs. In fact, some forms of synaptic plasticity rely on differential recruitment of glutamate receptors to synaptic sites (Barry and Ziff, 2002; Malenka, 2003).

3. Synaptic Plasticity

The brain's aptitude to modify itself in response to experience lies in the ability of synaptic transmission to undergo reversible changes. Synaptic plasticity refers to the processes by which synaptic connections are altered in response various stimuli. Substantial evidence indicates that the number and strength of synaptic connections can be changed by neuronal activity (Bailey and Kandel, 1993; Bliss and Collingridge, 1993; Malenka, 2003). These changes must be stabilized or consolidated in order for memory to persist. Temporary reversible changes are referred to as short-term memory (STM), and the persistent changes as long-term memory (LTM). While there is considerable evidence indicating that LTM requires gene expression and protein synthesis (Kandel, 2001), these

molecular changes are transient and thus, cannot solely account for LTM (Dudai, 2002). Instead, it is believed that alterations in synaptic morphology are also required for LTM. Recent advances in imaging of neurons undergoing plasticity *in vivo* support this idea (Lendvai et al., 2000; Trachtenberg et al., 2002). The molecular mechanisms that translate patterns of neuronal activity into specific structural and functional synaptic modifications are only beginning to be elucidated.

3.1 Calcium Influx Induces Synaptic Plasticity

Calcium influx in postsynaptic cells through NMDA receptors, and possibly L-type voltage-gated calcium channels (VGCCs), is generally accepted as the triggering event in synaptic plasticity. In fact, the persistent enhancement of synaptic efficacy in response to a burst of synaptic activity, known as long-term potentiation (LTP), depends on postsynaptic NMDA receptors-mediated Ca^{2+} influx (Tsien, 2000). Since its discovery by Bliss and Lomo (1973), LTP has been the object of intense investigation as it is believed to provide an understanding of the cellular and molecular mechanisms by which memories are formed and stored (Bliss and Collingridge, 1993). Experimental induction of LTP by repetitive tetanic stimulation causes AMPA-receptor mediated depolarization of postsynaptic membranes, leading to Ca^{2+} influx through voltage-sensitive NMDA channels. The rise of intracellular Ca^{2+} is critical to induce LTP. In fact, whereas NMDA receptor antagonists and Ca^{2+} chelators have minimal effects on basal synaptic transmission, they were shown to completely block the generation of LTP (Collingridge et al., 1983; Lynch et al., 1983; Malenka et al., 1988). Moreover, it is thought that a threshold level of Ca^{2+} must be reached to trigger LTP and that increases in postsynaptic Ca^{2+} below that threshold can generate either a short-term potentiation (STP) that quickly

decays to baseline within minutes, or long-term depression (LTD), a long-lasting decrease in synaptic strength (Bear and Malenka, 1994; Yang et al., 1999). Therefore, it is clear that postsynaptic Ca^{2+} levels significantly influence synaptic plasticity.

3.2 Signal Transduction Mechanisms

What signaling pathways activated by Ca^{2+} are required to mediate synaptic plasticity? Overwhelming evidence implicates Ca^{2+}-sensitive kinases such as PKC and calcium/calmodulin-dependent kinases (CaMK), as well as cAMP/PKA signaling, in the enhancement of synaptic transmission (Lisman et al., 2002; Nguyen and Woo, 2003). Conversely, Ca^{2+}-dependent phosphatases such as calcineurin and protein phosphatases 1/2A were shown to decrease synaptic transmission (Morishita et al., 2001; Winder and Sweatt, 2001). Ca^{2+} levels therefore bi-directionally control synaptic efficacy by influencing the balance between the activity of protein kinases and phosphatases. These activities modulate synaptic efficacy, initially by regulating ionotropic glutamate receptor gating and trafficking through phosphorylation (Barry and Ziff, 2002; Malenka, 2003), and subsequently, by influencing gene transcription and protein synthesis (Kandel, 2001; West et al., 2001). Similarly to the short- and long-term forms of memory, an early translation-independent form of LTP (E-LTP) can be distinguished from the more slowly developing, protein synthesis-dependent late-phase LTP (L-LTP). Although it is still unclear how gene expression and protein synthesis contribute to the persistence of memory (Dudai, 2002; Steward and Schuman, 2001), some of the proteins synthesized, such as neurotrophins, are though to mediate structural changes in synapses (Poo, 2001).

3.3 Expression Mechanisms

How are signaling events translated into an increase or reduction in synaptic transmission? The determination of the molecular events underlying the expression and maintenance of synaptic plasticity has been one of the most contentious subjects in neuroscience. Multiple mechanisms have been proposed, both pre- and postsynaptically, including enhanced neurotransmitter release (Liu, 2003), alterations in postsynaptic glutamate receptor number and properties (Barry and Ziff, 2002; Malenka, 2003), and structural changes in synaptic morphology and connectivity (Lamprecht and LeDoux, 2004), but their relative contribution to memory formation remains unclear.

Presynaptic regulation of synaptic transmission. The strength of synaptic transmission can be determined both pre- and postsynaptically, through the magnitude of neurotransmitter release, and the number and properties of receptors available for activation, respectively. However, technical and conceptual difficulties have hampered the determination of the relative contribution of quantal (single vesicle) transmission to the modification of synaptic strength during plasticity. For example, providing all postsynaptic receptors are activated by a single package of transmitter, modifications in quantal content are unlikely to significantly impact synaptic transmission. A number of recent studies indicate that this is not the case for a single quanta of transmitter (Ishikawa et al., 2002; Liu et al., 1999; Mainen et al., 1999; McAllister and Stevens, 2000). However, the simultaneous fusion of multiple vesicles can saturate postsynaptic receptors (Auger et al., 1998; Oertner et al., 2002; Wadiche and Jahr, 2001). Synapses in the cortex and hippocampus have a low probability of release, and receptor activation is therefore frequently determined by the release of a single vesicle of transmitter (Gil et al., 1999;

Murthy et al., 1997). These findings indicate that presynaptic quantal changes might affect synaptic transmission.

Quantal size can be modulated by regulating the amount of neurotransmitter released per quantal event. This can be achieved by changing the number or efficacy of vesicular neurotransmitter transporters (Ahnert-Hilger et al., 2003; Wojcik et al., 2004). In fact, quantal content is thought to fluctuate by as much as 30% (Bruns et al., 2000; Karunanithi et al., 2002). The probability of successful action potential-mediated quanta release (P_r) also contributes to quantal transmission. In fact, LTP and LTD were shown to increase and decrease P_r, respectively (Hashimoto and Kano, 1998; Kullmann and Siegelbaum, 1995). P_r is controlled by many factors including the number of AZs per synapse, the size of the readily releasable pool of SVs, the amount of Ca^{2+} influx through presynaptic channels and the SV release machinery (Calakos et al., 2004; Castillo et al., 2002; Finley et al., 2002; Harris and Sultan, 1995; Schikorski and Stevens, 1997). Furthermore, P_r can also be affected by altering the number of presynaptically active terminals. In fact, LTP was demonstrated to be accompanied by an increase in the number of presynaptic terminals (Engert and Bonhoeffer, 1999; Zakharenko et al., 2001).

Glutamate receptor gating and trafficking. If one assumes that the amount of neurotransmitter released is fixed, changes in the number and properties of postsynaptic receptors are central to determining the efficacy of synaptic transmission. Compelling evidence for a postsynaptic regulation of plasticity was provided with the identification of postsynaptically "silent synapses" – which show an NMDA but no AMPA receptor response, and the demonstration that they could be converted to active synapses – which display AMPA-meditated currents (Durand et al., 1996; Isaac et al., 1995; Liao et al.,

1995). At resting potentials, NMDA receptors are minimally opened because of their strong voltage-dependence; synaptic transmission at silent synapses is thus recorded as a failure. The appearance of AMPA responses at such synapses during LTP, with no change in the NMDA response, strongly supports a postsynaptic modification consisting of a functional recruitment of AMPA receptors. Evidence supporting rapid delivery of AMPA receptors from nonsynaptic sites to the synapse via an exocytic mechanism was provided with the observation that postsynaptic perturbation of membrane fusion events could block LTP (Lledo et al., 1998), and that exocytosis in dendrites was regulated by CaMK activation (Maletic-Savatic et al., 1998). It is now clear that synaptic insertion and removal of AMPA receptor are closely coupled to synaptic strengthening and depression, respectively (reviewed in Barry and Ziff, 2002; Malenka, 2003). Mechanisms regulating the recruitment of AMPA receptors from the diffuse postsynaptic plasma membrane by lateral migration have also been suggested (Borgdorff and Choquet, 2002). Moreover, the composition of AMPA and NMDA receptor subunits was shown to affect plasticity-induced trafficking and synaptic transmission (Liu et al., 2004a; Passafaro et al., 2001; Shi et al., 2001).

Another mechanism accounting for the postsynaptic expression of plasticity is the differential phosphorylation of the AMPA receptor subunit GluR1. GluR1 was shown to be phosphorylated on multiple serine residues by kinases involved in plasticity such as CaMKII and PKA (Barria et al., 1997; Roche et al., 1996). In fact phosphorylation of GluR1 subunits was shown to increase the conductance of AMPA receptors (Derkach et al., 1999), AMPA receptor externalization (Esteban et al., 2003; Mangiavacchi and Wolf, 2004) and to be required for LTP and memory *in vivo* (Lee et al., 2003). Accordingly,

AMPA receptor dephosphorylation (Lee et al., 2000) and endocytosis (Matsuda et al., 2000) were shown to be involved in LTD.

Gene expression and protein synthesis. The above-mentioned findings suggest that the rapid modulation of synaptic transmission efficacy during the early phase of synaptic plasticity is likely mediated by both pre- and postsynaptic events. However, it remains unclear to what extent these changes contribute to the long-term maintenance of facilitated or depressed synaptic transmission. It is currently thought that use-dependent modulation of gene expression and protein translation confers long-term plastic changes. In fact, modulation of cAMP-response element (CRE)-regulated gene expression by CRE-binding protein (CREB) appears to be a universal requirement for this process (see Kandel, 2001; West et al., 2001 for review). However, this proposal led to a new set of conceptual challenges, specifically the ability of neuronal transcriptional and translational machineries to specifically target gene products to synaptic sites undergoing plastic changes (reviewed in Frey and Morris, 1997; Steward and Schuman, 2001). It is now clear that dendrites are equipped with local translation machinery (Casadio et al., 1999; Guzowski et al., 1999), and that synaptic activity can regulate the translation of locally available RNA (Aakalu et al., 2001; Martin et al., 1997). But how do newly synthesized gene products lead to persistence of memory? One possibility is that the same types of molecules that alter short-term synaptic efficacy, such as components of transmitter release machinery and postsynaptic receptors are synthesized (Goelet et al., 1986). A second possibility is that *de novo* protein synthesis contributes to synaptic growth, culminating in the remodeling of existing synapses or the emergence of new ones (reviewed in Lamprecht and LeDoux, 2004).

Structural modifications. It has long been believed that changes in the number or structure of synapses may represent a substrate of memory formation. Such changes were postulated to involve the establishment of new synaptic connections or the remodeling of existing ones to make them more efficacious (Ramon y Cajal, 1894; Tanzi, 1893). Substantial evidence has accumulated indicating that synaptic growth and remodeling are indeed correlated with some forms of long-term memory and potentiation (Andersen and Soleng, 1998; Bailey and Kandel, 1993; Colicos et al., 2001; Kleim et al., 2002; Weiler et al., 1995; Yuste and Bonhoeffer, 2001).

Most excitatory synapses in the brain terminate on spines (Gray, 1959), specialized dendritic protrusions that serve to compartmentalize postsynaptic signaling molecules, such as Ca^{2+}, and allow efficient responses to synaptic inputs (Nimchinsky et al., 2002; Tsay and Yuste, 2004). It has been proposed that modulation of the number and/or morphology of dendritic spines could contribute to alterations in excitatory synaptic transmission during learning. Indeed, there is evidence that induction of synaptic plasticity leads to changes in the number or shape of spines (Fifkova and Anderson, 1981; Fifkova and Van Harreveld, 1977; Geinisman et al., 2001; Knott et al., 2002; Leuner et al., 2003; Nikonenko et al., 2002; Trachtenberg et al., 2002). While some studies show that LTP enhances the number of spines making contact with a single presynaptic terminal (Toni et al., 1999), others have determined that they are not formed by splitting of existing synapses, indicating that other mechanisms, such as growth of new spines, must be involved (Fiala et al., 1998; Harris et al., 2003). Given the potential role of filopodia as spine precursors, neuronal activity was hypothesized to modulate the actin cytoskeleton and dendrite dynamics. Accordingly, LTP-like synaptic stimulation of hippocampal slice preparations was shown to increase the growth of filopodia (Maletic-

Savatic et al., 1999) and spines (Engert and Bonhoeffer, 1999) in an NMDA receptor-dependent manner. Dendritic motility was also shown to be modulated by experience *in vivo*, as evidenced by a reduction of filopodia dynamics on cortical dendrites upon sensory deprivation (Lendvai et al., 2000), and by increased spine sprouting and synapse formation in response to novel sensory experience (Trachtenberg et al., 2002). Together, these observations indicate that synaptic plasticity increases the number and motility of dendritic protrusions. Because these morphological changes last for many hours, they have been proposed to contribute to persistent changes in synaptic transmission. However, proofs of necessity, causality and sufficiency are lacking.

How does synaptic plasticity regulate spine morphology? Time-lapse recordings of hippocampal neurons expressing GFP-labeled actin revealed that the actin cytoskeleton changes its configuration continuously under control conditions and that cytochalasin-D, a drug that interferes with actin polymerization, blocks the motility of dendritic protrusions (Fischer et al., 1998). These observations indicate that actin dynamics play a central role in the development and motility of dendritic spines (Matus et al., 2000; Matus, 2000). Consistent with this, LTP induction *in vivo* increases the content of F-actin in hippocampal spines for at least five weeks after stimulation (Fukazawa et al., 2003), activity-induced NMDA signaling increases actin dynamics (Maletic-Savatic et al., 1999), and interfering with actin polymerization suppresses LTP maintenance (Krucker et al., 2000). Spine morphology is profoundly influenced by glutamate (Hering et al., 2003; Matus et al., 2000; Matus, 2000; Tashiro et al., 2003; Tashiro and Yuste, 2003). Whereas AMPA receptor signaling has been associated with decreased actin dynamics and stabilization of spine motility (Fischer et al., 2000; Kirov and Harris, 1999; McKinney et al., 1999), NMDA receptor signaling was shown to induce spine formation

and motility (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). Importantly, both mechanisms rely on Ca^{2+} influx, via VGCCs in the case of AMPA receptor mediated spine stabilization, and via itself in the case of NMDA receptor induced spine motility, but were proposed to differ in the levels of Ca^{2+} influx (Matus, 2000; Tashiro et al., 2003). This effect is consistent with the observation that different cytoplasmic Ca^{2+} levels in neuronal growth cones can produce opposite turning responses to the same stimulus (Hong et al., 2000; Zheng, 2000). Although it remains unclear exactly how changes in intracellular Ca^{2+} influence the actin cytoskeleton of growth cones and dendritic filopodia, there is considerable evidence implicating the Rho family of GTPases (reviewed in Luo, 2002; Matus, 2000; Nakayama and Luo, 2000). Of particular interest, the activity of Rac1 and RhoA were shown to promote the formation and reduce the number of spines, respectively, and were proposed to act antagonistically in spine formation and growth (Luo et al., 1996; Nakayama et al., 2000; Scott et al., 2003; Tashiro et al., 2000; Wong et al., 2000). Moreover, blockade of Rac1 was recently reported to induce long, thin spines and to inhibit spine head growth morphing and stability (Tashiro and Yuste, 2004), suggesting that Rac1 activation is an important mediator of the morphological changes that spines undergo during synaptic plasticity. The identification of Ca^{2+}-dependent signaling events responsible for the differential activation of Rho GTPases and the regulation of actin dynamics within spines will provide valuable insight as to the relative importance of structural plasticity to long term memory formation.

How do structural changes in spine morphology contribute to the modulation of synaptic transmission? Dendritic spines are typically composed of a bulbous head with a thin neck connecting it to the parent dendrite. Spines display at least two major types of

motility: extensive length changes including both extension and retraction, and morphological changes in their heads (Dunaevsky et al., 1999). Morphological changes in spines might act to enhance neuronal connectivity, by making a wider selection of axons available to the dendrite (Stepanyants et al., 2002), or by increasing the amount of dendritic membrane available for synaptic contact (Fiala et al., 2002; Harris et al., 2003; Toni et al., 1999). Large spine heads typically have larger PSDs, increased AMPA to NMDA receptor ratios, and are connected by presynaptic AZs with larger readily-releasable SV pools (Harris and Stevens, 1989; Schikorski and Stevens, 1997; Takumi et al., 1999). Accordingly, glutamate sensitivity was shown to be heightened in spines with large heads (Matsuzaki et al., 2001). In addition, structural modification of spines was suggested to alter their biochemical properties, namely their ability to compartmentalize Ca^{2+} (Tsay and Yuste, 2004). Indeed, variations in the length of the spine neck influence the ability of spines to transfer Ca^{2+} transients into the parent dendrite (Majewska et al., 2000a; 2000b; Volfovsky et al., 1999). Thus, the longer the spine neck, the more independent the spine is from the dendrite and the lesser its effect on dendrites. Spine independence, together with the rather small head volume, may result in the ability of the spine to maintain a high concentration of Ca^{2+} for longer periods, which might be relevant for Ca^{2+}-dependent synaptic plasticity.

