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Molecular mechanisms underlying the response of commissural axons to netrin-1

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Molecular Mechanisms Underlying the Response
of Commissural Axons to Netrin-1

by

Christine Mirzayan

DISSERTATION

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To my grandmother, Valia, who lived her life with great composure, grace and integrity.

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This thesis would not have been possible without help from a large number of colleagues, collaborators and friends whom I'd like to thank. Following the first year rotations, I joined the T4 replication group in Bruce Albert's lab for one year. Partially due to its small size, the T4 community seemed sheltered from the politics too common in science today and that year felt like a journey to the heydays of biology of the late 60's. This atmosphere was in large part reflective of Bruce's own affable personality and I came to have the deepest respect for his generosity, energy, and wisdom. In addition, I owe lots of thanks to Jack Barry. Jack and I would "talk shop" daily: going over the details of experiments, discussing what can and cannot be concluded, and deciding what questions to pursue. For a budding scientist like myself, Jack's careful guidance was a dream come true. He is also a dear friend who, along with the other colorful characters of the Albert's lab, made that year exceedingly fun.

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Molecular Mechanisms Underlying the Response of Commissural Axons to Netrin-1

by

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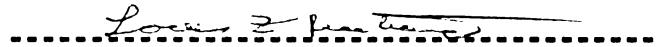

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Abstract

Netrin-1, expressed in floor plate cells of the ventral spinal cord forms a diffusible gradient which attracts commissural axons of the dorsal spinal cord. To understand how such gradients may form, I have characterized netrin-1's diffusion properties *in vitro*. Soluble and cell associated netrin-1 protein seem to exist in equilibrium, suggesting that netrin-1 continually binds to cells as it diffuses dorsally from the floor plate. Furthermore, free gycosaminoglycans extract netrin-1 protein from tissue culture cells, suggesting that cell associated proteoglycans may mediate cell binding. Proteoglycan mediated cell binding may seem like an unfavorable feature for a protein whose function depends on its ability to diffuse. However, cell binding may be important for facilitating the establishment of a far ranging netrin-1 gradient.

Once the netrin-1 gradient does form within the spinal cord, netrin-1 affects the growth of commissural axons in two distinct ways. It functions as a permissive cue by allowing axons to enter the ventral spinal cord, and as an instructive cue by guiding them to the floor plate. Using the "outgrowth" and "turning" assays to mimic these functions, I have studied how commissural axons detect and respond to netrin-1. Binding to DCC does

not seem sufficient for eliciting outgrowth. Rather, to elicit outgrowth, DCC molecules may homodimerize or heterodimerize with other netrin-1 receptor(s).

Is dimerization also required for the chemoattractant activity of netrin-1? It does not seem to be. Although netrin-1(VI-V), a netrin-1 mutant deleted of domain C, is partially defective for eliciting outgrowth, it can elicit turning of commissural axons. Furthermore, under conditions where anti-DCC antibodies effectively block netrin-1 elicited outgrowth activity, turning is only partially blocked. These results suggest that the permissive and instructional activities of netrin-1 may be mediated by separate signaling pathways: a DCC and dimerization dependent pathway which causes commissural axons to grow into unfavorable environments, and a DCC and dimerization independent pathway required for detecting the direction of the netrin-1 gradient.

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Chapter I

Introduction

The functions of the adult nervous system including perception, motor control and learning depend on the elaborate and extensive network of connections made between nerve cells. These connections form during embryonic development and occur in three distinct stages (for reviews see Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996). First, thin neuronal extensions, known as axons, travel large distances through varied terrain to reach their target. Second, axons recognize their target, stop growth, and make synaptic connections. Finally, these connections are fine-tuned by retraction and reformation in an activity dependent manner. In the last 6-8 years, our understanding of these three processes has greatly improved. The advances in the field of axon guidance have predominantly been due to the identification of molecular guidance cues and axonal receptors which recognize and respond to these cues. While in many cases, the importance of these cues *in vivo* has been determined by analyzing axon guidance defects in knock-out animals, for the most part, our understanding of their mechanisms of action has come from *in vitro* culture studies where the response of neurons can be assessed directly.

Axons extend in a precise and stereotypical pattern

In the 1941, Weiss proposed the resonance hypothesis to explain how precise neuronal connections are generated (Weiss, 1941). This theory states that axons migrate and select targets randomly but that these connections are selectively retained in an activity dependent manner. As a classic test of this model, Roger Sperry cut the optic nerve of newts, rotated their eye 180°C and allowed the nerves to regenerate (Sperry, 1943). He discovered that these animals regained their sight but that their vision was inverted, indicating that retinal ganglion axons regenerated to their original target cells despite the fact that these connections were now maladaptive. Thus, contrary to the resonance model, this experiment suggested that the migration paths of axons were preimposed. To explain this result, Sperry proposed the chemoaffinity hypothesis (Sperry, 1963) which states axons contain specific chemical labels on their surface which direct guidance. Moreover, synaptic

connections form only when the pre and post synaptic neuron contain labels which appropriately match.

Though Sperry's chemoaffinity model is flawed in certain details, its major premise has since been verified. Receptors on axons detect cues within their environment to navigate accurately, specifically, and stereotypically from embryo to embryo. As an example, in the peripheral nervous system, motor axons of zebrafish (Eisen et al., 1986; Eisen et al., 1989), chick (Landmesser, 1978; Lance-Jones and Landmesser, 1980a; Lance-Jones and Landmesser, 1980b; Landmesser, 1980) and *Drosophila* (Halpern et al., 1991) innervate correct muscle cells from the outset. In vertebrates, motor axons leave the spinal cord ventrolaterally, migrate through the anterior sclerotome, giving rise to a segmental pattern of ventral roots. Those destined for the limb, converge in the plexus then defasciculate into specific motorneuron pools which grow towards target cells within the limb. Innervation into target muscle cells is specific even when subpopulations of motor axons are deleted (Lance-Jones and Landmesser, 1980a) or when muscle cells are deleted or duplicated (Cash et al., 1992; Chiba et al., 1993).

Like migrations in the PNS, axonal migrations within the central nervous system are also precise. For example, visual space perceived in the retina is topographically mapped onto the brain. To achieve this, retinal ganglion axons born next to one another in the retina extend axons and migrate over long distances before synapsing onto neighboring cells within the optic tectum (lateral geniculate nucleus in mammals) (for reviews, see Bonhoeffer and Grierer, 1984; Holt and Harris, 1993). Olfactory neurons are faced with an even more complex guidance problem. Despite the fact that they are dispersed randomly within each of the four zones of the olfactory epithelium, neurons that express the same odorant receptor extend axons to only two topographically fixed loci among 1800 glomeruli in the olfactory bulb (Mombaerts et al., 1996). Amazingly, activity does not appear to be required for either of these guidance events, suggesting that these axons contain different

receptors or signaling molecules that allow them to respond differently while navigating through the same environment.

General themes in axon guidance

Axons make precise pathway choices by constantly growing, retracting, and changing direction in response to cues present in their environment. In general, growth is directed by the growth cone, an enlarged structure at the leading edge of the axon. However, guidance cues can sometimes be detected by the axon shaft. For example, cortical neurons extend past their target, the basilar pons, but sprout a collateral branch interstitially in response to a diffusible cue secreted by the pons (O'Leary and Terashima, 1988). As development progresses and axonal pathways become longer and more complex, axons begin to travel along preexisting axon tracts called fascicles (Raper et al., 1983; Bastiani et al., 1984; Goodman et al., 1984; Dodd and Jessell, 1988; Klose and Bentley, 1989). This strategy of later axons following previously laid down paths simplifies the problem of axon guidance. But, it also emphasizes how important it is that axons navigate to their targets correctly from the start.

The axon's task of reaching its target is further simplified since its trajectory is often divided into short segments (for review, see Tessier-Lavigne and Goodman, 1996). This allows axons to move in steps, from one intermediate target onto the next until they reach their final target. As an example, sensory axons in the developing grasshopper limb bud (Ti1 pioneer axons) travel from the distal tip of the limb bud to the CNS (Keshishian and Bentley, 1983). To do this, they first extend in the proximo-distal axis until they reach an inhibitory boundary of specialized epithelial cells. At this border, they turn and migrate ventrally, then turn proximally onto the Cx1 cell prior to reaching the CNS (O'Connor et al., 1990). This Cx1 guidepost cell acts as an intermediate target for the Ti1 axons and ablation of this cell results in the failure of these axons to reach the CNS (for review see (Bentley and O'Connor, 1992). In a similar way, floor plate cells at the ventral midline of

the developing spinal cord act as an intermediate target for commissural axons of the dorsal spinal cord (Bovolenta and Dodd, 1990). Commissural axons initially extend circumferentially from the dorsal spinal cord, then ventromedially towards the floor plate. Only upon crossing the floor plate do they turn longitudinally towards their final targets in the brain. In the Danforth short-tail mutant which is missing a floor plate at caudal levels, commissural axons are undirected and only a few turn rostrally towards the brain. Many project out of the spinal cord, forming an abnormal ventral root. Others cross the midline to the contralateral side but fail to turn rostrally (Bovolenta and Dodd, 1991b; Bovolenta and Dodd, 1991a). These results suggest that commissural axons must first reach their intermediate target, the floor plate, in order to reach their final targets within the brain.