4. Molecules involved in CNS Synapse Formation and Plasticity

In the vertebrate NMJ, the extracellular-matrix protein agrin, which is secreted by presynaptic motoneurons, induces postsynaptic differentiation (reviewed in Burden 2002; Sanes and Lichtman, 1999). In the CNS, a role for agrin in synapse formation remains controversial (Bose et al., 2000; Ferreira, 1999; Li et al., 1999; Serpinskaya et al., 1999).

Evidence suggests that cell-cell contact is sufficient to induce the assembly of highly organized synaptic sites (Ziv and Garner, 2001). Signals that influence the formation of filopodia and enhance the probability of axo-dendritic contact are therefore likely to play a central role in initiating central synapse formation. So, what are these signals, and how do they induce the asymmetric differentiation and organization of pre- and postsynaptic specializations? In recent years, a number of molecules have been proposed to participate in the assembly of synaptic contacts. Of interest, extracellular-matrix (ECM) and cell-adhesion molecules (CAMs) were found to play a central role in CNS synaptogenesis as they were shown to regulate both initial and later stages of synapse assembly (Dityatev and Schachner, 2003; Pavlov et al., 2004; Yamagata et al., 2003).

4.1 Axo-dendritic Matchmakers

Glutamate. Substantial evidence implicates excitatory neuronal transmission and ionotropic glutamate signaling in the regulation of the density and motility of axonal and dendritic filopodia. Activity-dependent release of glutamate from extending axons is well positioned to influence filopodia motility in both axons and dendrites (Kraszewski et al., 1995; Sabo and McAllister, 2003). However, exactly what its effects are remain controversial. While some studies indicate that neuronal activity and glutamate stimulate the motility and growth of filopodial protrusions (Engert and Bonhoeffer, 1999; Fiala et al., 2002; Maletic-Savatic et al., 1999; Toni et al., 1999; Wong et al., 2000), others show that glutamate may in fact inhibit axonal and dendritic motility (Chang and De Camilli, 2001; Fischer et al., 2000). Similar to the dependence of axonal growth cone motility on intracellular Ca^{2+} and cyclic nucleotide levels (Song and Poo, 1999), Tashiro and colleagues (2003) proposed a developmentally-regulated control of filopodia motility and

synapse formation by glutamate; glutamate enhances filopodia motility in young hippocampal slices, and inhibits motility once synaptic contacts have been established. Although highly speculative, the authors propose that changes in receptor composition and sensitivity to glutamate might be responsible for the 'switch' in filopodia responsiveness.

Neurotrophins. Members of the neurotrophin (NT) family of secreted factors have been implicated in numerous neurodevelopmental processes, including neuronal survival, axonal guidance, synapse formation, maturation and plasticity (McAllister, 1999). Of particular interest is the involvement of NTs in the development of neuronal connectivity and synaptogenesis. BDNF, for example, promotes axonal and dendritic growth and branching *in vitro* and *in vivo* (Alsina et al., 2001; Cohen-Cory and Fraser, 1995; Lom et al., 2002; McAllister et al., 1995). Local nerve growth factor (NGF) or BDNF stimulation induces axonal sprouting within 30 minutes (Gallo and Letourneau, 1998). Transgenic mice overexpressing BDNF show a two-fold increase in synapse density in the superior cervical ganglia, while mice deficient in BDNF have lower synapse numbers (Causing et al., 1997). Similarly, mice lacking the NT receptors TrkB and TrkC display reduced axonal branching and synapse density (Martinez et al., 1998). Although it is hard to identify which of the many of synaptic functions attributed to NTs is responsible for these defects (Poo, 2001), it is tempting to speculate that a decline in axo-dendritic contacts is to blame. Also, NTs' ability to be secreted in response to synaptic activity (Balkowiec and Katz, 2002; Blochl and Thoenen, 1996; Griesbeck et al., 1999) makes them good candidates to mediate the morphological changes that lead to the formation of new synapses during LTP (Engert and Bonhoeffer, 1999; Korte et al., 1998).

4.2 Contact-Mediated Synaptic Adhesion and Presynaptic Specialization

Following initial contact, a stable synaptic adhesion site is established and both axonal and dendritic compartments differentiate into pre- and postsynaptic specializations, respectively. Some of the major factors involved in contact-mediated synaptic adhesion and induction of presynaptic differentiation are outlined here.

Cadherins. Cadherins are a family of Ca^{2+}-dependent CAMs localized at adherens junctions (Perez-Moreno et al., 2003). Classic cadherins mediate strong homophilic adhesion through cytoplasmic interactions with β-catenin, which in turn associates with α-catenin and the actin cytoskeleton. Cadherins localize at pre- and postsynaptic sites and are thought to constitute the 'synaptic glue' that stabilizes both initial axo-dendritic contacts and mature synaptic structures (Benson et al., 2001). Consistent with this, synaptic activity influences the adhesive strength of N-cadherin (Tanaka et al., 2000), and disruption of cadherin adhesion impairs LTP (Bozdagi et al., 2000; Tang et al., 1998). Beyond their adhesive functions, cadherins and β-catenins were reported to organize pre- and postsynaptic specializations; they induce the clustering of SVs at presynaptic AZs (Bamji et al., 2003) and the postsynaptic localization of PSD elements (Honjo et al., 2000). Although it is still unclear whether cadherins have instructive or permissive roles in initiating synapse formation and function, disruption of cadherin-mediated adhesion affects spine morphology and synaptic strength (Togashi et al., 2002). These observations suggest a role for N-cadherin in synaptic adhesion and maturation, and provide a direct link between synapse structure and function. The study of cadherin function in synaptogenesis is further complicated by the large number non-classical cadherin family members such as protocadherins and cadherin-related neuronal receptors (CNRs). Their

different patterns of expression and extensive alternative splicing led to the proposal that they act as synapse specific 'addresses' (Colman, 1997; Fannon and Colman, 1996; Shapiro and Colman, 1998), in a manner reminiscent to the 'lock and key' hypothesis proposed by Sperry (1963).

Integrins. Integrins are heterodimeric Ca^{2+}-independent cell surface receptors composed of noncovalently associated α and β subunits. Integrins promote cell-cell and cell-matrix adhesion by binding, respectively, to several different ECM ligands, including laminins, collagen, fibronectin and tenascins, and members of the Immunoglobulin (Ig) superfamily of CAMs, such as ICAMs (Aplin et al., 1998; Milner and Campbell, 2002). Integrins provide a physical link between extracellular ligands and the intracellular cytoskeleton, either via direct interaction with actin filaments or through the activation of molecules that affect the cytoskeleton such as the focal-adhesion kinase (FAK) and Rho GTPases (Chen et al., 1995; Clark et al., 1998). In the CNS, integrins have been implicated in axon pathfinding and outgrowth (Hoang and Chiba, 1998; Murase and Horwitz, 2002; Pasterkamp et al., 2003; Stevens and Jacobs, 2002; Yebra et al., 2003), as well as LTP stabilization, but not induction (Chan et al., 2003; Staubli et al., 1990; 1998; Xiao et al., 1991). The observations that inhibition of integrin-mediated adhesion blocks hippocampal kindling in rats (Grooms and Jones, 1997) and short term facilitation in *Drosophila* (Rohrbough et al., 2000) indicate that the contribution of integrins to synaptic plasticity is not restricted to LTP. Furthermore, integrins have also been implicated in pre- and postsynaptic maturation, but do not appear to be required for synapse formation (Chavis and Westbrook, 2001).

ECM molecules. Considering the implication of the ECM molecule agrin and the integrin family of ECM receptors in synapse maturation and function (see above), ECM molecules were hypothesized to modulate synaptogenesis and synaptic efficacy. Although no ECM molecules have yet been demonstrated to be required for synapse formation or plasticity, several ECM components were reported to affect synaptic functions. For example, laminin- and fibronectin-mediated interactions between neurons and the ECM are required for LTP maintenance *in vitro* (Chun et al., 2001; Nakagami et al., 2000). Heparin-binding growth associated molecule (HB-GAM), which has been implicated in neurite outgrowth and axon guidance during development, was shown to participate in synaptogenesis and induction of NMDA-dependent LTP (Amet et al., 2001; Lauri et al., 1998; Rauvala and Peng, 1997). In addition, the ECM molecule Narp, whose expression is upregulated during LTP, organizes postsynaptic differentiation (see below). Finally, several other ECM components such as Tenascins, heparin sulfate proteoglycans and chondroitin sulfate proteoglycans have been implicated in synaptic development and plasticity (Dityatev and Schachner, 2003; Pavlov et al., 2004; Strekalova et al., 2002). ECM molecules have been suggested to mediate these functions by: 1) regulating cellular motility and morphology, thus contributing to structural alterations that are associated with synaptic plasticity (del Pozo et al., 2000; Ethell et al., 2000; 2001; Ethell and Yamaguchi, 1999; Tanaka et al., 2000; Wenk et al., 2000), 2) coordinating trans-synaptic signaling, membrane receptor composition and clustering via their cell-surface receptors (Chavis and Westbrook, 2001; Hirbec et al., 2002; O'Brien et al., 1999; Son et al., 2000), and 3) defining the physical parameters of the extracellular space, thereby regulating the diffusion and signaling of soluble molecules in the ECM (Bukalo et al., 2001; Evers et al., 2002; Lauri et al., 1998; Sinnarajah et al., 1999).

Neuroligins and neurexins. Neuroligins are postsynaptic receptors that bind to the β-neurexin family of presynaptic cell surface proteins. The cytoplasmic tail of both receptors interacts with PDZ-domain proteins (Missler et al., 1998). Neurexins comprise a family of three genes that undergo extensive alternative splicing and were consequently proposed to define synaptic specificity (Missler and Sudhof, 1998). Moreover, their asymmetric cellular distribution makes them ideal candidates to mediate synapse specification and adhesion. Scheiffele and colleagues (2000) reported that ectopic expression of neuroligin in non-neuronal cells was sufficient to induce β-neurexin-dependent presynaptic assembly in contacting axons *in vitro*. They later showed that neuroligin-mediated β-neurexin multimerization is required to mediate presynaptic SV recruitment (Dean et al., 2003). These studies are the first to identify CAMs possessing intrinsic synaptogenic activity. Importantly, these data support the idea that axo-dendritic contact is sufficient to induce SV recruitment and presynaptic assembly. Moreover, these studies imply the requirement for postsynaptic clustering of neuroligin receptors to induce presynaptic differentiation. How this is achieved remains to be elucidated.

IgCAMs. Members of the Ig-superfamily of cell-adhesion molecules, such as NCAM/fasciclin II, L1, sidekicks and nectins have been implicated in synaptogenesis, even though none are synapse-specific, or absolutely required for synapse formation or function (Davis et al., 1997; Schachner, 1997; Takai and Nakanishi, 2003; Yamagata et al., 2002; 2003). The only exception to this statement is synaptic cell-adhesion molecule (SynCAM), which was recently identified as a brain-specific IgCAM localized both pre- and postsynaptically (Biederer et al., 2002). SynCAM shows Ca^{2+}-independent homophilic binding via its extracellular domain and associates intracellularly with PDZ

proteins such as CASK, Mint1 and syntenin. Overexpression of SynCAM in neuronal and non-neuronal cells is sufficient to induce functional presynaptic assembly in contacting hippocampal axons *in vitro*, indicating that SynCAM-mediated adhesion is a powerful synaptogenic inducer (Biederer et al., 2002). Whether SynCAM also mediates postsynaptic differentiation remains to be addressed.

4.3 Organizers of Postsynaptic Differentiation

In addition to the contact-mediated induction of presynaptic assemblies, a number of molecules capable of organizing postsynaptic receptor complexes have been characterized, including neuronal activity-regulated pentraxin (Narp), ephrinB and its receptor EphB, and cholesterol.

Narp. Narp is a secreted molecule that accumulates at synaptic junctions and promotes the postsynaptic clustering of AMPA receptors by binding to their extracellular domains (O'Brien et al., 1999). Conversely, expression of a dominant-negative form of Narp in cultured spinal neurons suppresses AMPA receptor clustering (O'Brien et al., 2002). These experiments imply a central role for Narp in organizing PSD specializations *in vitro*. It is unclear whether Narp acts by decreasing the turnover rate of synaptically inserted AMPA receptors or by aggregating extrasynaptic receptors. Narp overexpression also enhances the number of excitatory synapses *in vitro* (O'Brien et al., 1999), suggesting that Narp might also have synaptogenic actions. It is interesting to note that Narp-mediated modulation of postsynaptic receptor organization does not influence presynaptic assembly (O'Brien et al., 2002), indicating that the formation of pre- and postsynaptic assemblies can occur independently.

EphrinB/EphB. EphrinBs are small transmembrane ligands for EphB receptor tyrosine kinases (reviewed in Kullander and Klein, 2002). In addition to its role in regulating axonal guidance, the ephrinB/EphB receptor system was recently implicated in the development, maturation and function of synapses. Specifically, EphB receptors were reported to bind to and cluster NMDA receptors on the surface of cultured hippocampal neurons upon addition of ephrinB (Dalva et al., 2000). Notably, this effect requires ephrinBs to be in a pre-aggregated form, suggesting that, under physiological conditions, EphB-mediated NMDA receptor clustering is preceded by presynaptic ephrinB clustering. The distribution of PSD-95 and AMPA receptors remained unaltered, indicating that ephinBs are not general inducers of postsynaptic differentiation (Dalva et al., 2000). EphrinB-EphB interactions have been implicated in the development of dendritic spines through the phosphorylation of the cell-surface heparan-sulfate proteoglycan syndecan (Ethell et al., 2001) and the regulation of small GTPases (Irie and Yamaguchi, 2002; Penzes et al., 2003). In addition, activated EphB receptors were reported to increase synapse density and to enhance Ca^{2+}-influx through NMDA receptors (Takasu et al., 2002). Although EphB2-knockout mice display deficits in activity-dependent synaptic plasticity, the structure and number of their synapses is unaltered, arguing against a major role for EphrinB/EphB in coordinating synapse development (Grunwald et al., 2001; 2004; Henderson et al., 2001).

Cholesterol. Cholesterol was recently identified as a glial-derived factor essential for synapse assembly, maturation and function *in vitro* (Hering et al., 2003; Mauch et al., 2001). Exactly how it mediates these effects remains elusive. However, although cholesterol has many biological functions, its requirement for the formation of lipid-

enriched plasma membrane microdomains distinct from the bulk fluid bilayer (Smaby et al., 1996) has been of particular interest. These so-called 'lipid-rafts' are insoluble in non-ionic detergents such as Triton X-100, and are thus termed detergent-insoluble, glycolipid enriched complexes (DIGs) (London and Brown, 2000). DIGs are enriched in signaling molecules and were proposed to function as platforms to organize efficient signal transduction (Langlet et al., 2000; Simons and Toomre, 2000), clathrin-dependent and independent endocytosis (Helms and Zurzolo, 2004; Subtil et al., 1999), constitutive and regulated exocytosis (Salaun et al., 2004; Wang et al., 2000), polarized sorting of proteins to axons, dendrites, pre- or postsynaptic sites (Craven et al., 1999; Dotti et al., 1991; Dotti and Simons, 1990; El Husseini et al., 2000a; 2001; Hering et al., 2003; Ledesma et al., 1999), neurite outgrowth and guidance (Guirland et al., 2004; Niethammer et al., 2002), ion channel clustering (El Husseini et al., 2000b; Hering et al., 2003; Takimoto et al., 2002) and pre- and postsynaptic plasticity (El Husseini et al., 2002; Hurley et al., 2000; Pfrieger, 2003; Veit et al., 2000). In particular, several components of the postsynaptic receptor complex such as AMPA receptors (Suzuki et al., 2001), NMDA receptors (Hering et al., 2003), PSD-95 (Perez and Bredt, 1998) and GRIP (Bruckner et al., 1999) are found associated with lipid rafts. Disruption of lipid rafts by cholesterol depletion results in AMPA receptor destabilization, dendritic spine collapse and the loss of synaptic connections (Hering et al., 2003). Therefore, although clearly involved in numerous cellular processes, cholesterol has a central role in the formation of functional postsynaptic receptor structures.

5. Netrins

Netrins are a family of secreted proteins originally identified for their ability to guide growing axons during neural development. Extensive characterization of netrin function revealed their ability to act as both chemoattractants and repellents. This bifunctionality is mediated by the recruitment of distinct netrin receptor complexes and the differential activation of intracellular signaling cascades. Netrins have since been attributed several functions during development, such as neuronal guidance, cellular adhesion and tissue morphogenesis. In addition, netrins continue to be expressed into adulthood, suggesting novel functions for the netrin family of secreted molecules.

5.1 Netrin Structure and Function

The netrin family of guidance molecules. Netrins form a family of phylogenetically conserved proteins structurally related to the laminin family of ECM proteins (Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994). The first netrin characterized, UNC-6, was identified as the protein encoded by the *unc-6* gene of the nematode *Caenorhabditis elegans* (Ishii et al., 1992). *Unc-6* mutants had defects in the trajectories of dorsally and ventrally projecting axons (Hedgecock et al., 1990). Many orthologs have since been cloned in other invertebrate species, such as *netrin-A* and *netrin-B* in *Drosophila melanogaster*, and in vertebrates, including *netrin-1*, *netrin-3*, *netrin-4/β-netrin* and *netrin-G1* in mammals (for review, see Manitt and Kennedy, 2002). Similar to the uncoordinated (unc) phenotype of *unc-6* mutants, netrins were identified in vertebrates for their ability to guide the circumferential migration of commissural and trochlear motoneuron axons toward and away from the ventral floor plate in the developing spinal cord, respectively (Colamarino and TessierLavigne, 1995; Kennedy et al., 1994; Serafini

et al., 1994; 1996). These findings led to the proposal that gradients of netrin protein secreted by floor plate cells differentially guide the extension of axons toward or away from the ventral midline during CNS development.