In addition to being a source of guidance information, intermediate targets can also change the properties of navigating axons. For instance, as discussed in detail below, commissural axons are deflected towards the floor plate at least partly in response to the diffusible chemoattractant, netrin-1. However, *in vitro*, commissural axons which have crossed the floor plate appear to lose their chemotropic response towards an ectopic floor plate, suggesting that the receptors or intracellular signals activated by netrin-1 have been downregulated or blocked (Katsumata et al., 1996). This would not be unprecedented since expression of TAG-1 and L1, both receptors of the Ig family, change after commissural axons cross the floor plate (Dodd et al., 1988). Similarly, in flies, the protein levels of Fas II, Derailed, and Robo I are modulated on commissural axons as they cross the midline (Lin et al., 1994; Callahan et al., 1995). In the case of Robo I, upregulation appears to be critical for the proper migration of axons after crossing. Axons in embryos lacking the Robo I gene aberrantly cross the midline multiple times suggesting that Robo I may be a receptor for a midline derived inhibitory signal. Thus, commissural axons on the ipsilateral side which do not express Robo I protein will not be able to detect this inhibitor and would freely cross the floor plate; however, upon reaching the contralateral side, these axons upregulate Robo I expression, will now be able to detect the floor plate derived

inhibitor and would therefore remain on the contralateral side (Kidd et al., 1997a; Kidd et al., 1997b). In this way, an axon's response to guidance cues can change en route to its target and receptors and signaling molecules may be modulated so that the axon becomes less responsive to old guidance cues and more responsive to guidance information laying ahead.

Evidence for the existence of diffusible guidance cues

Many types of molecules affect the guidance of developing axons. For instance, cues can attract axons or repel their growth. They can be cell associated or substrate bound cues affecting local guidance events, or diffusible cues which alter growth of distant axons. Ramon y Cajal (Cajal, 1892) was the first to suggest that diffusible cues may participate in modulating axonal growth. A century later, the existence of chemotropic guidance cues was verified with the advent of collagen gels, which are capable of stabilizing long range gradients. Ebendal, the first to use such gels for studies of axonal growth, gained evidence for the existence of a diffusible repellent from ventral spinal cord explants which blocked sympathetic axons (Ebendal and Jacobson, 1977). Later, Lumsden and Davies (1983) showed that maxillary whisker epithelium secreted a diffusible factor, independent of NGF, which could attract sensory axons from the trigeminal ganglion. As predicted initially by Cajal himself, floor plate cells produced a diffusible factor(s) which could elicit axon outgrowth of commissural axons from dorsal spinal cord explants and could reorient axons within the dorsal cord (Tessier-Lavigne et al., 1988). Finally, explants of basilar pons secreted a diffusible factor which elicits branching of cortical axons (Heffner et al., 1990). Since these studies, collagen gels have become a standard means of identifying diffusible cues in embryonic tissue. Among others (Fitzgerald et al., 1993; Pini, 1993; Colamarino and Tessier-Lavigne, 1995a), limb bud (Ebens et al., 1996), caudal septum (Hu and Rutishauser, 1996), optic chiasm (Wang et al., 1996), and ectoderm, dermomyotome, and notochord (Keynes et al., 1997) have all been found to secreted

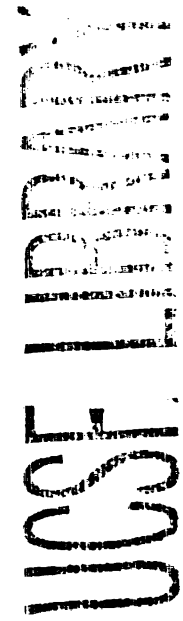
factors which attract or repel developing axons from a distance. Of these, only a handful of chemotropic cues have been identified at a molecular level. Netrin-1 was the first chemotropic cue identified, and will be discussed in detail. But first, we turn our attention to other cues which are capable of functioning chemotropically *in vitro*: semaphorin III, HGF, and the neurotrophins, NGF, NT3, and BDNF.

Chemotropic cues other than netrin-1

Semaphorin III: Sema III, also called collapsin-1, is a secreted member of a phylogenetically conserved family of guidance cues, the semaphorins (Kolodkin et al., 1993). Recombinant sema III protein inhibits axonal growth *in vitro* by causing growth cones to collapse in culture (Luo et al., 1993). However, it has a more moderate affect on growth cones when it is presented locally. *In vitro*, sensory axons which encounter a bead coated with sema III protein at an oblique angle, simply turn away from the bead on contacting it (Fan and Raper, 1995). In addition, recombinant sema III presented from transfected cell aggregates repel NGF but not NT3 responsive sensory axons from a distance, suggesting that sema III may also function as a long range chemorepellent (Messersmith et al., 1995). One site where sema III may function as a chemorepellant is in the developing spinal cord. Sensory axons enter the spinal cord through the dorsal root entry zone in a group but then segregate into different populations: NT3 responsive axons grow ventrally and NGF remain dorsal. Sema III expression is limited to the ventral half of the spinal cord, suggesting that it may function as a molecular sieve, limiting the extension of NGF responsive sensory axons, but allowing NT3 responsive neurons to continue their ventral trajectory. However, this activity is not required for the proper migration of sensory axons *in vivo* because there are only minor pathfinding defects within the spinal cord of embryos deficient for sema III expression (Behar et al., 1996; Taniguchi et al., 1997). Recently, neuropilin-1, a transmembrane receptor expressed on a subset of sensory axons, was identified as a sema III binding protein (He and Tessier-Lavigne,

1997; Kolodkin et al., 1997). Antibodies to neuropilin-1 block both the growth cone collapse and repellent activities of sema III, indicating that it is a receptor or a component of a receptor complex that mediates the effects of sema III.

HGF: Grafting and ablation experiments in amphibians, fish, and birds have suggested that limb bud secretes a diffusible factor capable of attracting developing motor axons (Detwiler, 1934; Hamburger, 1939; Okamoto and Kuwada, 1991a; Okamoto and Kuwada, 1991b). More recently, co-culture experiments in collagen gels have shown that limb bud can also attract spinal motor axons *in vitro* (Ebens et al., 1996). HGF, a growth factor capable of mediating the survival, proliferation, mobility and branching of various cells, is a strong candidate for this chemoattractant or it can mimic its effect (Ebens et al., 1996). Limb buds from HGF deficient embryos do not attract motor axons *in vitro* (Ebens et al., 1996), and motor axons from embryos deficient in expression of its receptor, c-Met, cannot respond to HGF. In spite of these experiments, the functions of HGF and c-Met in motor axon guidance are still poorly understood since motor axons have only minor guidance defects in embryos lacking HGF (Ebens et al., 1996).



Neurotrophins: In a classic experiment 18 years ago, Gunderson and Barret showed that growth cones of dissociated chick sensory neurons can turn rapidly towards a source of NGF (Gundersen and Barrett, 1979). More recently, Xenopus spinal axons have been shown to turn towards BDNF and NT3 proteins released in a pulsatile fashion from a pipette (Song et al., 1997). Interestingly, in the presence of a competitive analog of cAMP or of an inhibitor of protein kinase A, this attractive response to BDNF is changed to a repulsive response suggesting that c-AMP acts as a regulatory switch in growth cone turning. *In vivo*, the neurotrophins seem to be important cues for the selection and invasion of axon targets. For instance, sympathetic fibers of mice hetorozygous mutant for the NT3 gene invaded the pineal gland, but failed to branch within it (ElShamy et al.,

1996). In addition, sympathetic axons invade peripheral tissues in response to NGF. If NGF is misexpressed within these sympathetic axons, the numbers and patterns of fibers which innervate the target are defective (Hoyle et al., 1993). Interestingly, this effect is reversed if NGF in also overexpressed within the target tissue (in this case in the pancreas), indicating that target selection may depend on the presence of a gradient of NGF.

The netrin family of guidance cues

As shown in collagen gel co-cultures, the floor plate secretes a diffusible factor which elicits axon outgrowth of commissural axons into collagen (Tessier-Lavigne et al., 1988; Placzek et al., 1990). The floor plate also secretes a diffusible chemoattractant which deflects commissural axons from their dorsal-ventral trajectory within dorsal spinal cord explants *in vitro*. Evidence for such a floor plate secreted chemoattractant activity is provided by embryological manipulations where segments of the spinal cord are rotated around the dorsal-ventral axis (Yaginuma and Oppenheim, 1991), or where an ectopic floor plate is grafted on the dorsal-lateral edge of the spinal cord (Tessier-Lavigne et al., 1988; Placzek et al., 1990). In both of these experiments, commissural axons project towards the new location of the floor plate.

As we will see in chapter II, salt extracts of embryonic chick brains contained an activity which mimicked the outgrowth promoting activity of floor plate cells. This activity was purified biochemically and found to reside in two proteins, netrin-1 and netrin-2 (Serafini et al., 1994; chapter 2). Subsequently, aggregates of netrin transfected cells were shown to function as chemoattractants for commissural axons (Kennedy et al., 1994). Netrin-1 is expressed at high levels in the floor plate indicating that it contributes, at least in part, to the chemoattractant activity of floor plate.

A role for the netrins in the guidance of developing axons was suggested from their strong homology to the UNC-6 gene in *C. elegans*, known to be required for circumferential migrations throughout the length of the body. The vertebrate netrins,

UNC-6 and netrins subsequently identified in frogs, flies, zebrafish and human defined a phylogenetically conserved family of molecules related in the N-terminal two-thirds to domains VI-V of the laminin B2 chain (Serafini et al., 1994; Mitchell et al., 1996; de la Torre et al., 1997). Interestingly, UNC-6 is expressed only at the ventral midline but is required for both ventral and dorsal migrations, suggesting that UNC-6 is a bifunctional guidance cue, attracting some cells and repelling others (Hedgecock et al., 1985). This model was confirmed when netrin-1, which was initially identified based on its ability to attract ventrally migrating commissural axons, was also shown to repel dorsally directed trochlear motor neurons (Colamarino and Tessier-Lavigne, 1995a) and cranial motor axons such as the trigeminal, facial, and glossopharyngeal nuclei (Varela-Echavarria et al., 1997). However, the functions of netrin-1 and UNC-6 seem to differ in a fundamental way. In the nematode, UNC-6 functions as a global guidance cue for nearly all circumferential migrations and both dorsally directed and ventrally directed migrations are affected in unc-6 mutants. In vertebrates, netrin-1 functions as a global attractant, causing outgrowth of alar axons in the spinal cord, mesencephalon, metencephalon and myelencephalon into collagen (Shirasaki et al., 1996). Moreover, it is required for formation of commissures in the spinal cord and in the brain (e.g, the corpus collosum, hippocampal commissure, and anterior commissure) (Serafini et al., 1996). However, the repellent function of netrin-1 in vertebrates appears to be less extensive. Ventrally projecting spinal motor axons, abducens axons, and axons which form the posterior commissure are unresponsive to netrin-1 *in vitro*, and while netrin-1 is capable of repelling trochlear motor neurons in vitro, it does not appear to be required for the migration of these axons *in vivo* (Serafini et al., 1996; Shirasaki et al., 1996; Varela-Echavarria et al., 1997).

In addition to netrin-1's role at the midline, netrin-1 also functions in other regions of the developing nervous system. In *Drosophila*, netrins are expressed in subsets of muscle cells (Mitchell et al., 1996). When netrins A or B are misexpressed in all muscle cells, motor axons project aberrantly, suggesting that netrins are important for target

selection. In rodents, netrin-1 functions as a short range cue to allow retinal ganglion axons entry into the optic nerve where it is expressed (Deiner et al., 1997).

While these studies clearly identify netrin-1 as an important axon guidance molecule, our understanding of how netrin-1 functions at a molecular level is poor. In nematodes, three classes of mutations in *unc-6* selectively affect function (Wadsworth et al., 1996). The first, a deletion of domain v-2, affects the growth of dorsally migrating but not ventrally migrating cells. The second, a point mutation in domain VI, affects ventrally migrating but not dorsally migrating cells. The third, also a point mutation in domain VI, affects neuronal but not mesodermal cell migrations. Likewise, little is known about how a cell's responsiveness (either attractive or repulsive) to netrin-1 is mediated. Two transmembrane proteins, UNC-5 and UNC-40, were found to be required for migrations of cells and axons away from the UNC-6 signal or towards the UNC-6 signal, but not for both (Hedgecock et al., 1990). UNC-5, whose vertebrate homologues are high affinity receptors for netrin-1 (Leonardo et al., 1997), is required in dorsally migrating cells. Moreover, when UNC-5 is expressed ectopically in longitudinally migrating touch neurons, these neurons turn away from the UNC-6 signal, suggesting that UNC-5 is a repellent receptor (Leung-Hagesteijn et al., 1992; Hamelin et al., 1993; Leonardo et al., 1997). UNC-40, whose vertebrate homologues DCC and neogenin are also high affinity receptors for netrin-1, is predominantly required to grow towards UNC-6 at the ventral midline (Hedgecock et al., 1990; Chan et al., 1996; Keino-Masu et al., 1996). However, UNC-40 does not appear to act only as a receptor for attraction. UNC-40 on its own is not sufficient for attraction (as it is expressed in cells that don't require netrin-1 for their function) and UNC-40 is sometimes required in cells which migrate away from UNC-6 (Hedgecock et al., 1990). Finally, *unc-40* mutants exhibit some defects absent in the *unc-6* mutant embryos suggesting that unc-40 can signal in response to ligands other than UNC-6 (Hedgecock et al., 1990). Since these proteins affect attraction and repulsion to varying degrees and since specific mutations within UNC-6 affect different functions selectively, it

is likely that netrin-1 activates different signaling pathways within different neurons. In this thesis, I have concentrated on delineating the mechanism by which netrin-1 protein influences the growth of spinal commissural axons. Whether this mechanism will be similar amongst other neurons which respond positively to netrin-1, such as retinal ganglion neurons and cortical efferents, remains to be seen.

Netrin-1 function in the spinal cord

Commissural axons, born in the dorsal spinal cord, extend axons circumferentially until they reach the nascent motor column, then are deflected ventramedially towards floor plate cells at the midline (Colamarino and Tessier-Lavigne, 1995b). Netrin-1 is secreted by the floor plate, forms a gradient which extends dorsally (Kennedy et al., 1996), and is required for the proper migration of commissural axons to the ventral midline (Kennedy et al., 1994; Serafini et al., 1996). In mice defecient for netrin-1 expression, commissural axons have two obvious migration defects (Serafini et al., 1996). First, commissural axons grow poorly into the ventral spinal cord. Second, the few axons that do invade the ventral cord have difficulty reaching the floor plate and wander, growing towards the ventricular zone or into the motor column. These defects suggest that netrin-1 plays a dual role in modulating commissural axon growth: it functions as a permissive cue which allows them entry into the ventral spinal cord and it provides instructive information to direct axons to the floor plate.

Both the permissive and instructive functions of netrin-1 can be recapitulated *in vitro* in the outgrowth and turning assays, providing us with a simple way to probe the response of commissural axons. In the outgrowth assay, dorsal spinal cord explants are cultured in a three dimensional collagen matrix. Normally, the axons prefer to remain in the dorsal cord explant and avoid the collagen. However, netrin-1 makes the collagen matrix a more permissive environment and allows commissural axons to grow out as fasciculated bundles (Serafini et al., 1994). DCC, an UNC-40 related receptor for netrin-

1, is expressed on commissural axons and is involved in mediating this response since antibodies to DCC block axon outgrowth (Keino-Masu et al., 1996). However, we do not yet know whether DCC is one component of a larger receptor complex or whether DCC binding alone is sufficient for outgrowth. Motor neurons express DCC but are not responsive to netrin-1 *in vitro* and do not grow towards the floor plate *in vivo*, suggesting that other outgrowth receptors exist (Ebens et al., 1996; Keino-Masu et al., 1996).

In the turning assay, netrin-1 is presented from transfected cell aggregates at the cut edge of a dorsal spinal cord explant. Commissural axons, which normally grow dorsal-ventrally, turn at right angles towards the netrin-1 source, suggesting that commissural axons grow up increasing gradients of netrin-1 (Kennedy et al., 1994). However, little is known as to how axons might detect and respond directionally to gradients of netrin-1. For example, while we suspect that pure netrin-1 protein can attract commissural axons because it can cause turning of dissociated retinal ganglion axons (de la Torre et al., 1997), we cannot be certain that COS-1 cells or 293 cells do not produce cofactors which are required for turning of commissural axons. It is also unclear how the outgrowth and turning activities are related to one-another. Does netrin-1 activate different signaling pathways in commissural neurons or are the activities functionally linked? The fact that DCC antibodies fail to block turning of commissural axons towards netrin-1 producing cells, but block axon outgrowth, suggests that outgrowth and turning are mediated by separate pathways. However, we cannot rule out that this failure to block turning may reflect the antibody's inability to penetrate the dorsal spinal cord explant, where turning occurs (Keino-Masu et al., 1996).

Finally, despite the potent chemoattractant activity of netrin-1 transfected COS-1 cells, netrin-1 is not a very soluble protein. The majority of netrin-1 associates tightly with COS cell membranes and only 5% collects in the conditioned medium (Kennedy et al., 1994). How is netrin-1 then able to diffuse and form long range gradients within the spinal cord?

The aim of my thesis has been to explore some of these issues in greater depth. As is common in biology, more questions have arisen than answers. Still, I have succeeded in characterizing the bioactivities and biochemical properties of netrin-1 further, have generated tools to address mechanistic questions more easily, and have used these tools to gain insight into how netrin-1 functions.

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Chapter II

The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6

I joined Marc Tessier-Lavigne's lab in the summer of 1993. At this time, the netrin proteins had been purified and sequenced by Edman degradation, and Tim Kennedy and Tito Serafini, two post-docs in the laboratory, were involved in cloning the cDNAs of these genes. I joined in these efforts. Specifically, I isolated a clone containing the 5' end of the netrin-2 gene from a cDNA library of E10 chick brains (made by Tim Kennedy). Also, I isolated a full length clone of the netrin-1 gene from a library generated by 3' RACE of partial netrin-1 clones (also made by Tim Kennedy). Finally, by generating exonuclease digested subclones of these, I participated in sequencing the netrin-1 and netrin-2 DNAs.