Netrins have since also been implicated in the development of neural circuits in the hippocampus (Barallobre et al., 2000), corpus collosum (Serafini et al., 1996), thalamus (Braisted et al., 2000), cortex (Metin et al., 1997) and the retina (Deiner et al., 1997). In addition, netrins direct the migration of neuronal precursors (Alcantara et al., 2000; Hamasaki et al., 2001; Przyborski et al., 1998; VarelaEchavarria et al., 1997) and glial precursors in the developing optic nerve (Sugimoto et al., 2001) and spinal cord (Jarjour et al., 2003; Tsai et al., 2003).

Netrin structure. Netrin genes encode ~70-80 kDa proteins made up of three domains (VI, V and C) and an amino terminal signal peptide characteristic of secreted proteins. Like most ECM and cell-adhesion molecules, the domains of netrin family members are found in functionally divergent proteins. Domain VI and domain V, which is made up of three consecutive epidermal growth factor-like repeats (V-1, V-2 and V-3), are similar to domains VI and V of the ECM glycoprotein laminin (Ishii et al., 1992). The carboxy-terminal basic domain (C domain) is also conserved; sequence similarities have been found in the complement and tissue inhibitors of metalloproteinases (TIMPs) protein families (Banyai and Patthy, 1999; Ishii et al., 1992; Sandoval et al., 2000). The distinct biological activities of netrin proteins are thought to be mediated by these domains (Lim and Wadsworth, 2002; Wadsworth et al., 1996). Domains VI and V, for example, are thought to mediate interactions with netrin receptors and thus, were proposed to dictate guidance decisions. Specifically, domains VI, V-2 and V-3 were shown to be required for

dorsal migration, and domains VI and V-3 for ventral migration in *C. elegans* (Lim and Wadsworth, 2002). The C domain has strong binding affinity for heparin (Kappler et al., 2000; Serafini et al., 1994), a polysaccharide commonly found in ECM proteoglycans, suggesting that netrins, once secreted, might not be freely soluble but rather associated with ECM components. This type of interaction was proposed to serve as a mechanism to either locally concentrate netrins, present netrins to cell-surface receptors, or influence the ability of netrins to diffuse and act as short- or long-range cues (Kennedy, 2000). Although originally characterized as a soluble diffusible cue, analysis of the subcellular distribution of netrin-1 in embryonic and adult brain tissue revealed that the majority of netrin-1 protein is ECM- or cell-membrane-associated (Manitt et al., 2001; Manitt and Kennedy, 2002; Serafini et al., 1994). Furthermore, consistent with their homology to the basal lamina protein laminin (Colognato and Yurchenco, 2000), several lines of evidence suggest that netrins are secreted baso-laterally and deposited primarily in basement membrane structures. In *C. elegans*, UNC-6 is secreted to the body wall basement membrane (Hedgecock et al., 1990; Wadsworth et al., 1996). Immuno-histological analyses in vertebrates revealed the presence of netrin family members in the basal lamina of a variety of tissues (Koch et al., 2000; Liu et al., 2004b; Yebra et al., 2003), including the spinal cord pia, a basement membrane-like structure in the CNS (MacLennan et al., 1997). These observations are consistent with the idea that netrins are not freely soluble but rather associated with cell-membranes or ECM structures.

5.2 Netrin Receptors

Candidate netrin receptors were first identified in *C. elegans* based on the observation that *unc-40* and *unc-5* mutants produced guidance errors that appeared to be

subsets of the *unc-6* phenotype. While *unc-5* mutations prevented dorsal migration away from *unc-6* producing cells, *unc-40* mutants showed defects in both trajectories (Hedgecock et al., 1990). Cloning of *unc-40* and *unc-5* indicated that they encode transmembrane proteins belonging to the Ig superfamily of cell adhesion molecules (Chan et al., 1996; Leung-Hagesteijn et al., 1992). Based on these observations, UNC-40 and UNC-5 were proposed to function as receptors for the ventrally secreted cue UNC-6, with UNC-40 being required for both attractive and repulsive responses, and UNC-5 being primarily required for repulsive responses. In recent years, the adenosine receptor A2b as well as the integrin receptors $\alpha6\beta4$ and $\alpha3\beta1$ were reported to function as netrin receptors (Corset et al., 2000; Yebra et al., 2003). Because the nature of these interactions is either controversial (Stein et al., 2001) or still preliminary, this review will focus on the "classical", better characterized, UNC-40/deleted in colorectal cancer (DCC) and UNC-5 families of receptors.

DCC receptor family. The *dcc* gene was originally isolated as a possible tumor suppressor (Fearon et al., 1990), but the role of DCC in tumor progression is unclear. Members of the DCC family of transmembrane receptors include DCC and neogenin in vertebrates, UNC-40 in *C. elegans* and Frazzled in *Drosophila* (Chan et al., 1996; KeinoMasu et al., 1996; Kolodziej et al., 1996; Vielmetter et al., 1994). The extracellular domain of DCC proteins is composed of four Ig-like domains and six fibronectin type III (FNIII) repeats. Direct netrin-DCC binding was recently demonstrated between the fifth FNIII repeat of DCC and domains VI and V of netrin-1 (Geisbrecht et al., 2003). The cytoplasmic tail of DCC is devoid of any obvious catalytic activity and contains three conserved proline-rich domains termed P1, P2 and P3 (Kolodziej et al., 1996).

Strong evidence indicates that DCC is responsible for the chemoattractant response to netrin (Chan et al., 1996; KeinoMasu et al., 1996; Kolodziej et al., 1996): antibodies that block DCC function (DCCfb) prevent the floor plate's ability to promote the directed outgrowth of commissural axons (KeinoMasu et al., 1996), and *dcc* knockout mice (Fazeli et al., 1997) have commissure defects similar to those observed in *netrin-1* knockouts (Serafini et al., 1996). These findings are consistent with the reported functions of UNC-6 and UNC-40 in *C. elegans* (Hedgecock et al., 1990).

Neogenin, the second member of the DCC family identified in vertebrates, was shown to bind netrin-1 (KeinoMasu et al., 1996) and to be widely expressed in and outside the CNS (Gad et al., 1997; Vielmetter et al., 1994). Up until recently, no function for neogenin had been demonstrated either *in vitro* or *in vivo*. Within the last year, two papers implicated neogenin in both neural and non-neural organogenesis (Mawdsley et al., 2004; Srinivasan et al., 2003). Srinivasan and colleagues identified a novel function for netrin-1 in mammary gland morphogenesis. They proposed that netrin-1 acts as a short-range attractant through neogenin (DCC is not expressed in the developing breast epithelium) providing an adhesive, rather than a guidance function required for proper organization of the gland's epithelial buds. More recently, Mawdsley and coworkers suggested a guidance function for neogenin in the migration of neuroectodermal and mesodermal cells leading to neural tube formation and somitogenesis in zebrafish. Finally, neogenin was recently reported to function as a receptor for the guidance cue repulsive guidance molecule (RCM) in retinal axons, opening new research avenues in the guidance field (Matsunaga et al., 2004).

UNC-5 receptor family. The UNC-5 family of receptors includes *C. elegans* UNC-5, *Drosophila* Dunc5 and four vertebrate homologs UNC5H1-4 (Ackerman et al., 1997; Engelkamp, 2002; Keleman and Dickson, 2001; Leonardo et al., 1997; Leung-Hagesteijn et al., 1992; Przyborski et al., 1998). Although UNC-5 receptors also belong to the Ig superfamily of transmembrane receptors, they are structurally distinct from DCC receptors. Their shorter extracellular domain is composed of two Ig-like domains followed by two thrombospondin type 1 (Tsp-1) repeats. The intracellular domain contains a death domain (DD), which is found in apoptosis-regulating proteins and is likely involved in protein-protein interactions, a DCC-binding domain (DB) and a ZU5 domain, a sequence found in the gap-junction protein ZO-1 (Hofmann and Tschopp, 1995; Leonardo et al., 1997).

In *C. elegans*, the trajectories of axons extending away from the UNC-6-expressing ventral midline were disrupted in *unc-5* mutants (Hedgecock et al., 1990), suggesting that UNC-5 is required for the chemorepellent response to netrins. Consistent with this, direct binding of UNC-5 Ig repeats with netrin-1 domains VI and V was demonstrated (Ackerman et al., 1997; Geisbrecht et al., 2003; Leonardo et al., 1997). *Drosophila Dunc5* is expressed by a subset of motoneurons that exit the CNS without crossing the netrin-expressing midline (Keleman and Dickson, 2001). In addition, ectopic expression of *unc-5* in neurons that are normally insensitive or attracted to netrins in *Drosophila* and *C. elegans* causes these axons to be repelled by them (Hamelin et al., 1993; Keleman and Dickson, 2001). Together, these observations suggest that UNC-5 receptors mediate the chemorepellent response to netrin (Ackerman et al., 1997; Hamelin et al., 1993; Leonardo et al., 1997; Leung-Hagesteijn et al., 1992).

Receptor interactions. Although both DCC and UNC-5 receptor families can signal independently of one another (Keleman and Dickson, 2001; Merz et al., 2001), the axonal response to netrin is likely mediated by the balanced action of both. In fact, many neurons express both *dcc* and *unc-5* genes (Chan et al., 1996; Leonardo et al., 1997). Studies in vertebrates and invertebrates indicate that DCC/UNC-40 is responsible for netrin-induced attraction and outgrowth (Chan et al., 1996; KeinoMasu et al., 1996), while DCC/UNC-40 and UNC-5 families both contribute to axon repulsion from netrin (Hamelin et al., 1993; Hedgecock et al., 1990; Hong et al., 1999; Merz et al., 2001). Moreover, biochemical evidence suggests that DCC and UNC-5 homologs interact to form receptor complexes (Hong et al., 1999). In *Drosophila melanogaster*, it was recently reported that UNC-5 requires frazzled, the fly homolog of DCC, to mediate long- but not short-range repulsion by netrin (Keleman and Dickson, 2001). Together, these observations indicate that models accounting for the bifunctional action of netrin, with one receptor mediating attraction and the other repulsion are oversimplified.

The cytoplasmic domains of DCC and UNC-5 receptors are key effectors in initiating attractive or repulsive responses to netrin (Bashaw and Goodman, 1999; Hong et al., 1999; Stein et al., 2001), both of which appear to function by recruiting signaling/adaptor molecules. While attractive responses require netrin-induced dimerization of the P3 region of DCC, repulsion arises from the association of DCC and UNC-5 cytoplasmic domains P1 and DB, respectively (Hong et al., 1999; Stein et al., 2001). In the absence of netrin, the extracellular domains of DCC and UNC-5 prevent these interactions (Hong et al., 1999). Netrin-induced extracellular domain association was proposed to induce conformational change of the intracellular regions to allow cytoplasmic signaling. Interestingly, transmission electron microscopy shadowing of

recombinant β-netrin protein suggests that netrins exist as dimers formed by the antiparallel alignment of domains V and C, the latter being required for dimerization (Koch et al., 2000).

5.3 Signaling Downstream of Netrin Receptors

In recent years, considerable progress was made in the characterization of the mechanisms by which netrin signaling controls the actin cytoskeleton. Work from our laboratory indicated that DCC localizes at the tip of growth cone filopodia and that netrin-DCC signaling in HEK293 cells increases filopodia number and cell-surface area via the activation of the Rho GTPases Cdc42 and Rac1, respectively (Shekarabi and Kennedy, 2002). Netrin-mediated Cdc42 activation led to actin reorganization in Swiss 3T3 fibroblasts expressing DCC (Li et al., 2002b), suggesting that netrin-1 influences motility by activating Rho GTPases. The exact mechanism by which netrin mediates these effects is unclear, but the adaptor protein Nck, by interacting with the proline-rich domains of DCC, and PAK1 have been suggested to couple DCC signals to Rho GTPases (Li et al., 2002a; Shekarabi et al., 2004). Further evidence linking netrin-DCC signaling and the cytoskeleton was provided from genetic analyses in *C. elegans* of genes whose mutations interacted with netrin receptor gain-of-function mutants. Gitai and colleagues (2003) identified the Rac homolog CED-10, the actin-binding protein UNC-115, and the actin polymerization nucleation factor UNC34 (the worm homolog of Enabled in *Drosophila* and Mena in mammals) as UNC-40 signaling components. UNC-34 acts downstream of the first proline-rich domain of UNC-40 in both axon attraction and repulsion, and CED-10 and UNC-115 signal downstream of P2 (Gitai et al., 2003).

Together, these findings imply that DCC signals via Rho GTPases to modulate actin cytoskeleton dynamics.

How UNC-5 signals netrin-mediated repulsion is unknown. Netrin was shown to induce tyrosine phosphorylation of UNC-5 in transfected cell lines (Tong et al., 2001). A physiological role for UNC-5 phosphorylation was suggested following the observation that mutation of UNC-5 tyrosine phosphorylation sites compromised UNC-6-medited axonal guidance in *C. elegans* (Killeen et al., 2002). Genetic screens in *C. elegans* led to the identification of *max-1* (Huang et al., 2002) and *unc-34* (Yu et al., 2002). *Max*-1 encodes a multidomain cytoplasmic protein required for UNC-5-, but not UNC-40, dependent repulsive guidance to netrin, but it remains unclear exactly how MAX-1 participates in UNC-5 signaling (Huang et al., 2002). UNC-34 was proposed to act as a repressor of UNC-5-induced repulsion (Yu et al., 2002), consistent with its involvement in DCC-mediated attraction to UNC-6 (Gitai et al., 2003).

5.4 Modulation of Netrin Signaling

The response of a growth cone to netrin is further complicated by the state of intracellular signal transduction pathways. Several studies report that the intracellular levels of calcium and cyclic nucleotides, such as cAMP and cGMP, play a key role in determining the response of *Xenopus* growth cones to netrin-1 (reviewed in Song and Poo, 1999). Specifically, axons are attracted to netrin-1 gradients *in vitro* when growth cone cAMP levels are high, but repelled when cAMP levels are low (Song et al., 1997; 1998; Ming et al., 1997). PKA activation using pharmacological agents, as well as electrical activity-induced Ca^{2+}-influx were similarly demonstrated to influence netrin-1 guidance *in vitro* (Ming et al., 1997; 2001). A physiological role for this regulatory

mechanism was demonstrated for the guidance of *Xenopus* retinal ganglion cell axons; netrin-1-mediated guidance of retinal axons into the optic nerve changes from attraction to a repellent action, pushing developing axons along the optic pathway (Shewan et al., 2002). This developmentally regulated switch in the chemotropic response to netrin-1 was associated with a decrease in axonal cAMP levels. Laminin (Hopker et al., 1999), A2b receptors (Corset et al., 2000; Shewan et al., 2002) and netrin-1 itself (Ming et al., 1997) have been shown to alter intracellular cAMP levels *in vitro*, and therefore were proposed to regulate growth cone responsiveness to netrin-1. By analogy, Nishiyama and coworkers (2003) recently proposed a model where UNC-5 switches DCC-mediated netrin-1 attraction to repulsion by increasing the ratio of cGMP to cAMP, leading to PKG activation, PKA inactivation and decreased intracellular Ca^{2+} levels. Whether this model applies to vertebrate growth cones *in vivo* is unknown, but the observation that netrin-1 is unable to induce detectable cAMP changes in commissural growth cones *in vitro* (Bouchard et al., 2004) hints at different signaling cascades in vertebrates. Instead, we and others have recently shown that PKA (Bouchard et al., 2004) and PKC activities (Williams et al., 2003) differentially influence the cell surface presentation of DCC and UNC-5H1 receptors, respectively, thereby providing a novel mechanism to explain the ability of cAMP and Ca^{2+} to modulate growth and guidance. Nevertheless, these findings indicate that extracellular factors, by influencing the intracellular cyclic nucleotide and Ca^{2+} levels, play a key role in modulating the response of growth cones to guidance cues.

Other factors proposed to modulate netrin-mediated guidance include MAPK cascade signaling (Forcet et al., 2002), phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI_3K) activation (Ming et al., 1999), localized protein synthesis in growth cones (Campbell and Holt, 2001), growth cone desensitization (Ming et al., 2001), silencing by

the action of other guidance cues/receptors, such as Slit/Robo (Stein and Tessier-Lavigne, 2001) and differential cell surface expression of its receptors DCC and UNC-5 by regulated exocytosis (Bouchard et al., 2004), endocytosis (Williams et al., 2003) and cleavage by metalloproteinases (Galko and Tessier-Lavigne, 2000).

5.5 Novel Functions for Netrins

During development in vertebrates, netrin RNA transcripts are widely distributed both in the CNS (Kennedy et al., 1994; Livesey and Hunt, 1997) and in peripheral tissues, including heart, aorta, spleen, kidney, testes, ovary and epithelial tissues such as lung, mammary gland and gut (Kennedy et al., 1994; Koch et al., 2000; Salminen et al., 2000; Wang et al., 1999). Although netrin function has been most investigated in the CNS as a guidance cue, several recent papers indicate that netrins mediate adhesion (Koch et al., 2000; Srinivasan et al., 2003; Yebra et al., 2003) and participate in organogenesis (Liu et al., 2004b; Mawdsley et al., 2004; Srinivasan et al., 2003).