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The Netrins Define a Family of Axon Outgrowth-Promoting Proteins Homologous to C. elegans UNC-6

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Summary

In vertebrates, commissural axons pioneer a circumferential pathway to the floor plate at the ventral midline of the embryonic spinal cord. Floor plate cells secrete a diffusible factor that promotes the outgrowth of commissural axons in vitro. We have purified from embryonic chick brain two proteins, netrin-1 and netrin-2, that each possess commissural axon outgrowth-promoting activity, and we have also identified a distinct activity that potentiates their effects. Cloning of cDNAs encoding the two netrins shows that they are homologous to UNC-6, a laminin-related protein required for the circumferential migration of cells and axons in C. elegans. This homology suggests that growth cones in the vertebrate spinal cord and the nematode are responsive to similar molecular cues.

Introduction

The axons of developing neurons extend along stereotyped trajectories to their targets by detecting specific cues in their local environments. Axonal growth cones appear to be guided through the combined action of attractive cues, which encourage axon extension, and repulsive cues, which discourage or prevent axonal growth (Dodd and Jessell, 1988; Goodman and Shatz, 1993). Many of these cues are short range, influencing growth cones in the immediate vicinity of the cells that present the cues (Hynes and Lander, 1992). There is also evidence for the existence of diffusible chemoattractants, secreted by target cells, that attract axons at a distance (reviewed by Tessier-Lavigne and Placzek, 1991), and of diffusible chemorepellents that are secreted by cells in regions that axons avoid (Fitzgerald et al., 1993; Pini, 1993).

The molecular identity of cues that direct axon guidance events is largely unknown. In particular, no endogenous diffusible chemoattractants for developing axons have yet been identified. Nerve growth factor (NGF) can act as a chemoattractant for regenerating sensory axons in cell culture (Gundersen and Barrett, 1979) but does not appear to be involved in guiding developing axons as they first grow to their targets (Davies, 1987) or in guiding regenerating axons in vivo (Diamond et al., 1992). Neurotransmitters can induce growth cone turning in vitro (Zheng et al., 1994), but their involvement in axon guidance in vivo remains to be established.

One region of the vertebrate central nervous system where evidence for the operation of chemotropic mechanisms has been obtained is the developing spinal cord. Commissural neurons that differentiate in the dorsal spinal cord extend axons along a stereotyped dorsoventral trajectory that leads them to the floor plate, an intermediate target at the ventral midline of the spinal cord (Ramón y Cajal, 1909; Holley, 1982; Wentworth, 1984; Dodd et al., 1988; Yaginuma et al., 1990). Experiments in vitro (Tessier-Lavigne et al., 1988; Placzek et al., 1990a) and in vivo (Weber, 1938; Placzek et al., 1990b; Yaginuma and Oppenheim, 1991) have demonstrated that the floor plate secretes a chemoattractant for developing commissural axons during the period that these axons grow to the floor plate, suggesting that chemotropism contributes to the ventral guidance of these axons to the floor plate during normal development.

Floor plate cells have two long-range effects on commissural axons in vitro (Tessier-Lavigne et al., 1988; Placzek et al., 1990a). First, they promote the outgrowth of these axons from explants of embryonic dorsal spinal cord into collagen gels. Second, they attract commissural axons by reorienting their growth within dorsal spinal cord explants. Both the outgrowth and the orienting effects of the floor plate can occur at a distance, with the floor plate influencing the growth of axons over hundreds of micrometers. However, the factor(s) that mediate the outgrowth and orienting activities of the floor plate are unknown. Moreover, it is not known whether the two activities of floor plate cells are mediated by a single factor or by distinct outgrowth-promoting and chemotropic molecules. In fact, it is unclear whether, in general, molecules that promote the outgrowth of developing axons can also orient the axons when present in gradients. In the only case examined to date, gradients of the extracellular matrix (ECM) molecule laminin promoted the growth of sympathetic axons but failed to orient their growth (McKenna and Raper, 1988).

As a first step toward identifying the molecular mechanisms involved in guidance of commissural axons, we have focused on the identification of molecules that can elicit commissural axon outgrowth into collagen gels. Although many known neurite outgrowth-promoting molecules, neurotrophic factors, and growth factors have been tested (Placzek et al., 1990b; and unpublished data), none have been found to have this activity. We report here the discovery of an activity in embryonic brain that promotes the outgrowth of commissural axons into collagen gels, and the purification from this tissue of two proteins that possess this outgrowth activity, which we have named netrin-1 and netrin-2. We have also identified a distinct

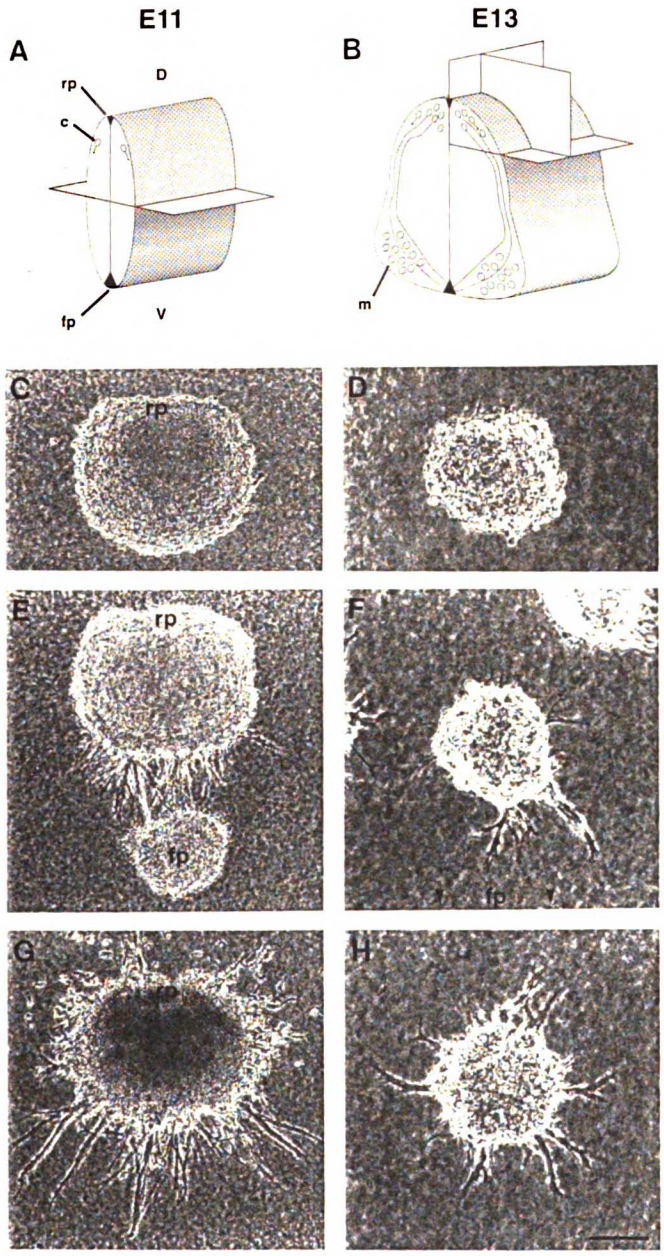

Figure 1. Floor Plate Cells and Embryonic Brain Extract Elicit Commissural Axon Outgrowth from E11 and E13 Rat Dorsal Spinal Cord Explants

(A and B) Diagrams illustrating the dissection of E11 (A) and E13 (B) rat dorsal spinal cord explants from short lengths of dissected spinal cords (see Experimental Procedures). The planes indicate the cuts required to generate the explants. The E11 and E13 spinal cords are not drawn exactly to scale. c, commissural neuron; D, dorsal; fp, floor plate; m, motor neuron; rp, roof plate; V, ventral.

(C, E, and G) Phase micrographs showing E11 explants cultured for 40 hr alone (C), with floor plate (E), or with 0.19 mg/ml high salt extract of embryonic (E10) chick brain membranes (G). Note that the E11 explant in (G) is phase-dark in its center, because the extract is slightly toxic for explants at this age. The extract also induces migration of cells away from the dorsal region of the explant. E11 explants are shown with the roof plate (dorsal surface) oriented toward the top of the photographs.

(D, F, and H) Phase micrographs showing E13 explants cultured for 16 hr alone (D), with a floor plate explant (F), or with the same concentration of high salt extract as in (G). In (F), the floor plate explant is just beyond the field of view across the bottom of the photograph (arrowheads).

Scale bars are 140 μm in (C), (E), and (G), and 70 μm in (D), (F), and (H).

activity in embryonic brain that potentiates the effects of netrins in promoting commissural axon outgrowth. Molecular cloning of cDNAs encoding the two netrins shows that they define a family of proteins that are homologs of the Caenorhabditis elegans protein UNC-6 (Ishii et al., 1992), which regulates the circumferential guidance of mesodermal cells and axons during C. elegans development (Hedgecock et al., 1990). In the following article (Kennedy et al., 1994 [this issue of *Cell*]), we investigate the relation of the netrins to both the outgrowth and orientation activities previously described for floor plate cells.

Results

Salt Extracts of Embryonic Brain Membranes Contain a Commissural Axon Outgrowth-Promoting Activity

Floor plate cells secrete a factor (or factors) that promotes

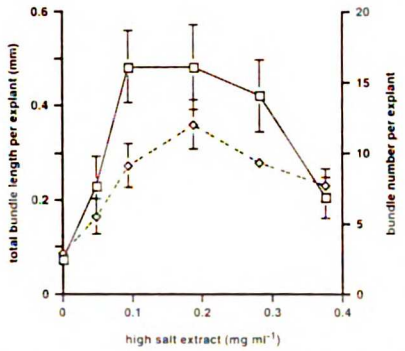

Figure 2. Quantification of Axon Outgrowth from E13 Dorsal Spinal Cord Explants in Response to High Salt Extracts of E10 Chick Brain Membranes

Axon outgrowth was quantified by counting bundles (for bundle number, open diamonds) or measuring bundle lengths and summing (for total bundle length, open squares) for each E13 dorsal spinal cord explant. Characteristic changes in these parameters were observed with increasing extract concentration, including a decrease at high concentrations due to an inhibition of outgrowth. In addition, the thickness of the bundles increased in characteristic fashion with increasing concentration, so that the morphology of the bundles was a good predictor of activity (data not shown; see Experimental Procedures). Values shown are means (± standard errors) of three to eight individual explants cultured for 16 hr in the presence of an indicated concentration of E10 chick brain membrane high salt extract.

the outgrowth of commissural axons from embryonic day 11 (E11) rat dorsal spinal cord explants (Figures 1A, 1C, and 1E; Tessier-Lavigne et al., 1988; Placzek et al., 1990a). Outgrowth is observed after ~24 hr and is profuse by 40 hr (Tessier-Lavigne et al., 1988; Placzek et al., 1990a; Figure 1E). To facilitate the characterization of commissural axon outgrowth-promoting activities, we examined whether the floor plate also promotes the outgrowth of axons from E13 rat dorsal spinal cord explants (Figure 1B), which can be dissected more easily owing to the larger size of the E13 spinal cord. As with E11 rat dorsal spinal cord explants, outgrowth of thick axon bundles was observed from E13 explants cultured with floor plate (Figure 1F). Outgrowth was observed after ~12 hr and was profuse by 16 hr, whereas over the same time period, little or no outgrowth was observed from E13 explants cultured alone (Figure 1D). Floor plate-conditioned medium mimicked the effect of floor plate tissue in promoting axon outgrowth from E13 explants (data not shown). Many of the axons that emerged from E13 explants in response to floor plate were TAG-1$^+$ (data not shown), identifying them as commissural axons (Dodd et al., 1988). Thus, the outgrowth of axons from E13 dorsal spinal cord explants provided an alternative and more convenient commissural axon outgrowth assay.

Using this assay, we first characterized the outgrowth-promoting activity in floor plate homogenates. Homogenates of E13 floor plate evoked robust axon outgrowth from E13 dorsal spinal cord explants (data not shown). When homogenates were fractionated into soluble and membrane fractions, all detectable activity was associated with the membrane fraction (data not shown), even though activity can be secreted in diffusible form by cultured floor plate cells (Tessier-Lavigne et al., 1988; Placzek et al., 1990a). The activity associated with floor plate membranes could be solubilized by exposure to 1 M NaCl and was indistinguishable in its effects from floor plate-conditioned medium (data not shown), suggesting that the activity can exist in both diffusible and membrane-associated forms.

It was not possible to purify the commissural axon outgrowth activity in floor plate cells, owing to the small size of the floor plate. We therefore searched for a more abundant source of a commissural axon outgrowth activity by screening salt extracts of membranes derived from several neural tissues. An activity was detected in extracts of embryonic brain and whole spinal cord from E13–P3 rats and E5–E13 chicks (Figures 1G and 1H; data not shown). A characteristic dose–response curve was obtained when the number of bundles per explant and the

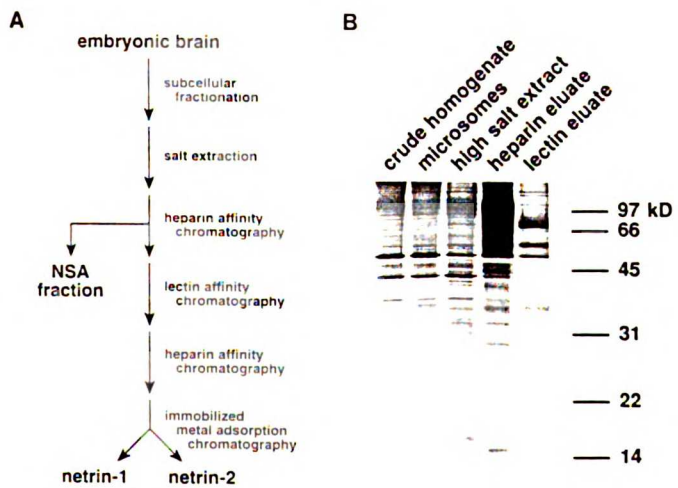

Figure 3. Netrin Purification Scheme and SDS-PAGE Protein Profiles of Active Fractions

(A) Major steps in the purification of the netrins are indicated (see Experimental Procedures for details). The fraction containing NSA is generated during the first heparin affinity chromatography step (see text for details).

(B) Silver-stained SDS-PAGE gel (12.5%) displaying the protein composition of several of the active fractions at early stages of the purification. The "heparin eluate" refers to the active fraction obtained during the first heparin affinity chromatography step. The following amounts of each fraction were subjected to SDS-PAGE: crude homogenate, 0.025 μl; microsomes, 0.033 μl; high salt extract, 1.0 μl; heparin eluate, 10 μl; and lectin eluate, 25 μl. Positions of molecular weight standards are indicated (phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 22 kDa; and lysozyme, 14 kDa).

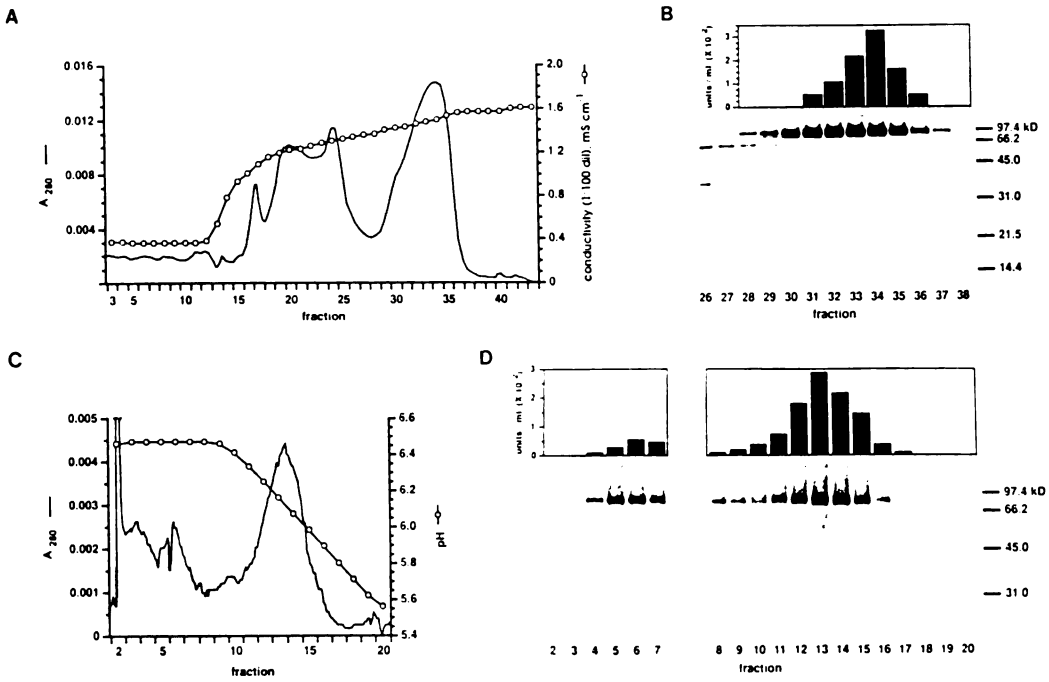

Figure 4. Purification of the Netrins to Apparent Homogeneity

(A and B) Cofractionation of outgrowth activity with two major proteins during the second heparin affinity chromatography step. (A), absorbance at 280 nm (measuring protein content) and conductivity (measuring ionic strength) of the 500 μl fractions generated by elution with a salt gradient (starting at fraction 12) during the second heparin affinity chromatography step. Conductivity was measured on 1:100 dilutions of the fractions. (B), outgrowth activity in the E13 assay (top portion of figure) was observed in a subset of the fractions described in (A) and cofractionated with a doublet of protein bands (70–80 kDa, not well resolved with this gel) eluting at a conductivity of ~1.5 mS/cm (~1.35 M NaCl) (bottom portion of figure). Of each fraction, 100 μl was TCA precipitated and subjected to SDS–PAGE (12.5% gel) and silver staining.