Genetic analysis in *Drosophila* identified a role for netrin as a muscle-derived short-range cue regulating nerve-muscle synaptogenesis (Mitchell et al., 1996; Winberg et al., 1998). A similar phenotype was observed in flies lacking frazzled (Kolodziej et al., 1996). Furthermore, modulating the amount of netrin expressed by muscle cells influenced the number of synaptic connections established by motoneurons (Winberg et al., 1998). These findings suggest that netrin can act as a short-range cue to modulate the formation of neuromuscular junctions, possibly by influencing local target selection and axonal branching. However, a role of netrin and its receptor DCC in the regulation of CNS synaptogenesis remains uninvestigated.

We recently reported that netrin-1 protein levels in the adult vertebrate CNS are similar to that of the embryo, and that netrin-1 is located at the interphase between myelinating oligodendrocytes and axons, consistent with netrin mediating an adhesive interaction between axons and myelin sheaths (Manitt et al., 2001). Netrin receptors continue to be expressed in the adult CNS as well (Manitt et al., 2004), indicating that the actions of netrin are not restricted to embryonic development. Interestingly, several other guidance cues and receptors used by neurons during embryonic development to establish neural networks are expressed in the adult nervous system, where they are thought to mediate plastic changes (Dityatev and Schachner, 2003; Gavazzi, 2001; Koeberle and Bahr, 2004; Pavlov et al., 2004; Poo, 2001). Together, these observations lead us to investigate the function of netrin-1 and DCC in the adult CNS and their possible involvement in synapse formation and synaptic plasticity.

Chapter 2

Netrin-1 signaling through DCC enhances synapse number and function

INTRODUCTION

Accurate targeting of extending axons and dendrites during development is ensured by guidance cues in the environment that regulate the dynamics of actin-rich processes such as filopodia and lamellipodia (Gallo and Letourneau, 2004; Huber et al., 2003; Kim and Chiba, 2004). Similar mechanisms were proposed to regulate synaptogenesis and synaptic plasticity, the processes by which synaptic connections are altered in response to various stimuli. In fact, there is evidence that initial contact between dynamic actin-based filopodia extensions from both axonal and dendritic compartments is sufficient to initiate the formation of functional synaptic sites (Chang and De Camilli, 2001; Fiala et al., 1998; Jontes and Smith, 2000; Ziv and Garner, 2001; Ziv and Smith, 1996), and that activity-dependent actin reorganization contributes to synaptic plasticity (Fukazawa et al., 2003; Krucker et al., 2000; Lendvai et al., 2000; Trachtenberg et al., 2002). Moreover, several guidance cues and receptors used by neurons during embryonic development to establish neural networks are expressed in the adult nervous system and have been proposed to be involved in plastic changes (Dityatev and Schachner, 2003; Gavazzi, 2001; Koeberle and Bahr, 2004; Manitt and Kennedy, 2002; Pavlov et al., 2004; Poo, 2001).

Netrins are a family of secreted molecules structurally related to the laminin family of extracellular matrix proteins (Manitt and Kennedy, 2002). In vertebrates, netrin-1 was first characterized for its ability to guide the circumferential migration of commissural axons to the floor plate in the developing ventral spinal cord (Kennedy et al., 1994; Serafini et al., 1994). Netrin-1 signals through the receptor Deleted in Colorectal Cancer (DCC) (KeinoMasu et al., 1996), which localizes at the tip of growth cone filopodia in commissural neurons (Shekarabi and Kennedy, 2002). DCC signals to

members of the Rho family of GTPases such as Rac1 and Cdc42 to influence cytoskeletal dynamics and filopodia extension (Li et al., 2002b; Shekarabi and Kennedy, 2002). We recently reported that netrin-1 and DCC continue to be expressed in the adult vertebrate spinal cord (Manitt et al., 2001; 2004), indicating that netrin-1 function might not be restricted to embryonic development. These observations lead us to investigate the function of netrin-1 and DCC in the mature CNS and their possible involvement in synapse formation and synaptic plasticity.

Here, we report that both netrin-1 and DCC are expressed by neurons in the cortex and hippocampus of adult mice, and that they distribute to synaptosomes *in vivo*. Exogenous bath application of netrin-1 to primary cortical neurons in culture increased the density of axonal and dendritic filopodia. We measured a concurrent increase in axonal and dendritic branching, suggesting that netrin-1 functions to enhance the likelyhood of axo-dendritic contact. Accordingly, netrin-1 increased the density of synaptic connections and enhanced pre- and post-synaptic function, as determined by immunocytochemical and electrophysiological analyses. Although these changes require functional signaling through DCC, netrin-DCC signaling is not necessary for synapse formation *in vitro*. Together, these data suggest a novel function for netrin-1 and DCC in initiating synaptogenesis and influencing synaptic plasticity.

RESULTS

Netrin-1 and DCC are expressed in the adult CNS.

Netrin-1 and *dcc* are widely expressed in developing neuronal tissues such as the spinal cord, striatum, midbrain, cortex, hippocampus and cerebellum (Livesey and Hunt, 1997). We recently reported that *netrin-1* and *dcc* continue to be expressed in the adult nervous system (Manitt et al., 2001; 2004). Consistent with this, *in situ* hybridization analysis revealed that both *dcc* (Figure 1A-B) and *netrin-1* (Figure 1C-D) are expressed by neurons throughout the cortex and in the hippocampal formation in adult mice. Positive DCC *in situ* hybridization signals were strongest in layers II, III and V of the cerebral cortex, but weaker than levels in the CA1, CA3 and dentate gyrus regions of the hippocampus. Netrin-1-specific signal showed a similar, yet more subtle layering pattern in the cortex and modest expression in the hippocampus. Brain sections incubated with netrin-1 and DCC sense probes produced no signal, confirming the specificity of the signal (data not shown).

To verify that netrin-1 and DCC protein are expressed in cerebral cortex, cortices were isolated from mice at various developmental stages, from embryonic day 15 (E15) to adult (A) mice, and protein homogenates processed for western blot analysis. Membranes were immunoblotted for netrin-1 and its receptor DCC, as well as for the AMPA receptor subunit GluR1 to illustrate the development of synaptic connections. Actin served as a loading control (Figure 2A). Netrin-1 protein levels were highest at E15 and remained relatively constant during postnatal development (postnatal day (P) 0 through P20) and in adult cortical extracts. DCC followed a similar pattern of expression until the second postnatal week, and declined thereafter. Despite significant

downregulation, DCC protein was still detected in adult cortex, consistent with our findings in the spinal cord (Manitt et al., 2004).

Netrin-1 and DCC localize to synaptic sites in vivo.

The cellular distribution of netrin-1 and DCC *in vivo* was examined by subcellular fraction analysis of adult mouse cortex. The homogenate (H) was first fractionated into nuclear pellet (P1), crude synaptosome (P2), microsome (P3) and soluble protein (S) fractions. P2 was then fractionated into the plasma membrane, endoplasmic reticulum and golgi complex fraction (P2B) and the synaptosome fraction (P2C). P2C was further separated into the crude synaptic membrane fraction (CSM) and the synaptic vesicle fraction (SVF), which comprises both pre- and postsynaptic vesicles. Treatment of CSM with 0.5% Triton X-100 for 15 min at 4°C yielded Triton-soluble (TS) and Triton-resistant (TR) fractions. Triton X-100 insoluble membrane compartments typically comprise post-synaptic densities (Allison et al., 2000; Cohen et al., 1977; Cotman et al., 1971; Orosz et al., 1975), lipid rafts (Simons and Toomre, 2000) and cytoskeleton-associated complexes (Caroni, 2001; Yin and Janmey, 2003). The distribution of netrin-1 and DCC was examined in comparison to synaptophysin, a presynaptic integral membrane protein enriched in synaptic vesicles, and PSD-95, a postsynaptic scaffold protein enriched in postsynaptic densities. DCC was detected in synapse-enriched fractions (P2C), but was more abundant in other fractions such as P3 and P2B, indicating that DCC does not specifically localize to synapses (Figure 2B). At synaptic sites, DCC was enriched in the vesicular pool (SVF), and membrane DCC partitioned exclusively to detergent-resistant membranes (TR).

Netrin-1 was most abundant in synapse-rich fractions such as P2C (Figure 2B). Netrin-1 was enriched in the vesicular pool at synapses (SVF), but was hardly recovered from P3, suggesting that netrin-1 containing vesicles reside primarily at synapses. A significant netrin-1 signal was also detected in triton-insoluble synaptic membrane, but not in the soluble protein fraction (S), confirming previous reports that netrin is not freely soluble but rather membrane-associated (Manitt et al., 2001; Manitt and Kennedy, 2002; Serafini et al., 1994). These observations suggest that netrin-1 and DCC exist mostly in intracellular vesicles, and that, once at the plasma membrane, they distribute to specialized membrane domains at, or near synapses. However, the distribution of netrin-1 and DCC at pre- and/or postsynaptic sites cannot be inferred from such an experimental approach.

Netrin-1 and DCC distribution in vitro

As a first step in investigating the function of netrin-1 and DCC in neurons, we established dissociated primary cultures of mouse cortical neurons. The cultures were maintained in serum-free medium to prevent the proliferation of glia (Brewer et al., 1993). Under these conditions, cortical neurons display typical features of mature pyramidal neurons such as well differentiated axonal and dendritic arbors, and functional synaptic connections (Evans et al., 1998). Western blot analysis of primary cortical culture lysates harvested after 1 to 18 days *in vitro* (DIV) revealed that these cultures express both netrin-1 and DCC (Figure 2C). Netrin-1 immunoreactivity remained relatively constant at all developmental stages, except at 3 and 5 DIV, where netrin-1 expression levels were noticeably increased. DCC protein levels were highest during the first 5 days *in vitro* and significantly declined by ~70% after a week in culture. The

expression levels of netrin-1 and DCC protein *in vitro* closely resemble the changes in the protein levels observed *in vivo*, making primary cortical cultures a suitable model to study the function of netrin-1 in neurons.

We next analyzed by immunofluorescence microscopy the localization of netrin-1 and DCC in cortical neurons following fixation. At 15 days *in vitro* (DIV), netrin-1 immunoreactivity was detected associated with neuronal processes labeled with MAP2, a cytoskeletal protein restricted to dendrites, as well as with MAP2-negative axons (Figure 3A-C; arrowhead), suggesting that netrin-1 distributes to both axonal and dendritic compartments in mature neurons. This being said, netrin-1 is a secreted protein that was shown to associate with extracellular matrix components and cell membranes (Manitt and Kennedy, 2002; Wadsworth, 2002). To determine whether netrin-1 is actively transported down axons and dendrites or merely associated with their surface, mature cortical neurons were transfected with vectors encoding green fluorescent protein (GFP), GFP-tagged netrin-1 (N-GFP) or Myc-tagged netrin-1 (N-Myc) chimeras. Cultures were then fixed and stained with antibodies against MAP2, GFP or Myc. At 20 hr post-transfection, control GFP staining was mostly detected in large processes identified morphologically and immunocytochemically as dendrites (Figure 3D-E). Thin processes of constant width (~1 μm) extending over long distances were also seen emerging from the soma of GFP transfected cells (Figure 3D). The lack of colocalization between GFP and MAP2 immunoreactivities identified these processes as axons (Figure 3F). GFP therefore distributes to both axons and dendrites. A strong N-GFP signal was detected in the cell body and throughout the dendritic arbor of transfected cortical neurons after 20 hr (Figure 3G). Unlike GFP-transfected cells, N-GFP or N-Myc were not detected in axons, even

after 48 hr of expression (not shown), indicating that netrin-1 is preferentially transported to dendrites.

The distribution of DCC in cultured cortical neurons was compared to that of the post-synaptic AMPA receptor subunit GluR1. DCC immunoreactivity distributed to both GluR1-positive dendrites and GluR1-negative processes (Figure 3H-J; arrowhead), suggesting that DCC localizes to both axons and dendrites. This was confirmed by the co-localization of DCC with the axonal marker NFM (not shown). At 7 DIV, before cortical neurons form synaptic connections, DCC was detected at the tip of filopodial extensions on axonal shafts (Figure 3K) and in dendrites (Figure 3L). In mature 15 DIV neurons, DCC immunoreactivity was also detected at synaptic sites (Figure 3M). Together, these observations suggest that the polarized secretion of netrin-1 from dendrites might act on DCC-expressing axons and dendrites *in vitro*.

Netrin-1 increases the number of axonal and dendritic filopodia and branches

We recently reported that netrin-1 signaling through DCC influences cytoskeletal dynamics and promotes the formation of filopodia in HEK293 cells (Shekarabi and Kennedy, 2002). To determine whether netrin-1 similarly induces filopodia formation in DCC-expressing neurites, dissociated cortical neurons cultured for 6 to 8 DIV were stimulated by bath application of netrin-1 at a concentration of 150 ng/ml, fixed and stained for GAP-43, a membrane protein associated with the cytoplasmic surface of growth cones and filopodia. GAP-43 staining was detected in both axons and dendrites of neurons cultured for up to 8 DIV, but was restricted to axons at later developmental stages, consistent with previous reports (Van Lookeren et al., 1992a; 1992b). Cortical axons treated with netrin-1 for 60 min showed a 25% increase in the density of GAP-43-

positive axonal filopodia compared to control untreated cells (Figure 4 A-D). This effect was detected as early as 15 min after netrin-1 stimulation and lasted for at least 24 hr (Figure 4D). To determine if the increase in filopodia density induced by netrin-1 requires DCC, cortical neurons were exposed to 10 μg/ml DCC function-blocking monoclonal antibodies (DCCfb), a concentration previously reported to antagonize DCC-mediated netrin-1 signaling (KeinoMasu et al., 1996), 15 min before the addition of netrin-1. Whereas application of DCCfb alone for 24 hr did not significantly affect the density of axonal filopodia compared to control, DCCfb abolished both initial and long-lasting increases in filopodia number induced by netrin-1, indicating that netrin-1 requires DCC to enhance axonal filopodia density (Figure 4D). It is well established that filopodia extensions from axon shafts can be stabilized to form interstitial axonal branches (Bastmeyer and O'Leary, 1996; Gallo and Letourneau, 1998). We investigated whether netrin-1-mediated enhancement of filopodia density could translate into *de novo* cortical axon branch formation. To do so, cortical neurons were incubated with netrin-1 for periods ranging from 15 min to 72 hr and the total number of branch points per axon was scored and normalized as a function of branches per 100 μm axon length. Under control conditions, cortical neurons cultured for 6 DIV averaged 1.0 branch, whereas neurons treated with netrin-1 for 24 hr and 72 hr averaged 1.5 branches, and 1.9 branches per 100 μm axon length, respectively (Figure 4E-F). Importantly, we did not detect any significant changes in axon outgrowth rate (Figure 4G), indicating that netrin-1 had a direct effect on axonal branching. Netrin-1-induced axonal branching was observed as early as 1 hr after stimulation, and required signaling through DCC receptors (Figure 4F).

Changes in the density of filopodia were not restricted to axons. In fact, we found that netrin-1 induced a rapid ~23% increase in the density of dendritic filopodia within 15

min of stimulation. The effect was most noticeable 1 hr post-stimulation; primary dendrites averaged 9.4 ± 0.4 filopodia compared to 7.0 ± 0.4 filopodia per 10 μm primary dendrite in control untreated cultures, an increase of ~34% (Figure 5A-C). Interestingly, no significant difference in the density of dendritic filopodia was observed 24 hr after netrin-1 stimulation when compared to control cultures, indicating that netrin-mediated remodeling of dendritic filopodia dynamics is short lasting and subject to regulatory mechanisms. To investigate whether netrin-1 also influences dendritic branch formation, dissociated cortical neurons were cultured for 14 DIV, stimulated with netrin-1 for 24 hr, fixed and immunostained for MAP2 to label dendrites (Figure 5D-E). A profile of dendritic arbor complexity was established using the method of Sholl (1953). Under control conditions, cortical neurons exhibited on average 6.5 ± 0.2 primary dendrites, which branched most extensively within a 30 μm radius from the cell body (Figure 5F). Whereas netrin-1 did not affect the number of primary dendrites (6.7 ± 0.3), it significantly enhanced dendrite branching. Netrin-1-induced increases in dendritic branch complexity was reduced to control upon concomitant incubation of DCCfb with netrin-1.

Netrin-1 increases the number of synaptic contacts in vitro.