(C and D) Purification and separation of the netrins by IMAC on a Zn^{2+}-charged resin. (C), absorbance at 280 nm and the pH of the 1 ml fractions generated during the IMAC step. Fractions are from a different purification run than that shown in (A) and (B). (D), outgrowth activity in the E13 assay (top portion of figure) was observed in a large number of the fractions generated in (C) and cofractionated with each of two separately eluting proteins (bottom portion of figure), one of 75 kDa, which eluted isocratically from the column at pH ~6.5 (fractions 4–8; netrin-2), and one of 78 kDa, which eluted during the application of a decreasing pH gradient at pH ~6.1 (fractions 10–17; netrin-1). Of each fraction, 200 μl was TCA precipitated and subjected to SDS–PAGE (10% gel) and silver staining.

summed lengths of these bundles were quantified in response to serial 2-fold dilutions of each extract, with inhibition of outgrowth observed at high concentrations (Figure 2; data not shown). The specific activity of the extracts (measured as described in Experimental Procedures) decreased with increasing age, and was comparable for P3 rat and E13 chick brain (data not shown). Activity was not detected in extracts of brain or spinal cord from several later developmental stages or from the adult, nor in the embryonic or adult nonneural tissues that were tested (listed in Experimental Procedures).

Purification of the Axon Outgrowth Activity to Apparent Homogeneity Yields Two Proteins

Embryonic chick brain was used in preference to rat brain as a source of commissural axon outgrowth activity for purification, because it is a more economical and easily obtained tissue. The procedure used to purify the outgrowth activity is summarized in Figure 3A. Differential centrifugation experiments showed that the microsomal membrane fraction contained almost all of the salt-extractable activity (data not shown). A low salt wash (~500 mM NaCl) followed by a high salt extraction (~1 M NaCl) of this fraction was used to prepare a soluble form of the activity for column chromatography. This activity was bound to a heparin–Sepharose (HS) CL-6B column and was eluted with 2 M NaCl. We also observed that the flowthrough fraction from this column, which had little outgrowth-promoting effect on its own, potentiated the effects of the eluted outgrowth activity (see discussion below and Figures 7–9).

The activity in the eluate from this first column was bound to a wheat germ agglutinin (WGA) agarose column and eluted with N-acetylglucosamine (Figure 3B), indicating that the active component(s) is likely to be glycosylated. The activity in this eluate was bound to a heparin–Sepharose high performance (HSHP) column and eluted in a peak centered at ~1.35 M NaCl, where it cofractionated with two major proteins of ~70–80 kDa (Figures 4A and 4B). Active fractions from this column were pooled

Table 1. Purification of Netrin-1 and Netrin-2

Fraction	Volume (ml)	Total Protein[a] (mg)	Total Activity[b] (Units)	Specific Activity[b] (Units/mg)	Purification[b,c] (-fold)	Yield[b] (%)
Crude homogenate	720	13000	NA[d]	NA[d]		
Low speed supernatant	640	7200	41000	5.7	[1]	[100]
High salt extract	200	140	13000	93	16	32
Heparin affinity (I)	30	1.7	3000	1800	320	7.3
IMAC pool 1 (netrin-2)[e]	3.0	0.003	80[e]	30000[e]	5000[e]	0.2[e]
IMAC pool 2 (netrin-1)	6.0	0.010	690	69000	12000	1.7

Values shown are for one purification run (~2,000 E10 chick brains).

[a] Peterson method used for all fractions except IMAC pools, for which protein gold assay was used.

[b] Owing to the semiquantitative nature of the quantification method used (see Experimental Procedures), all unit measurements and values calculated using these measurements are subject to a 2-fold uncertainty.

[c] Values for fold purification would increase by approximately a factor of two if all activity in the crude homogenate were recovered in the low speed supernatant and the purification were normalized to a specific activity for the crude homogenate estimated from its protein content.

[d] Not assayed.

[e] The activity measurement for the IMAC pool 1 was performed on fractions from a purification run different from the run used for protein measurement (the latter was the same run used for all other protein and activity measurements shown in this table); the activity value was scaled down based on a comparison of absorbance profiles for the two IMAC runs. This difference, together with the inherent uncertainty of the semiquantitative estimate of activity (see footnote b and Experimental Procedures), makes it possible that the specific activities of the two netrins purified from brain are more similar than indicated here (the specific activities of the recombinant proteins are equal; see Figure 7).

and subjected to immobilized metal adsorption chromatography (IMAC) (Figures 4C and 4D). Outgrowth-promoting activity cofractionated with each of the two major proteins of 75 kDa and 78 kDa. Silver staining of overloaded sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels of the active fractions (Figure 4D) indicated that the two proteins have been purified to apparent homogeneity. Because they guide axons (see the following paper, Kennedy et al., 1994) the proteins of 78 and 75 kDa have been termed netrin-1 and netrin-2, respectively; the root "netr" derives from the Sanskrit ("one who guides"). From 2000 E10 chick brains (one purification run), ~10 μg of netrin-1 is obtained after an ~10,000-fold purification with an ~1.7% yield, and about a third as much netrin-2 is obtained after a similar purification with a lower yield (the values for netrin-2 are less certain; see Table 1).

The Netrins Are Homologs of UNC-6, a Laminin-Related Protein Involved in Axon Guidance in C. elegans

Purified netrin-1 and netrin-2 were cleaved with cyanogen bromide (CNBr), and amino acid sequence was obtained from several of the resulting peptides. In addition, purified netrin-1 and netrin-2 were sequenced directly to obtain N-terminal sequence. The unambiguous sequences obtained (four internal and one N-terminal for netrin-1, and two internal and one N-terminal for netrin-2; see Experimental Procedures) permitted the design of oligonucleotide primers that were used in polymerase chain reactions (PCR) with reverse-transcribed E10 chick brain poly(A)$^+$ RNA as a template to isolate fragments of the cDNAs encoding the two proteins.

An E10 chick brain cDNA library and an E2.5 chick spinal cord library were screened with probes designed from these fragments as well as from cDNAs isolated as the screen proceeded. Eleven partial *netrin-2* cDNAs were isolated, which provided the sequence of the entire mature netrin-2 polypeptide, but not the full signal sequence. The screen also yielded three partial *netrin-1* cDNAs, all of which had primed internally. The 3′ end of the *netrin-1* coding sequence was obtained by 3′ rapid amplification of cDNA ends (RACE), followed by the generation of a primer extension library (see Experimental Procedures). A cDNA containing the full coding sequence of *netrin-1* was obtained from this library. Southern blot analysis indicated that the *netrin-1* and *netrin-2* mRNAs are the products of distinct genes (data not shown).

The derived amino acid sequences of netrin-1 and netrin-2 (Figure 5) encode proteins that are likely to be secreted: the two proteins each have a predicted N-terminal signal sequence (von Heijne, 1985; only a portion of the full signal sequence for netrin-2 has been obtained), followed immediately by a predicted N-terminal sequence for the mature protein that is identical to the peptide sequence obtained by microsequencing. Neither mature protein appears to contain a hydrophobic stretch that could function as a transmembrane domain or as a signal to direct addition of a glycosylphosphatidylinositol (GPI) lipid anchor (Kyte and Doolittle, 1982; Moran and Caras, 1991a; Moran and Caras, 1991b). The deduced sizes of the mature proteins are 581 and 566 amino acids for netrin-1 and netrin-2, respectively. The two amino acid sequences show 72% identity.

The netrins are homologous to the product of the *unc-6* gene of C. elegans (Figure 5) (Ishii et al., 1992), which is required for circumferential guidance of growth cones and migrating mesodermal cells in the nematode (Hedgecock et al., 1990). Over the entire length of the mature proteins, netrin-1 and netrin-2 are 50% and 51% identical to UNC-6, respectively. The N-terminal two-thirds of the netrins and UNC-6 are homologous to the N-termini of the polypeptide chains (A, B1, and B2) of laminin, a large (880 kDa) heterotrimeric protein of the ECM (Beck et al., 1990). The homologous region corresponds to domains VI and V of the laminin chains (Figure 6) (Sasaki et al., 1988). As described

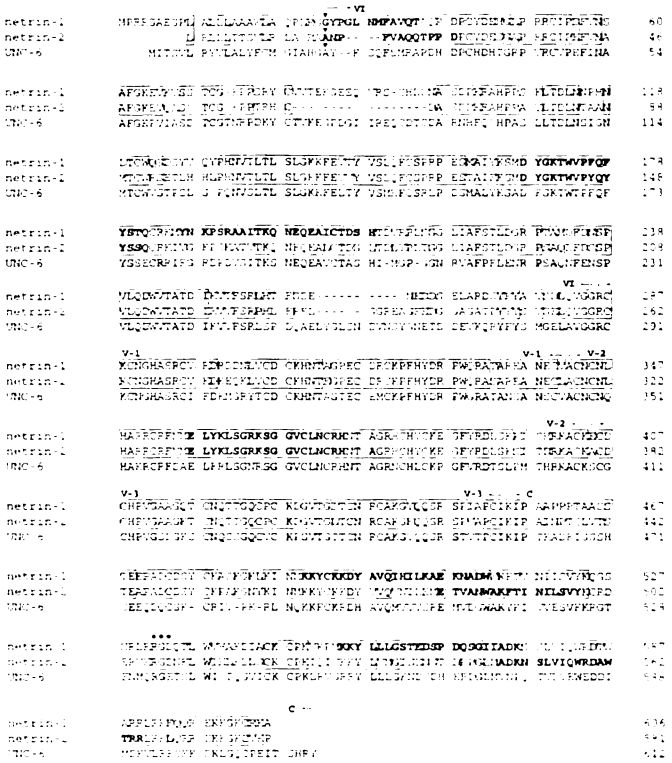

Figure 5. The Predicted Amino Acid Sequences of Netrin-1, Netrin-2, and UNC-6

The predicted amino acid sequences for the two netrins and UNC-6 (Ishii et al., 1992) are shown aligned (see Experimental Procedures). Line boxes around netrin-1 and netrin-2 sequences delineate identity between the two proteins, whereas shaded boxes indicate identity among all three proteins. Sequences obtained by microsequencing CNBr-generated peptides of the purified proteins are shown in bold. The arrowheads at positions 26, 16, and 22 in netrin-1, netrin-2, and UNC-6, respectively, indicate the first amino acid of the mature polypeptides (obtained by microsequencing for netrin-1 and -2, and predicted for UNC-6 [Ishii et al., 1992]). The bracketing arrows above the sequences delimit the stretches comprising domains VI and V of the three proteins (homologous to domains VI and V of individual laminin polypeptides; see text for details) and the C-terminal region shared among the netrins and UNC-6. V-1, V-2, and V-3 refer to the first, second, and third EGF repeats comprising domain V of the three proteins. The starred sequence (RGD in netrin-1 and netrin-2) is a recognition sequence for several members of the integrin family of receptors (Hynes, 1992).

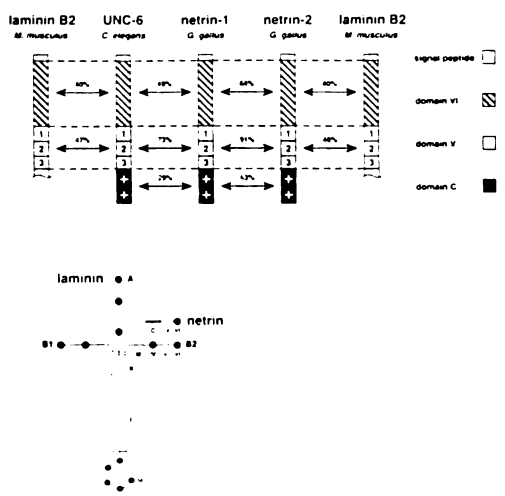

Figure 6. Comparisons of the Sequences and Structures of Netrins, UNC-6, and Laminins

(A) Diagram displaying the percent identity in amino acid sequence between homologous domains (see Figure 5 and text) of netrin-1, netrin-2, UNC-6, and murine laminin B2 proteins. Within the domains V, the three individual EGF repeats are indicated; laminin B2 possesses four such repeats in its domain V (Sasaki and Yamada, 1987). The plus symbols indicate the predicted high net positive charge of the domains C at neutral pH.

(B) Diagram illustrating the relative sizes of and relationship between the netrins and the cruciform laminin heterotrimer (after Beck et al., 1990). The portions of the laminin molecule represented by closed circles are globular, and those portions of the molecules indicated by line segments have a more extended conformation. A, B1, and B2 refer to the three laminin polypeptides comprising the heterotrimer; numerals I through VI and the letter G refer to named domains of the laminin and netrin polypeptides, whereas C refers to the domain C of the netrins. By analogy with the conformation of the various domains of laminin, domain VI of the netrins is predicted to be globular, and domain V extended. For convenience, domain C is depicted as a linear segment, although its conformation is unknown. The figure is drawn only approximately to scale.

for UNC-6, the netrins are most closely related in these domains to the B2 chain, although they also show some hallmarks of the B1 chain (see Ishii et al., 1992). Despite the homology to the B1 chain, phylogenetic analysis of these domains (see Experimental Procedures) suggests that the netrins and UNC-6 diverged from an ancestral laminin B2-like molecule after the emergence of separate laminin B1 and B2 chains (data not shown). The remaining C-terminal thirds (domains C) of netrin-1 and netrin-2 are homologous to each other (63% identity) and to domain C of UNC-6 (29% identity for both netrin-1 and netrin-2),

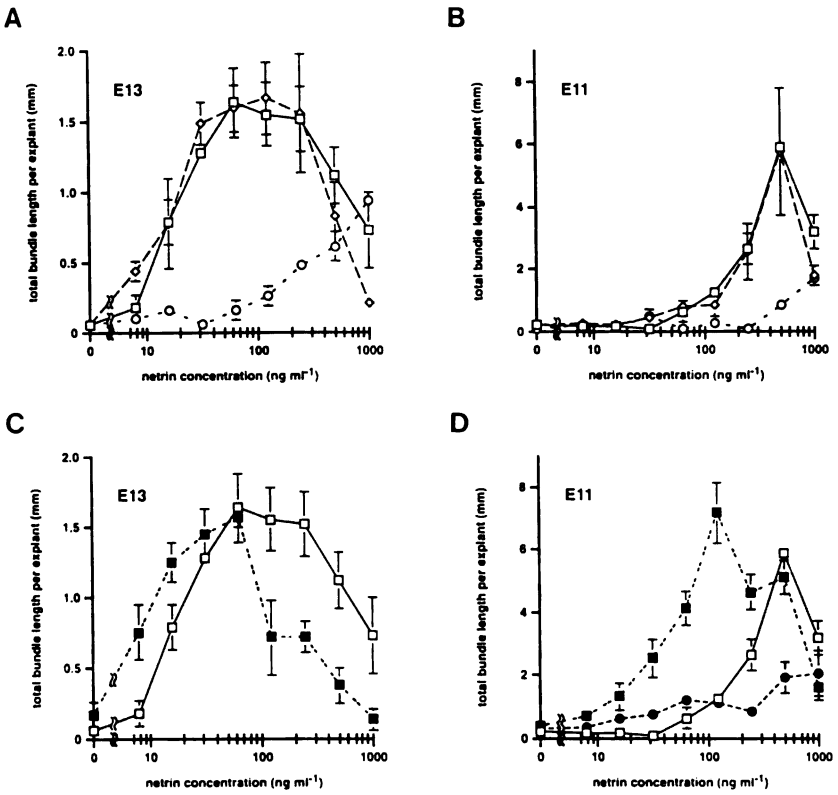

Figure 7. Quantification of Axon Outgrowth from E13 and E11 Dorsal Spinal Cord Explants in Response to Recombinant Netrins and NSA

Graphs show the total bundle length per E13 explant (A and C) or E11 explant (B and D) (mean ± standard error; n = 4) elicited by salt extracts of transfected COS cells containing recombinant tagged netrin-1 or netrin-2. Curves in (A) and (B) compare the effects of netrin-1 (open squares) and netrin-2 (open diamonds). Curves in (C) and (D) compare the effects of netrin-1 alone (open squares) with the effects of netrin-1 and NSA (50–60 μg/ml NSA-containing fraction (closed squares); NSA potentiates the effects of netrin-2 to a similar extent; data not shown). To obtain the indicated concentrations of the netrins, 2-fold serial dilutions of salt extracts of COS cells in which netrin concentration had been quantified (see Experimental Procedures) were used. A control extract was prepared from COS cells transfected with the parent vector alone. For each netrin concentration, the control extract (alone, open circles; with NSA, closed circles) was used at a concentration equal to that of the netrin-1-containing extract. The specific activities of the netrins deduced from the dose–response curves shown here probably underestimate the true potencies of the netrins (see text and Figure 9 legend).

but diverge completely from the laminin sequence. Thus, netrin-1, netrin-2, and UNC-6 define a novel family of secreted proteins that are relatives of laminins.

The greatest identity between the netrins and UNC-6 is found in domain V. This domain is composed of several epidermal growth factor (EGF) repeat structures, as delineated by the conservation in number and spacing of their many cysteine residues (Blomquist et al., 1984; Engel, 1989); the netrins and UNC-6 each have three EGF repeats (Figure 6, top). The domains C of netrin-1 and netrin-2 are enriched in basic residues (predicted isoelectric points of 10.6 for the netrin-1 domain C and 10.5 for the netrin-2 domain C), and each contains the motif RGD (see Figure 5), a known recognition sequence for several members of the integrin family of adhesion/signaling receptors (Hynes, 1992).

Recombinant Netrins Have Outgrowth Activity

To determine whether the proteins responsible for the outgrowth-promoting activity of embryonic chick brain had been purified, cDNAs encoding the netrins were cloned into a mammalian expression vector and expressed in COS cells. In these experiments, the netrins were modified at their extreme C-termini by addition of an epitope from the c-Myc protein that is recognized by a monoclonal antibody, 9E10 (Evan et al., 1985; Munro and Pelham, 1987). High salt (1 M NaCl) extracts of COS cell monolayers transfected with the netrin expression constructs promoted axon outgrowth from E13 rat dorsal spinal cord explants (Figures 7A, 8A, and 8B; data not shown). Salt extracts from cells transfected with the tagged netrin-1 construct had at least as much activity (as assessed in serial dilution experiments) as salt extracts prepared in