Initial contact between axons and dendrites is sufficient to initiate assembly of a functional synaptic site (Fiala et al., 1998; Ziv and Smith, 1996). Factors that modulate the dynamics of branches and filopodia are therefore likely to influence synapse formation, simply by altering the probability of chance encounters between axons and dendrites. We investigated whether netrin-1 stimulation enhances the formation of synaptic connections. Cortical neurons cultured for 2 weeks *in vitro* were stimulated by bath application of netrin-1 for 24 hr, fixed and processed for synapse visualization by

immunocytochemistry. Synapses were identified as synaptophysin immunoreactive puncta closely apposed to GluR1 immunoreactive dendrites (Figure 6A) (O'Brien et al., 1997; Rao et al., 1998). Compared to control untreated cells, netrin-1 induced a 23.4 ± 3.7 % increase in the density of presynaptic puncta adjacent to dendritic processes. This effect was prevented in the presence of DCCfb (Figure 6A-C), consistent with the requirement for DCC to initiate actin cytoskeleton dynamics. Netrin-DCC signaling was reported to increase cell spreading (Shekarabi and Kennedy, 2002) and it has been suggested that increases in dendritic thickness can influence the number of synaptic connections by augmenting the target surface area (Jontes and Smith, 2000). In fact, our data confirm that larger dendrites bear greater number of synapses (not shown). We found that exogenous addition of netrin-1 and/or DCCfb did not appreciably affect dendritic thickness (Figure 6D), indicating that netrin-1-mediated increase in synapse density is likely a reflection of enhanced axonal and dendritic sprouting. In addition, we determined that netrin-1 does not appreciably alter the levels of several major synaptic proteins, indicating that the observed increase in synaptic density is due to the reorganization of synaptic components (Figure 6E).

DCC signaling is not required for synapse formation and stabilization in vitro

Our findings suggest that exogenous addition of netrin-1 enhances synapse number in a DCC-dependent manner. However, although netrin-1 is produced by cortical neurons in culture (see Figure 2), addition of DCCfb alone for 24 hr does not significantly affect the density of synaptic connections (Figure 6C), suggesting that endogenous netrin-DCC signaling is not required for synapse formation. DCC was recently reported to participate in netrin-mediated adhesion (Shekarabi et al., 2004),

indicating that netrin-DCC interaction might be involved in synapse adhesion and stabilization. However, DCCfb antibodies do not prevent netrin-DCC binding (KeinoMasu et al., 1996), and thus do not reveal an endogenous function for netrin in adhesion and synapse maintenance. To investigate the requirement for DCC in synapse formation and/or adhesion, cortical neurons derived from cerebral cortices of E15 DCC knockout (-/-), DCC heterozygous (+/-), or wild type (+/+) littermates were cultured for 2 weeks *in vitro*, fixed and immunostained for synaptophysin and GluR1 to identify axo-dendritic synaptic boutons. We did not detect any difference in the density of synaptic contacts (Figure 7), suggesting that DCC is not required for synapse formation and maintenance *in vitro*.

Netrin increases the frequency and amplitude of miniature excitatory postsynaptic currents.

To address whether the netrin-1-mediated increase in synapse number leads to functional changes in synaptic transmission, we used whole-cell patch-clamp recording of dissociated cortical neurons cultured for 2 weeks *in vitro*. Cultures were stimulated or not by bath application of netrin-1 for 24 hr, with or without DCCfb. Under control conditions, cortical neurons displayed synaptic activity with spontaneous miniature excitatory postsynaptic currents (mEPSCs) occurring at a mean frequency of 5.3 ± 0.7 sec^{-1}, and with a mean peak amplitude of 18 ± 1.3 pA (Figure 8A). Exposure to netrin-1 significantly potentiated the mean mEPSC frequency to 11.3 ± 1.7 sec^{-1} and the mean amplitude to 24 ± 1 pA (Figure 8A-C). Although we cannot exclude that the ~36% increase in mEPSC amplitude appreciably contributes to the twofold increase in the frequency of mEPSCs detected above background noise, it is unlikely to be responsible

for the effect in its entirety. The potentiation of mEPSC frequency induced by netrin stimulation likely indicates that netrin-1 increases the probability of quantal release and/or the number of functional presynaptic sites. The latter is consistent with our immunocytochemical findings. Moreover, addition of DCCfb with netrin-1 to the culture medium for 24 hr blocked the increase in mEPSC frequency (5 ± 0.3 sec^{-1}) and amplitude (15.6 ± 1.3 pA), suggesting that netrin-1 modulates synaptic activity in a DCC-dependent manner. DCCfb alone did not influence either the frequency (4.5 ± 0.5 sec^{-1}) or amplitude (16.7 ± 1.2 pA) of mEPSCs compared to control, indicating that DCCfb acts specifically to block exogenous netrin-1 signaling.

Netrin-1 induces the cell surface expression of DCC.

Our findings suggest that endogenous netrin-1-DCC signaling is not appreciably involved in the formation and function of synaptic contacts *in vitro*. One possible explaination is that the cell surface presentation of netrin-1 and DCC is limiting in our dissociated culture conditions, thereby preventing the detection of an endogenous function for the netrin-DCC receptor complex using function-blocking antibodies. Our findings *in vivo* support a model where netrin-1 and DCC reside primarily intracellularly in the adult CNS. Furthermore, we recently reported that netrin-1 increases the cell surface expression of DCC in commissural neurons (Bouchard et al., 2004). In order to understand how netrin-1 secreted from dendrites might affect DCC-expressing neuronal processes, we tested whether netrin-1 enhanced the surface presentation of DCC in cortical neurons. Mature cortical neuron cultures were stimulated with netrin-1 for 15 min or 24 hr, and processed for cell surface protein expression using biotinylation, streptavidin pull-downs and western blot analysis. Whereas the surface expression of N-

Cadherin and the glutamate receptor subunits GluR1, GluR2/3 and NR1 remained unaltered, DCC surface expression was significantly increased after 15 min, but not 24 hr in the presence of netrin-1 (Figure 9). Interestingly, consistent with its distribution *in vivo*, netrin-1 was barely detected at cell surfaces under control conditions. Whereas exogenous netrin-1 addition at a concentration of 150 ng/ml did not appreciably increase the total levels of netrin-1 protein detected in whole cell lysates, it significantly contributed to the amount of netrin-1 protein found at cell surfaces (Figure 9). Synaptophysin was used as a negative control to assess cell membrane integrity during the biotinylation procedure. These results suggest that application of netrin-1 to cortical neurons increases the relative amount of netrin-1 at the plasma membrane and the cell surface expression of DCC, and provide a possible explaination for the lack of effect observed with DCC function-blocking antibodies alone.

DISCUSSION

It has become increasingly clear over the past few years that several guidance cues used by neurons during embryonic development to establish neuronal networks continue to be expressed in the adult central nervous system (CNS) (Gavazzi, 2001; Koeberle and Bahr, 2004; Manitt and Kennedy, 2002). However, little is known about the function of these molecules in the adult CNS. Here, we report that the guidance molecule netrin-1 and its receptor DCC are expressed by neurons in the cortex and hippocampus of adult mice. We show that netrin-1 enhances axonal and dendritic sprouting and increases the number and efficacy of synaptic connections in a DCC-dependent manner *in vitro*. Our data suggest a novel function for netrin-1 and DCC in initiating synaptogenesis and modulating synaptic plasticity.

Netrin-1 enhances synapse formation

It is well established that initial contact between axons and dendrites is sufficient to establish a functional synaptic site (Fiala et al., 1998; Ziv and Smith, 1996), and that dynamic actin-based filopodia extensions from both axonal and dendritic compartments are important in initiating this contact (Chang and De Camilli, 2001; Jontes and Smith, 2000). In addition, regulation of synapse number and morphology is thought to influence synaptic transmission efficacy (Bonhoeffer and Yuste, 2002; Lamprecht and LeDoux, 2004; Rao and Craig, 2000). Therefore, neuronal signals that influence cytoskeletal dynamics are likely to affect synapse formation and synaptic plasticity.

Netrin-1 mediates its guidance function during development by inducing actin reorganization and increasing filopodia number in neuronal growth cones, through the activation of the Rho GTPases Rac1 and Cdc42 (Li et al., 2002b; 2002a; Shekarabi et al.,

2004; Shekarabi and Kennedy, 2002). Accordingly, we find that netrin-1 increases the number of filopodia and branches in both axonal and dendritic compartments of cortical neurons in a DCC-dependent manner, suggesting that netrin-1 similarly affects Rho GTPases in cortical neurons. This effect is consistent with previous reports that demonstrated a role for netrin-1 in axonal branch formation *in vitro* and *in vivo* (Dent et al., 2004; Wadsworth et al., 1996).

In agreement with the hypothesis that increased axo-dendritic contact favors synaptogenesis, we detect a ~23% increase in the density of synaptic contacts in cortical neuron cultures 24 hr after netrin-1 stimulation. This effect requires signaling through DCC, but addition of DCC function-blocking antibodies alone, or culturing cortical neurons derived from DCC knockout pups does not reveal any appreciable defect in synapse number, indicating that DCC signaling is not required for synapse formation *in vitro*. This being said, the low levels of netrin-1 cell surface expression, as well as the limited number of contact sites between primary neurons cultured *in vitro* under low density conditions might limit our ability to detect endogenous functions for netrin-1-DCC signaling in the regulation of axonal and dendritic sprouting and synapse formation. We propose that netrin-1 serves as a positive regulator of synapse formation by influencing the likelihood of axo-dendritic contact and synaptogenesis. This modulatory function might be achieved via the regulated postsynaptic secretion of netrin-1 in the extracellular space and/or via the regulated plasma membrane presentation of DCC receptors pre- and postsynaptically.

A modulatory short-range function for netrin in synapse formation has previously been proposed. Genetic analysis in *Drosophila* identified a role for netrin as a muscle-derived short-range cue regulating nerve-muscle synaptogenesis (Mitchell et al., 1996;

Winberg et al., 1998). A similar phenotype was observed in flies lacking frazzled, the homolog of DCC, in motoneurons (Kolodziej et al., 1996). Interestingly, modulating the amount of netrin expressed by muscle cells influenced the number of synaptic connections established by motoneurons (Winberg et al., 1998). These findings ruled out a necessary function for netrin and DCC in nerve-muscle synaptogenesis, and suggested that netrin rather acts as a postsynaptic modulator of synaptic junction formation, possibly by influencing local target selection and axonal branching.

The developmental time course of netrin-1 and DCC expression in cortex *in vivo* further supports a role for netrin-1-DCC signaling in synaptogenesis. Whereas netrin-1 protein levels remain constant in postnatal and adult cortical extracts, DCC expression levels are high for the first two postnatal weeks and significantly decline thereafter, consistent with our findings in the spinal cord (Manitt et al., 2001; 2004). It is interesting to note that synaptogenesis occurs at a fast pace during early postnatal development in rodents – it is estimated that half the total number of synapses in the adult CNS is generated during the first two postnatal weeks (Hinds and Hinds, 1976; Smolen and Raisman, 1980). Moreover, dendritic filopodia are the prominent and most dynamic dendritic protrusion early during postnatal development, and they are progressively replaced by more stable spines as the animal ages (Dailey and Smith, 1996; Lendvai et al., 2000; Smith, 1999; Ziv and Smith, 1996). The tight developmental regulation of DCC protein expression levels suggests that it might function to modulate the plastic changes that occur during the maturation of the CNS. Indeed, the expression levels of DCC correlate nicely with filopodia formation and synaptogenesis *in vivo*. We therefore propose that high DCC protein expression levels induce dynamic cytoskeletal protrusions in both dendrites and axons, leading to synapse formation, and that downregulation of

DCC allows neuronal processes to bear less filopodia and be structurally more stable in the mature CNS.

The low levels of DCC protein expression in the adult CNS do not preclude DCC's involvement in synaptic plasticity. Activity-induced *de novo* synaptogenesis in the adult CNS is accompanied by filopodia extension from dendrites (Engert and Bonhoeffer, 1999; Harris et al., 2003; Maletic-Savatic et al., 1999). Although DCC protein levels are significantly decreased in the adult CNS, DCC might participate in filopodia extension via increased cell surface receptor presentation or localized upregulation of DCC protein expression within distinct neuronal populations.

Netrin-1 enhances synaptic transmission efficacy

Consistent with the observed increase in synapse density upon netrin-1 stimulation, we measured an increase in mEPSC frequency, which is typically attributed to presynaptic changes such as an increase in the number of presynaptic neurotransmitter release sites. However, mEPSC frequency also reflects quanta release probability and we cannot exclude at this point the possibility that netrin-1 modulates the probability of neurotransmitter release. Interestingly, we also detected a significant increase in mEPSC amplitude, which is often associated with postsynaptic modifications in synaptic transmission efficacy, such as increased postsynaptic receptor density. Although we did not detect any appreciable change in the cell surface biotinylated fraction of glutamate receptor subunits, further study is necessary to determine whether netrin-1 induces modifications in the postsynaptic density receptor composition or phosphorylation.

An alternative possibility to account for the increase in synaptic efficacy induced by netrin-1 is that structural changes mediated by DCC modulate synaptic strength. Of

particular interest, the activity of Rac1 and RhoA were shown to promote the formation and reduce the number of spines, respectively, and were proposed to act antagonistically in spine formation and growth (Luo et al., 1996; Nakayama et al., 2000; Scott et al., 2003; Tashiro et al., 2000). Moreover, blockade of Rac1 was recently reported to induce long, thin spines and to inhibit spine head growth morphing and stability (Tashiro et al., 2004), suggesting that Rac1 activation is an important mediator of the morphological changes that spines undergo during synaptic plasticity. Interestingly, DCC was reported to activate Rac1 but not RhoA in neurons (Li et al., 2002b; Shekarabi and Kennedy, 2002). Large spine heads typically have larger postsynaptic densities, increased AMPA to NMDA receptor ratios, and are connected by presynaptic active zones with larger readily-releasable synaptic vesicle pools (Harris and Stevens, 1989; Schikorsky and Stevens, 1997; Takumi et al., 1999). In addition, structural modification of spines alter their biochemical properties, namely their ability to compartmentalize Ca^{2+} (Tsai and Yuste, 2004). Indeed, variations in the length of the spine neck and volume of the spine head influence the ability of spines to maintain high concentrations of Ca^{2+} and to transfer Ca^{2+} transients into the parent dendrite (Majewska et al., 2000; Volfovsky et al., 1999). We propose that DCC modulation of spine morphology via Rac1 activation leads to larger spine heads and shorter necks, leading to better functional coupling between spine and parent dendrite, and thus larger postsynaptic depolarization currents.

Netrin-1/DCC distribution in neurons

We recently reported that *netrin-1* and *dcc* are expressed in the spinal cord of adult rats, suggesting that netrin function might not be restricted to embryonic development (Manitt et al., 2001; 2004). We confirmed by *in situ* hybridization and

western blot analyses that both netrin-1 and DCC are expressed by neurons throughout the cortex and in the hippocampal formation in adult mice. Subcellular fractionation of adult cortex revealed that both netrin-1 and DCC are present at synaptic sites. Consistent with our previous findings in commissural neurons (Bouchard et al., 2004) and our biotinylation data that suggests that endogenous netrin-1 is barely detectable at the cell surface *in vitro*, both netrin-1 and DCC reside mostly in intracellular vesicles *in vivo*. Interestingly, once at synaptic membranes, both proteins partition to triton X-100-resistent fractions. This is consistent with the recent observation that DCC is recruited to lipid rafts in cortical neuron growth cones upon netrin-1 stimulation (Guirland et al., 2004).

We determined by immunocytochemistry that DCC localizes to both axonal and dendritic processes, whereas netrin-1 is restricted to dendrites in cultured cortical neurons. The distribution of DCC is consistent with previous reports that axonal and dendritic growth cones respond to netrin-1 gradients (Furrer et al., 2003; Kennedy et al., 1994). Analysis of the endogenous staining pattern of netrin-1 is complicated by the fact that netrin-1 is a secreted protein that preferentially associates with extracellular matrix components and cell membranes (reviewed in Manitt and Kennedy; 2002; Wadsworth, 2002). Moreover, frazzled was shown to capture netrin and present it on cell surfaces in *Drosophila* (Hiramoto et al., 2000). Using GFP and Myc expression constructs, we determined that netrin-1 is restricted to dendrites *in vitro*, and propose that the faint endogenous netrin-1 immunoreactive signal detected along axons is cell-surface associated. The polarized distribution of netrin-1 in dendrites is consistent with its distribution to epithelial basement membranes in peripheral tissues (Koch et al., 2000; Srinivasan et al., 2003; Yebra et al., 2003), and the distribution of related family

members such as laminins, which, in epithelia, are secreted baso-laterally to basement membranes. In fact, baso-lateral secretory pathway in epithelia is thought to correspond to dendritic polarization in neurons (Caplan et al., 1987; Dotti et al., 1991; Dotti and Simons, 1990). In a manner similar to the action of postsynaptically released BDNF onto TrkB-expressing pre- and postsynaptic neurites (Huang and Reichardt, 2001; Kryl et al., 1999; Thoenen, 1995; 2000; Wu et al., 2004), we propose that, based on the distribution of netrin-1 and DCC in neurons, endogenous netrin-1 is released postsynapically to act on DCC-expressing neuronal processes both pre- and postsynaptically. Although it remains unclear whether netrin-1 is released in a constitutive or regulated manner, its accumulation in vesicles at synapses *in vivo* suggest that netrin-1 secretion might be regulated.

Physiological role for netrin-1 in synaptic plasticity – a conceptual model

Synaptic plasticity refers to the processes by which the number and/or strength of synaptic connections are altered in response various stimuli. Interestingly, it is thought that structural plastic changes that occur in the adult CNS are similar to the growth processes that occur during development (Lamprecht and LeDoux, 2004).

Netrins are bi-functional guidance cues that direct growth cone motility. The responsiveness of growth cones to netrins depends on the relative expression of two classes of netrin receptors – the DCC and UNC-5 receptor families. DCC is required for both attractive and repulsive responses to netrins, while UNC-5 is primarily required for repulsive responses. Our data show that DCC undergoes significant translational downregulation after the second postnatal week, suggesting an important regulatory role for DCC. Manitt and colleagues (2004) report that UNC-5 expression levels are increased

in the adult spinal cord compared to embryonic levels, and propose that netrin-1 is therefore primarily seen as an inhibitory guidance cue in the adult CNS. We propose that netrin-1 signals through DCC during postnatal development to promote cytoskeletal dynamics, and that in the adult CNS, netrin-1 is seen as a stabilizing cue.

We recently reported that DCC presentation at the cell surface is tightly regulated by cAMP levels, PKA and PKC activation in commissural and cortical neurons (Bouchard et al., 2004; Bouchard, unpublished observations). Here, we show that netrin-1 increases surface expression of DCC in cortical neurons, and propose that the presence of DCC at the cell surface allows endogenous and exogenous netrin-1 to signal as an axonal or dendritic filopodia-promoting cue. Moreover, Williams and coworkers (2003) reported that PKC activation leads to UNC-5 internalization (Williams et al., 2003). Importantly, cAMP, PKA and PKC are important mediators of long-term potentiation and plastic changes at synapses. We therefore propose that under physiological conditions, activity-dependent structural plasticity events involve the externalization of DCC and endocytosis of UNC-5, switching netrin-1 signaling from growth inhibition to promotion. We propose a mechanism where the cell surface presentation of DCC is the critical factor determining the ability of endogenous netrin-1 to induce morphological changes leading to enhanced synapse number and efficacy. This model, together with the low levels of netrin-1 detected at cell surfaces by subcellular fractionation and biotinylation, provide an explanation for the lack of effect observed with DCCfb alone. Collectively, these observations suggest a novel function for netrin-1 and DCC in synaptogenesis and synaptic plasticity.

MATERIALS AND METHODS

Reagents

All cell culture media and supplements were purchased from Invitrogen Canada (Burlington, ON). Anti-DCC (G97-449) monoclonal antibodies were purchased from PharMingen (Mississauga, ON) and anti-DCC function-blocking monoclonal antibodies (DCCfb, clone AF5) from Calbiochem (LaJolla, CA). Polyclonal antibodies against GAP-43 (AB5220), GluR1 (AB1504), GluR2/3 (AB1506) and NFM (AB1987) were obtained from Chemicon (Temecula, CA). Anti-MAP2 and anti-synaptophysin monoclonal antibodies were purchased from Sigma-Aldrich (Mississauga, ON). Monoclonal antibody against PSD-95 was obtained from BD Biosciences (Mississauga, ON). Monoclonal actin antisera was purchased from ICN Biomedicals (Aurora, OH), polyclonal anti-GFP (A-6455) from Molecular Probes (Eugene, OR) and polyclonal anti-NR1 from Upstate (Lake Placid, NY). Polyclonal netrin-1 antisera (11760) was characterized previously (Manitt et al., 2001). Anti-Myc (clone 9E10) monoclonal antibody and RFP expression vector were generously provided by Dr. Phil Barker (McGill). Recombinant netrin-1 protein was purified from a HEK293T cell line secreting netrin-1 as described (Serafini et al., 1994; Shirasaki et al., 1996). Netrin-1-GFP was obtained by cloning full-length netrin-1-Myc (Serafini et al., 1994) into pEGFP-N2 (BD Biosciences) using HindIII and PCR-generated BamH1 restriction sites.

In situ hybridization

In situ hybridization was carried out as described (Manitt et al., 2001; 2004). Sense and antisense cRNA probe pairs corresponding to 853 bases of rat *dcc*, nucleotides 3786-4639, and to 933 bases of rat *netrin-1*, nucleotides 882-1815, were used.

Subcellular Fractionation

Cortices from 10 adult CD1 mice were dissected and homogenized in ice cold homogenization buffer (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, Aprotinin, Leupeptine, Pepstatin) in a Teflon-glass homogenizer. Homogenates were subjected to differential centrifugation to various fractions according to the methods of Cohen et al. (1977), Ueda et al. (1979) and Carlin et al. (1980). Protein concentration was quantified using BCA (Pierce, Rockford, IL). 10 μg of proteins were resolved by SDS-PAGE and processed for western blot analysis. Results were visualized using chemiluminescence (NEN Life Science, MA).

Cortical Neuron Cultures

Cell cultures were prepared from cerebral cortices of E15 CD1 mice as previously described (Banker and Goslin, 1998; Brewer et al., 1993). Briefly, cortices were dissected in ice cold Hank-Balanced Salts Solution, diced and incubated for 15 min at 37°C in Hepes-buffered Minimum Essential Medium containing 0.25% Trypsin and 0.001% DNase I. The tissue was then washed once in cold Neurobasal supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gently triturated with a flamed Pasteur pipette to yield a suspension of single cells. Dissociated cells were plated at low density (2,500 cells/cm^2) for analyses of branching, filopodia and synapse number, at medium density (15,000 cells/cm^2) for biochemical and electrophysiological analyses, and at high density (40,000 cells/cm^2) for transfection. Cultures were maintained for 2 to 18 DIV in Neurobasal medium containing 1% B27, 0.5% N2, 2 mM glutamine, 1 unit/ml penicillin, and 1 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Penicillin, streptomycin and glutamine were omitted from the culture medium after one week in

culture. Under these conditions, cortical neurons formed large networks and developed functional synapses, while keeping glial cell contamination low (Brewer et al., 1993; Evans et al., 1998). Cortical neuron cultures were stimulated by adding 150 ng/ml netrin-1 directly to the culture media. DCCfb antibody (10 μg/ml) was added to the medium 15 min before netrin-1 stimulation. Cortical neurons were transfected using 0.5 μg of DNA and 0.25 μl Lipofectamine 2000 (Invitrogen) per cm^2.

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4, and blocked with 2% FBS/2% bovine serum albumine/0.1 % Tween-20 in 0.1 M PBS, pH 7.4, for 1 hr at room temperature. Antibodies were used in blocking solution overnight at 4°C at the following dilutions: anti-DCC (1:500), anti-GAP-43 (1:1000), anti-GFP (1:3000), anti-GluR1 (1:500), anti-MAP2 (1:6000), anti-Myc (1:500), anti-netrin (1:250), anti-NFM (1:3000) and anti-synaptophysin (1:500). After three washes in PBS, cells were labelled with Alexa 488 or Alexa 546 secondary antibodies (Molecular Probes) for 2 hr at room temperature in blocking buffer containing Hoechst 33258 (Sigma) to stain for nuclei. Images were captured using a Carl Zeiss Axiovert microscope, Magnafire CCD camera (Optronics, CA), and analysed using Northern Eclipse (Empix Imaging, Mississauga, ON) and NIH image analysis software (United States National Institutes of Health).

Electrophysiology

Electrophysiological recordings from 15 DIV cortical neurons were performed at room temperature after replacing the culture medium with Tyrode's Buffer (in mM: 140 NaCl,

5 KCl, 3 $CaCl_2$, 2 $MgCl_2$, 10 Hepes; adjusted to pH 7.4 with NaOH). Patch pipettes (4-7 MΩ) were filled with a solution consisting of (in mM): 105 CsF, 10 CsCl, 10 NaCl, 10 Hepes, 10 EGTA (pH 7.2 adjusted with CsOH). Whole-cell recording techniques were used to measure resting membrane potentials and spontaneous synaptic activity. Resting membrane potentials were measured in current-clamp mode after establishment of the whole-cell configuration. Spontaneous miniature excitatory postsynaptic currents (mEPSCs) were recorded in voltage-clamp mode at a holding membrane potential of –70 mV in the presence of 1 μm TTX (Sigma) to block Na^+-channels and 1 μm SR-95531 (Sigma) to block $GABA_A$ receptors. Data were acquired at 5 KHz and filtered at 0.5 KHz, using an Axiopatch 200B amplifier. Acquisition and analysis were performed with pCLAMP 8.0 (Axon Instruments Co., Foster City, CA). Miniature synaptic currents above our detection threshold of 9 pA were analyzed using MiniAnalysis (Synaptosoft Inc., Leonia, NJ) and verified visually.

Surface Receptor Biotinylation

12 DIV cortical neurons were washed three times with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++). Surface biotinylation was performed by adding 0.5 mg/ml EZ-Link Sulfo-NHS-LC-biotin (Pierce) in PBS++ for 30 min at 4°C. Excess biotin was quenched by washing the cells twice with 10 mM glycine in PBS++ at 4°C and twice with ice-cold PBS++. Cells were lysed in 1 ml RIPA buffer (150 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) for 15 min on ice. Biotinylated proteins were precipitated with streptavidin-agarose beads (Pierce) for 1 hr at 4°C and analyzed by western blot. Quantification was performed on scanned images of immunoblots using NIH image software.

Data Analysis and Quantification

All images used for quantification were taken by an observer blind to the experimental condition using the same exposure time to allow comparison of measurements within an experiment. Axons were defined as the longest neurite emerging from the cell body. Axonal branching analysis was performed according to Aoyagi et al. (1994), with all axonal processes greater than 20 μm in length being counted as branches. GAP-43 positive axonal filopodia were thresholded and skeletonized using NIH image software for clarity, and processes shorter than 15 μm were scored as filopodia. Filopodia and branch numbers were normalized as a function of filopodia/branches per 100 μm axon length. Dendritic filopodia were defined as GAP-43 positive protrusions from primary dendrites and normalized as a function of filopodia per 10 μm primary dendrite. Dendrite branching analysis was performed based on the method described by Sholl (1953). Neurons were overlaid with concentric rings set apart at 15 μm-equivalent intervals using the Northern Eclipse software and the number of dendrites intersecting each consecutive ring counted. Primary dendrites were defined as MAP2-positive processes over 15 μm in length extending from the soma. For quantitative measurements of synaptic puncta density, images of randomly selected neurons were captured. A thresholding function (Northern Eclipse) was used to include as many typical puncta as possible and exclude other structures. The number of immunoreactive presynaptic puncta closely apposed to proximal 30 μm segments of primary dendrites was scored for quantification. A measure of dendritic thickness was obtained by dividing the surface area of proximal dendritic segments by their length. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA for multiple comparisons followed by the Least Standard Deviation (LSD)

post hoc test using Systat (Chicago, IL). $p < 0.05$ was used as the criteria for statistical significance unless otherwise indicated.

FIGURES AND LEGENDS

Figure 1. Distribution of netrin-1 and DCC mRNA expression in the adult CNS.

Expression of *dcc* **(A-B)** and *netrin-1* **(C-D)** was detected in similar distributions of cells in the adult cortex and hippocampus by *in situ* hybridization. **(C)** and **(D)** are details of DCC and netrin-1 mRNA expression in CA1 and cortex, respectively. Differences in intensity of expression were detected in cortical layers and hippocampal formations, with stronger signals in layers II, III and V of the cerebral cortex for both DCC and netrin-1. DCC signal was strongest in CA1, and faint *netrin-1* hybridization was detected in the hippocampal formation. Hybridization of DIG-labeled probes was visualized by using a POD-conjugated DIG antibody, tyramide amplification, and DAB. Dorsal is at the top and ventral at the bottom. Brightfield optics. Cortical layers I-VI are indicated, cc = corpus callosum. Scale bar = 200 μm for (A) and (C), 50 μm for (B) and (D).

Figure 1

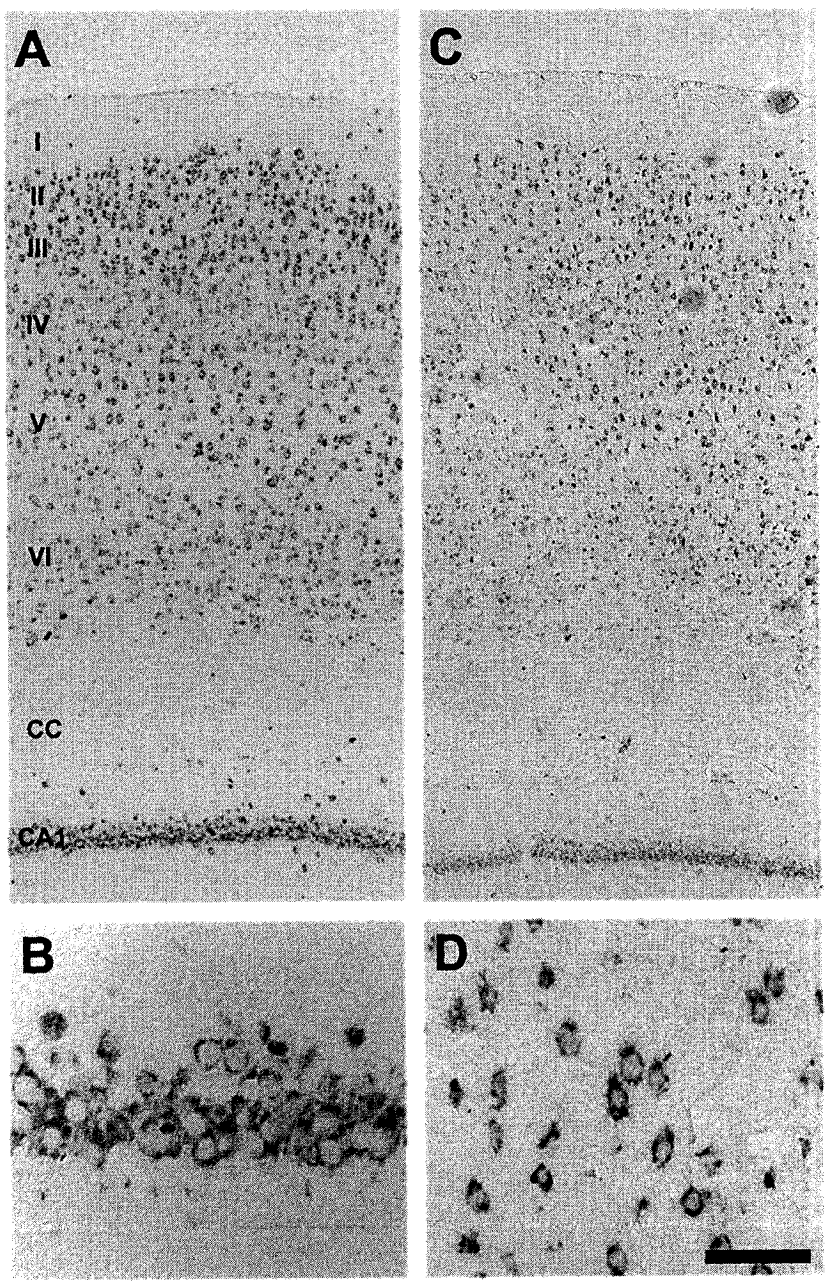


Figure 2. Netrin-1 and DCC protein expression *in vivo* and *in vitro*.

(A) Western blot analysis of netrin-1 and DCC protein expression levels in mouse cortex at various developmental ages ranging from embryonic (E) day 15, postnatal (P) day 0 through 20, and adult (A). GluR1 and Actin were used as controls. While netrin-1 protein levels remain constant throughout development and in the adult cortex, DCC levels significantly decline after the second postnatal week, but remain detectable in the adult CNS.
(B) Fractionation of adult mouse cortical lysates – see text for details. PSD-95 and synaptophysin were used as controls to illustrate synaptic enrichment and the specific separation of synaptic membrane and vesicular fractions. A doublet for netrin-1 has been reported before (Manitt et al., 2001).
(C) Western blot analysis of netrin-1 and DCC protein expression in primary cortical neurons cultured for 1 to 18 days in vitro (DIV). Note the similar pattern of protein expression levels *in vitro* and *in vivo*.

Figure 2

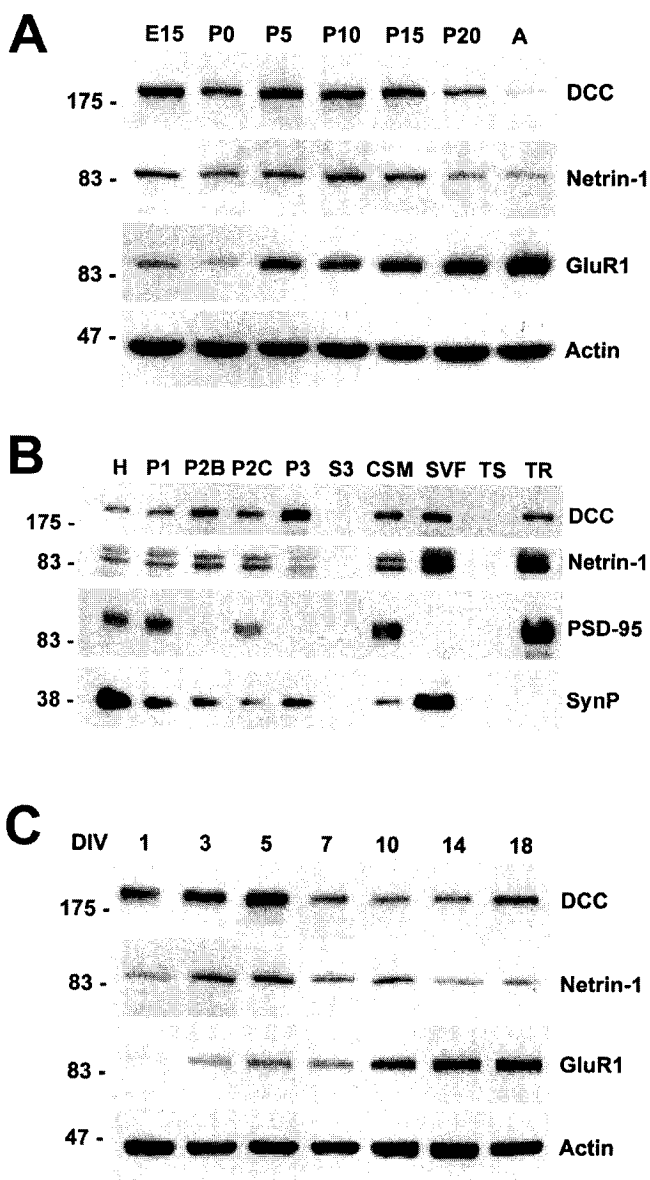

A
E15 P0 P5 P10 P15 P20 A
175 -
DCC
83 -
Netrin-1
83 -
GluR1
47 -
Actin
B
H P1 P2B P2C P3 S3 CSM SVF TS TR
175 -
DCC
83 -
Netrin-1
83 -
PSD-95
38 -
SynP
C
DIV 1 3 5 7 10 14 18
175 -
DCC
83 -
Netrin-1
83 -
GluR1
47 -
Actin

Figure 3. Cellular distribution of netrin-1 and DCC *in vitro*.

(A-G) Cellular distribution of netrin *in vitro*. **(A-C)** Primary cultures of cortical neurons were grown under serum free conditions for 15 DIV, fixed and stained for netrin-1 and MAP2. The specificity of the netrin-1 antibody (PN2) was demonstrated previously (Manitt et al., 2001). Endogenous netrin-1 **(A)** and MAP2 **(B)** immunoreactivity. **(C)** Merged image: netrin immunoreactivity (green) is seen in both MAP2-positive (red) dendrites and MAP2-negative axons (arrowhead). Scale bar represents 20 μm.
(D-G) 14DIV cortical neurons transfected with GFP **(D)** and netrin-GFP **(G)** for 24 hr. Scale bar is 50 μm. **(E-F)** Detail of neuronal segments labeled 'd' and 'a' in (D), respectively. **(E)** Upper panel: GFP. Middle panel: MAP2. GFP colocalizes with MAP2-positive (red) process (lower panel), indicating that GFP distributes to dendrites (labeled 'd'). **(F)** Upper panel: GFP. Middle panel: MAP2. Bottom panel: Lack of colocalization between GFP (green) and MAP2 (red), indicates that GFP also distributes to axons (identified 'a'). Scale bar = 10 μm. **(G)** Netrin-GFP localizes to dendrites (d), but not to neurites with axonal morphology, suggesting that netrin-1 distributes preferentially to dendrites.
(H-M) Cellular distribution of DCC *in vitro*. **(H-J)** 15 DIV cortical neurons stained for DCC (green) and the postsynaptic AMPA receptor subunit GluR1 (red). DCC distributes to dendrites in mature cortical neurons as indicated by the colocalization of DCC **(H)** with GluR1 **(I)**. **(J)** Merged image. DCC also localizes to GluR1-negative axons (arrowhead). Scale bar = 20 μm.
(K-M) DCC distributes to the tip of axonal filopodia **(K)**, dendritic filopodia **(L)** and synaptic sites **(M)** *in vitro* (arrows). Lower panels are details of images (K-M). Filopodia in (K) and (L) are revealed by staining for GAP-43 (green), a membrane-associated cytoplasmic present in axonal and dendritic filopodia in neurons grown for 8 DIV or less. DCC is shown in red. Presynaptic boutons in (M) are stained with VAMP2 (red) and DCC is shown in green. Note the presence of dendritic protrusions (arrowheads), some of which make contact with presynaptic boutons. Scale bar is 15 μm for images (K-M), and 5 μm for lower panel details.

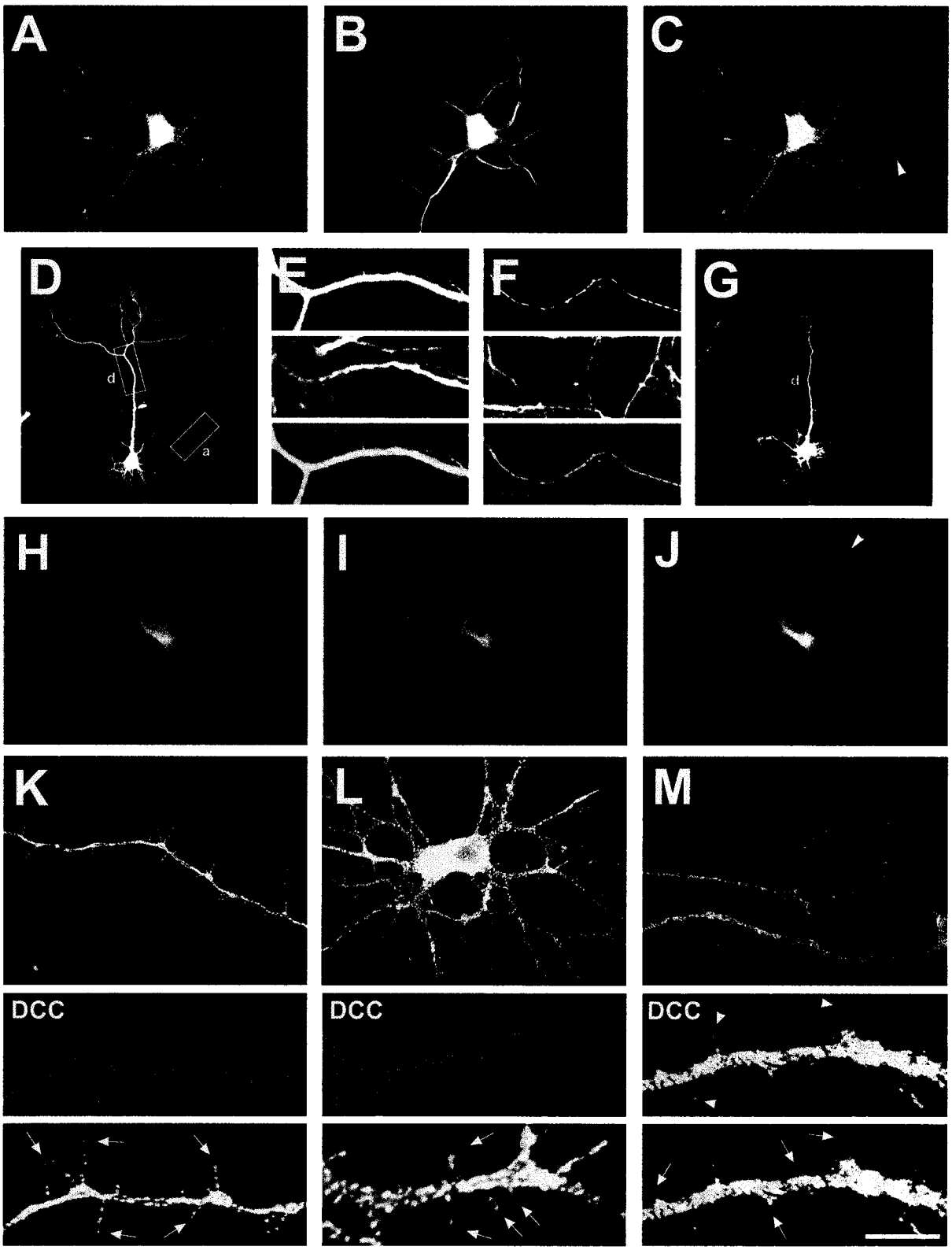

A
B
C
D
d
a
E
F
G
d
H
I
J
K
L
M
DCC
DCC
DCC

Figure 4. Netrin-1 increases axonal filopodia number and branching.

(A) Image of GAP-43 stained axons in the presence or absence of exogenous netrin-1 (150 ng/ml) for 1 hr. Netrin-1 stimulation increases the complexity of axon shafts. Scale bar = 30 μm.
(B) Shape of axonal filopodia extracted from the background by setting a threshold of fluorescence.
(C) Results of skeletalization that was used to distinguish individual axonal filopodia and estimate the filopodia density.
(D) Average number of filopodia per axon (n = 47 to 97 axons per condition) was normalized as a function of filopodia number per 100 μm axon length. Significant differences between netrin-1-treated and control cultures were found. Concomitant addition of DCCfb reduced netrin-1 changes to control levels. Error bars represent SEM, * $p < 0.005$, ** $p < 0.001$.
(E) Inverted immunofluorescence image of representative 6 DIV cortical neurons stained for GAP-43. Scale bar = 50 μm.
(F) Mean number of axonal branch points per axon (n = 74 to 183 axons per condition) was normalized as a function of branch points per 100 μm axon length. Addition of netrin-1 significantly increases axonal branching as early as 1 hr post-stimulation and DCCfb blocked this effect. * $p < 0.01$, ** $p < 0.001$.
(G) Mean axon length was not appreciably affected in these experiments ($p > 0.1$).

Figure 4

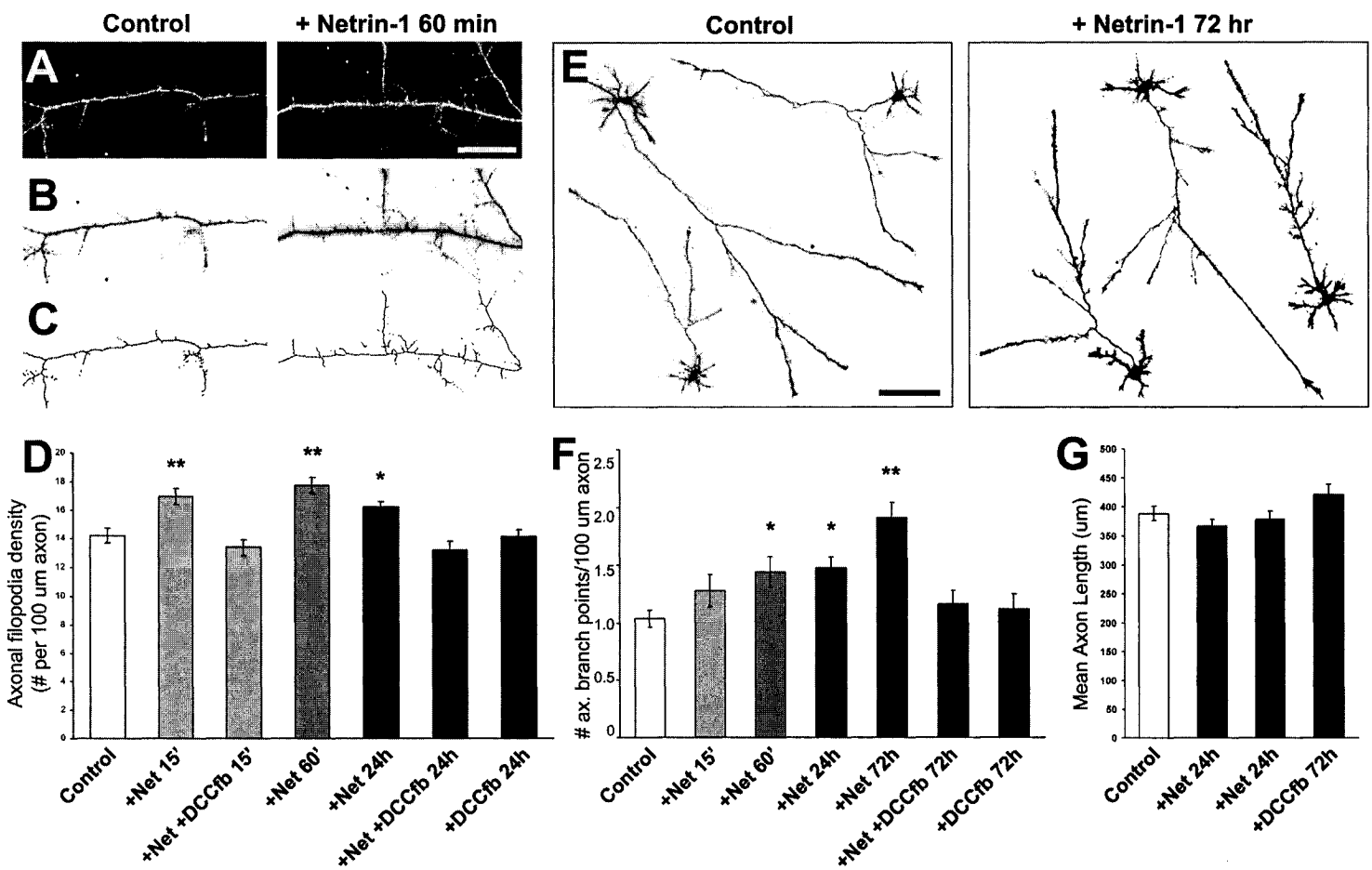

Control
+ Netrin-1 60 min
A
B
C
Control
+ Netrin-1 72 hr
E
D
Axonal filopodia density (# per 100 um axon)
**
**
*
Control
+Net 15'
+Net +DCCfb 15'
+Net 60'
+Net 24h
+Net +DCCfb 24h
+DCCfb 24h
F
ax. branch points/100 um axon
*
*
**
Control
+Net 15'
+Net 60'
+Net 24h
+Net 72h
+Net +DCCfb 72h
+DCCfb 72h
G
Mean Axon Length (um)
Control
+Net 24h
+Net 24h
+DCCfb 72h

Figure 5. Netrin-1 increases dendritic filopodia number and branching.

(A) Control untreated and netrin-1 stimulated **(B)** 8 DIV cortical neuron stained for GAP-43. Axons are labeled 'ax' and were distinguished from dendrites for quantification. Lower panel is a close up of a primary dendrite. Dendritic filopodia were identified as GAP-43 protrusions stemming from the parent primary dendrite. Scale bar = 20 μm for upper panels and 5 μm for lower panels.
(C) Average density of dendritic filopodia (n = 36 to 49 dendrites per condition) was normalized as a function of filopodia number per 10 μm primary dendrite length. Significant differences were found between control cultures and cultures stimulated with netrin-1 for 15 and 60 min, but not 24 hr. * $p < 0.01$, ** $p < 0.001$ compared to control.
(D) Sholl analysis of dendritic branching profile of control (white), netrin-1 stimulated for 24 hr (dark gray), netrin-1 stimulated for 72 hr (black), and netrin-1 stimulated for 72 hr in the presence of DCCfb (Light gray) cortical neurons (n = 62 neurons per condition). The first set of histograms represents the mean number of primary dendritic branches stemming from the soma; no significant difference was observed ($p > 0.4$). The average number of dendrites intersecting concentric rings set apart 15 μm-equivalent from the cell body is reported in subsequent sets of histograms. * $p < 0.05$, ** $p < 0.005$ compared to control.

Figure 5

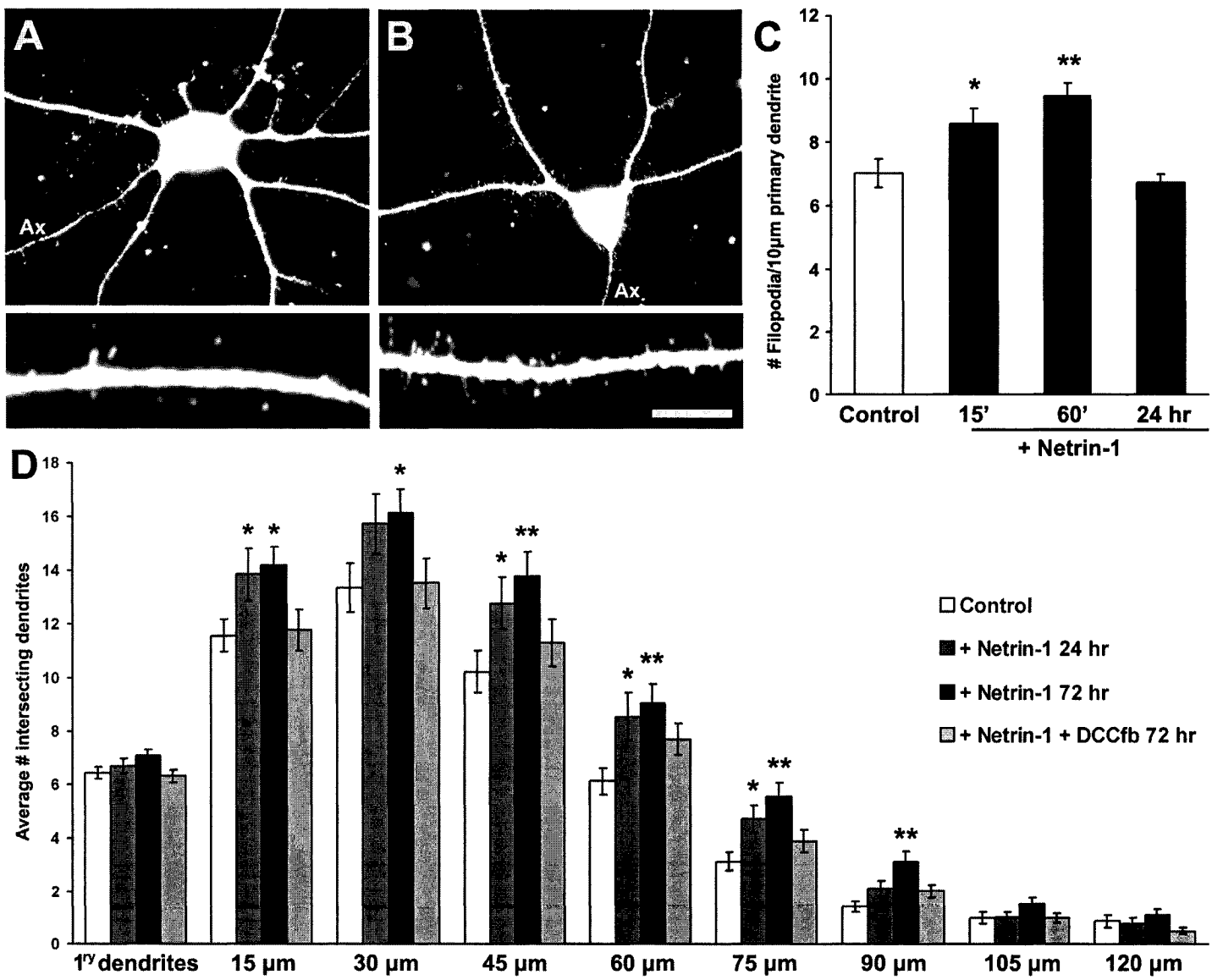

A
Ax
B
Ax
C
Filopodia/10µm primary dendrite
12
10
8
6
4
2
0
*
**
Control
15'
60'
24 hr
+ Netrin-1
D
Average # intersecting dendrites
18
16
14
12
10
8
6
4
2
0
1°dendrites
15 µm
30 µm
45 µm
60 µm
75 µm
90 µm
105 µm
120 µm
Control
+ Netrin-1 24 hr
+ Netrin-1 72 hr
+ Netrin-1 + DCCfb 72 hr

Figure 6. Netrin-1 increases the number of synaptic puncta *in vitro*.

(A-C) Immunocytochemical analysis of 15 DIV cortical neurons shows that 24 hr application of exogenous netrin-1 (150 ng/ml) significantly increases the number of immunoreactive synaptic puncta **(B)** when compared to control **(A)**. GluR1 staining is shown in green and was used as a dendritic marker. Synaptophysin stain (red) labels presynaptic boutons. Insets represent 30 μm segments of primary dendrites used for quantification. Isolated GluR1 positive neurons were selected randomly. Scale bar represents 15 μm in upper panels and 6 μm in lower panels.
(C) Average number of immunoreactive synaptic puncta per μm dendritic length (n = 57 to 138 dendritic segments per condition). * $p < 0.05$.
(D) Mean dendritic thickness, measured as the ratio of dendrite surface area to dendrite length, was not appreciably affected by netrin-1 stimulation.
(E) Western blot analysis of lysates obtained from cultured cortical neurons treated with netrin-1 with or without DCCfb for 24 hr did not reveal any significant changes in the protein expression levels of major synaptic proteins.

Figure 6

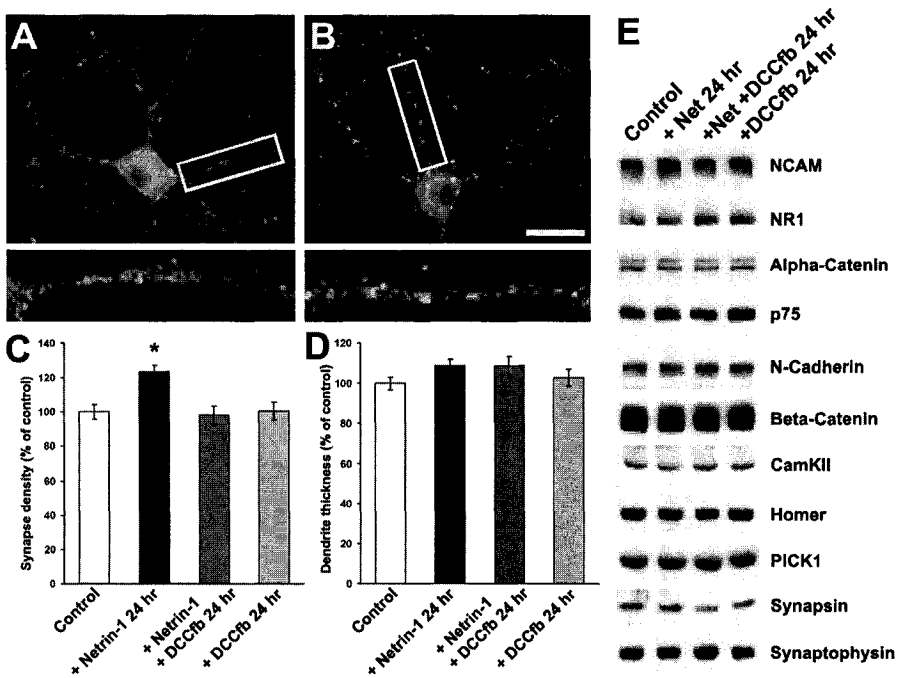

A
B
C
D
E
Synapse density (% of control)
Dendrite thickness (% of control)
Control
+ Netrin-1 24 hr
+ Netrin-1 + DCCfb 24 hr
+ DCCfb 24 hr
+ Net 24 hr
+Net +DCCfb 24 hr
NCAM
NR1
Alpha-Catenin
p75
N-Cadherin
Beta-Catenin
CamKII
Homer
PICK1
Synapsin
Synaptophysin

Figure 7. DCC signaling is not required for synapse formation *in vitro*.

15 DIV cortical neurons derived from DCC knockout (-/-), heterozygous (+/-) and wild type (+/+) littermates do not display any appreciable abnormality in synaptic density, revealed as the close apposition between GluR1-positive dendrites and synaptophysin-positive presynaptic boutons. Western blot analysis of cortical lysates confirm the absence of DCC protein in knockout pups. Scale bar = 10 μm in upper panels; 3 μm in lower insets.

Figure 7

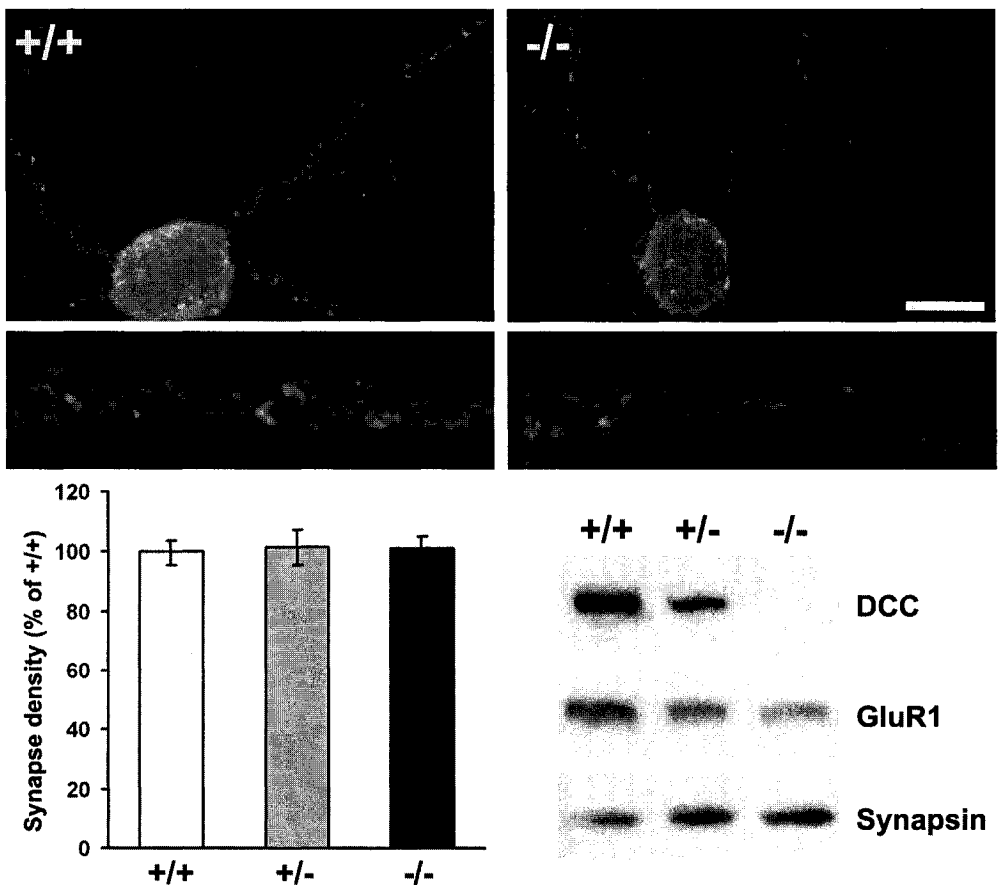


Figure 8. Netrin-1 increases the amplitude and frequency of spontaneous miniature excitatory post-synaptic currents (mEPSCs).

(A) Representative whole cell patch clamp recordings of cortical neurons grown for 14 DIV and treated or not with netrin-1 (150 ng/ml), with or without DCCfb (10 µg/ml) for 24 hr. Netrin-1 significantly increases both the frequency **(B)** and amplitude **(C)** of excitatory synaptic events in a DCC-dependant manner (n = 5 to 18 cells per condition). * $p < 0.05$, ** $p < 0.005$.

Figure 8

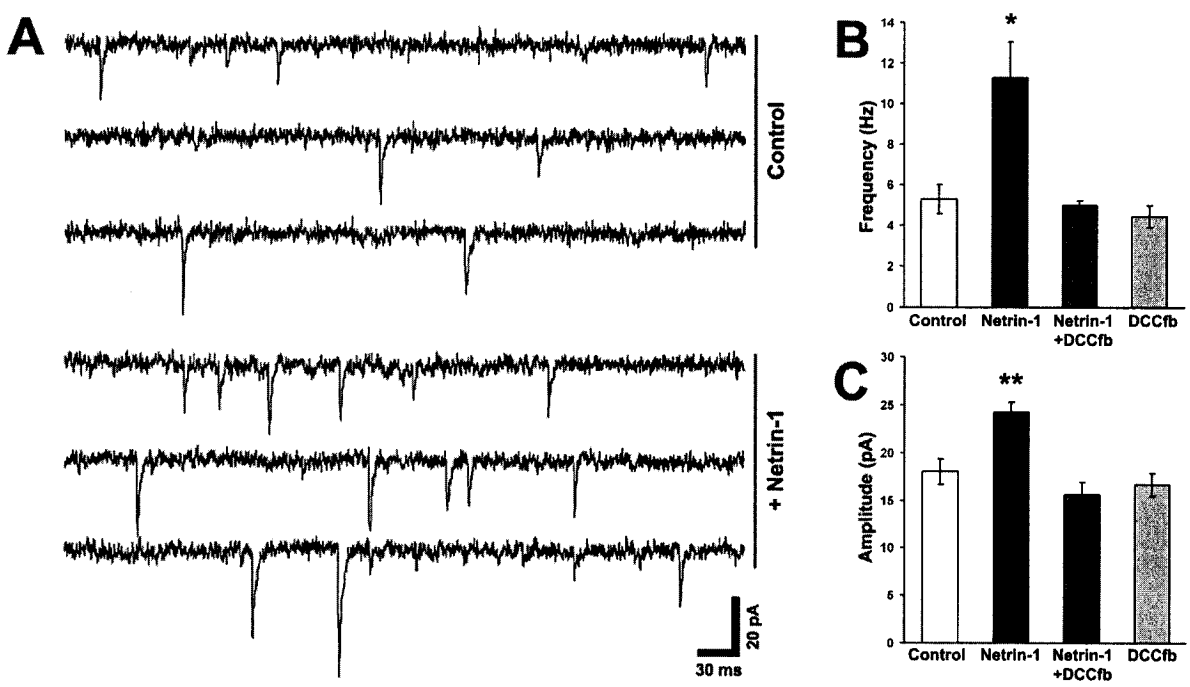
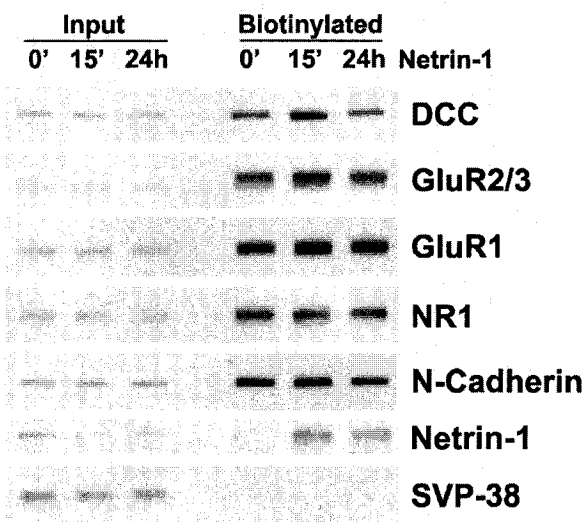

A
Control
+ Netrin-1
20 pA
30 ms
B
Frequency (Hz)
*
Control
Netrin-1
Netrin-1 +DCCfb
DCCfb
C
Amplitude (pA)
**
Control
Netrin-1
Netrin-1 +DCCfb
DCCfb

Figure 9. Netrin-1 increases the cell surface expression of DCC *in vitro*.

Cortical neurons were stimulated or not with netrin-1 for 15 min or 24 hr and cell surface protein expression levels were measured using by biotinylation and streptavidin pull-downs. First 3 lanes represent input lanes for 0 min, 15 min and 24 hr respectively, and the last 3 lanes represent the biotinylated pull-downs for each condition. Netrin-1 significantly increased cell surface DCC 15 min but not 24 hr after stimulation. Interestingly, very little endogenous netrin-1 was detected at the cell surface under control conditions. Although exogenous netrin-1 stimulation did not appreciably contribute to total input netrin-1 protein levels, it significantly contributed to the amount of netrin-1 protein found at cell surfaces. Membranes were stripped and reprobed for GluR1, GluR2/3, NR1 and N-Cadherin to control for the specificity of the netrin-1 effect, and for synaptophysin as a negative control to test for membrane permeability.

Figure 9


Input
0' 15' 24h
Biotinylated
0' 15' 24h
Netrin-1
DCC
GluR2/3
GluR1
NR1
N-Cadherin
Netrin-1
SVP-38

SUMMARY AND CONCLUSION

Since their characterization as the functional junction between two excitable cells over a century ago (see Shepherd and Erulkar, 1997 for discussion), synapses have fascinated neuroscientists as they ultimately dictate the connectivity and function of neural networks. This connectivity is achieved during development by genetically encoded guidance cues and perfected in the mature nervous system by activity-dependent plastic changes. Changes in synaptic transmission underlie complex cognitive processes such as learning, as well as pathological conditions such as epilepsy and dementia. It is therefore imperative to understand how synapses are assembled during development and subsequently altered throughout life to better comprehend CNS functions.

In this thesis, we demonstrate a novel function for the guidance cue netrin-1 and its receptor DCC in CNS neurons. We report that netrin-1-DCC signaling enhances the number and efficacy of synapses *in vitro*. Importantly, netrin-DCC signaling is not required for the formation or stabilization of synaptic contacts, indicating that netrin-1 plays a modulatory role at synapses. We propose that netrin-1 signals to the actin cytoskeleton in axons and dendrites, possibly through the Rho GTPases Cdc42 and Rac1 (Li et al., 2002b; Shekarabi and Kennedy, 2002), leading to the formation of filopodia and branches. These changes are thought to be responsible for the greater number of synaptic sites in netrin-1-treated cortical neuron cultures, simply by increasing the chances of cell-cell contacts required for synapse formation. We hypothesize that netrin-1 similarly induces morphological changes within DCC-expressing spines, leading to functional modifications in synaptic transmission efficacy. Specifically, we propose that, consistent with its reported function in cell lines (Li et al., 2002b; Shekarabi and Kennedy, 2002), netrin-DCC signaling activates the Rho GTPase Rac1 in cortical

neurons. Rac1 activation within dendritic spines is thought to increase synaptic transmission strength by reducing spine length and facilitating the transfer of Ca^{2+} from spines to dendrites (Majewska et al., 2000b; Tashiro and Yuste, 2004; Tsay and Yuste, 2004). The expression levels and distribution of netrin-1 and DCC protein *in vitro* closely resemble the patterns observed in cortical neurons *in vivo*. Together, these observations suggest a novel role for netrin-1 and DCC in promoting cytoskeletal rearrangements leading to synapse formation and synaptic plasticity.

It has become increasingly clear over the past few years that several guidance cues used by neurons during embryonic development to establish neuronal networks are expressed in the adult nervous system, where they are thought to mediate plastic changes such as regeneration and synaptic plasticity (Dityatev and Schachner, 2003; Gavazzi, 2001; Koeberle and Bahr, 2004; Manitt and Kennedy, 2002; Pavlov et al., 2004; Poo, 2001). Our investigation is consistent with such a proposal. However, further studies are required to determine the involvement of netrin-1 and DCC in activity-dependent synaptic plasticity. In particular, it would be interesting to determine if synaptic activity increases the synthesis and/or secretion of netrin-1, similarly to BDNF which, following activity-dependant synthesis and secretion, has been shown to mediate synaptic plasticity (Poo, 2001). Alternatively, the limiting factor to netrin-mediated plastic changes might be the presence of DCC at the plasma membrane. It would be of interest to investigate whether synaptic activity enhances the synthesis and/or cell surface presentation of DCC. In fact, we have preliminary evidence that, consistent with its modulatory role in growth cones during development (Bouchard et al., 2004), cAMP levels, PKA and PKC activation influence the surface presentation of DCC in cortical neurons. Together, these observations suggest that netrin-DCC signaling participates in the establishment of

neuronal connectivity not only during the development of the nervous system, but possibly during plastic changes in the adult CNS as well. The identification of molecular mechanisms regulating synapse formation and plasticity will provide important insight into normal brain functions and pathological CNS conditions.

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APPENDIX