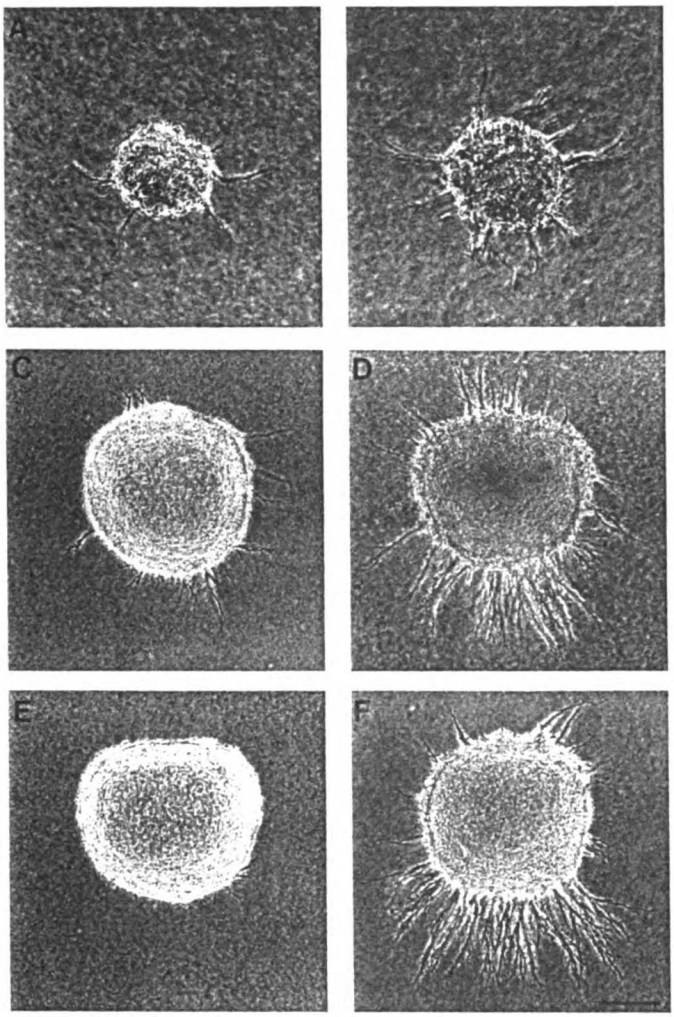

Figure 8. Axon Outgrowth from E11 and E13 Rat Dorsal Spinal Cord Explants Elicited by Recombinant Netrin-1

(A and B) Outgrowth elicited from E13 explants by 16 ng/ml (A) or 62 ng/ml (B) netrin-1 after 16 hr in culture. Maximal outgrowth is elicited by the higher concentration. Controls showed little outgrowth above background (see Figure 7A).

(C and D) Outgrowth elicited from E11 explants by 125 ng/ml (C) or 500 ng/ml (D) netrin-1 after 40 hr in culture. Controls showed little outgrowth above background (see Figure 7B).

(E and F) Outgrowth elicited from an E11 explant by NSA (50 μg/ml of the NSA-containing fraction) alone (E) or the same amount of NSA with 125 ng/ml netrin-1 (F) after 40 hr in culture. Control extract in the presence of NSA elicited only a small amount of outgrowth, comparable to that in (C) (see Figure 7D).

Explants were cultured in the presence of different concentrations of a salt extract of transfected COS cells expressing recombinant-tagged netrin-1 (explants shown here were among those used to derive the curves in Figure 7). Each E11 explant is shown with its roof plate toward the top of the photograph, as in Figure 1.

Scale bars are 70 μm in (A) and (B), and 140 μm in (D)–(G).

the same manner from cells transfected with an untagged netrin-1 construct (data not shown), indicating that the presence of the Myc epitope does not markedly alter outgrowth activity.

To compare the specific activities of recombinant netrin-1 and netrin-2, we measured the netrin content of salt extracts of the transfected monolayers by immunoblotting, using purified recombinant-tagged netrin-1 as a standard. Recombinant netrin-1 and netrin-2 in these salt extracts had identical specific activities (Figure 7A), evoking clear outgrowth above background from E13 explants at ~16 ng/ml (Figure 7A and 8A) and a maximal response at 62 ng/ml (Figure 7A and 8B). At high concentrations (>125 ng/ml), an inhibition of outgrowth was observed (Figure 7A), with axon bundles becoming progressively more stunted in appearance (data not shown). It is likely that the proteins are actually more potent than is indicated by these concentrations, because the dialysis that was required to prepare the extracts for assay leads to a loss of activity (discussed in Experimental Procedures and legend to Figure 9). Nevertheless, these results show that the two netrins have similar properties in the E13 outgrowth assay. Extracts prepared from control cells had a small amount of outgrowth activity (Figure 7A), suggesting the expression of a netrin-like activity by COS cells; this effect required 32- to 64-fold more extract than was required to elicit a comparable response using extracts from cells expressing recombinant netrins.

Recombinant netrin-1 and netrin-2 also promoted axon outgrowth from E11 rat dorsal spinal cord explants (Figures 7B, 8C, and 8D). As with floor plate, the axons that responded to each netrin were TAG-1$^+$ (data not shown), indicating that they derived from commissural neurons (Dodd et al., 1988). However, the netrins were markedly less potent on E11 explants than on E13 explants. Maximal outgrowth from E13 explants was observed at a con-

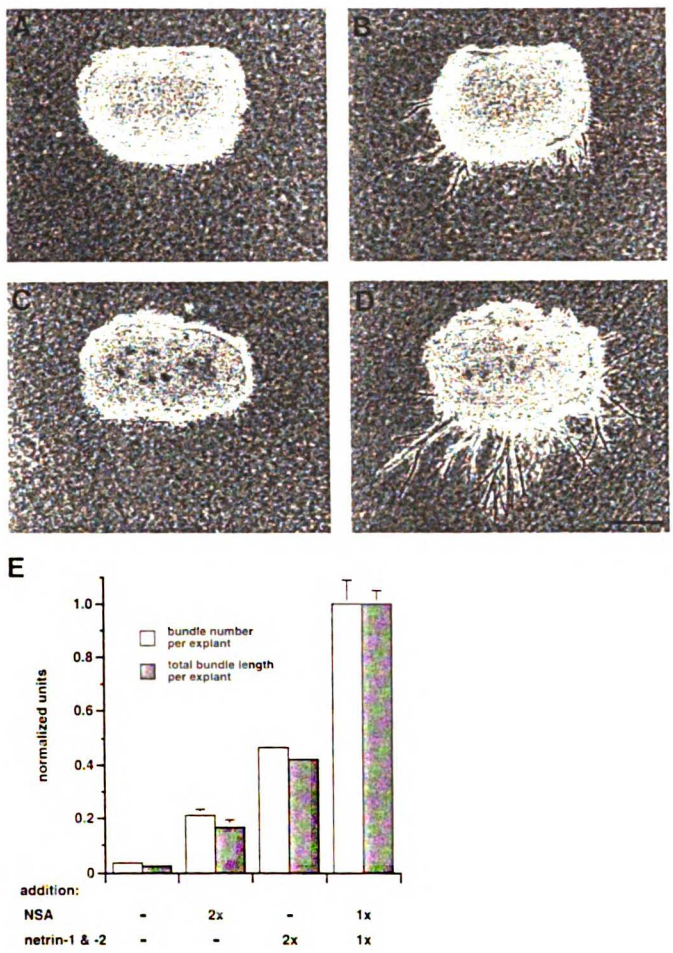

Figure 9. Demonstration of a Synergy between the Netrins and NSA

E11 explants were cultured for 40 hr alone (A), with a mixture of partially purified embryonic brain netrin-1 and netrin-2 (second heparin chromatography eluate; see Figures 4A and 4B) at 22 ng/ml (a 2× concentration of netrin mix) (B), NSA-containing fraction at 100 μg/ml (a 2× concentration of NSA) (C), or both the netrin mixture and the NSA-containing fraction (D), but at half the concentrations used in (B) and (C) (a 1× concentration of each). Explants are shown with the roof plate oriented toward the top of each photograph, as shown in Figure 1. In (E) are displayed the average values for three experiments (± standard errors) of mean bundle number and total bundle length (see Figure 2) from two to four explants cultured under the conditions of (A)–(D). The data have been normalized to the values obtained under conditions of maximal outgrowth (D).

Note that 11 ng/ml of the mix of brain-derived netrins elicited outgrowth in (D) comparable to that elicited by 31–62 ng/ml recombinant-tagged netrin-1 in Figure 7D (NSA was used in both cases). This difference was probably due to a loss of netrin activity during the dialysis that was required for assay of the recombinant netrin-containing extract; the brain-derived protein mix used here was not dialyzed (see Experimental Procedures, Preparation of Fractions for Assay).

centration of the netrins that evoked little or no outgrowth from E11 explants (Figure 7), and at least 8-fold more of each netrin was necessary to evoke a maximal response from E11 explants than from E13 explants (Figure 7 and Figure 8). Netrins purified from embryonic brain were likewise more potent on E13 than on E11 explants (data not shown). Extracts prepared from control COS cells had a small amount of outgrowth activity for E11 explants, with an ~8-fold higher concentration of these extracts being required to evoke a response comparable to that observed with extracts containing recombinant netrins (Figure 7B).

Embryonic Brain Contains a Synergizing Activity for the Netrins

The observation that E13 explants were much more sensitive to netrins than were E11 explants was surprising, because E13 explants did not appear to be much more sensitive than E11 explants to the outgrowth-promoting effects of the floor plate or floor plate–conditioned medium (data not shown), and because we did not observe a differential response of E13 and E11 explants to high salt extracts of embryonic brain membranes (see Figures 1G, 1H, and Figure 2, and data not shown). This suggested that during the course of the purification an additional activity had been separated from the netrins. As mentioned above, an activity that enhanced the effect of the netrins on E13 explants was observed in the flowthrough fraction from the first column used in the purification (see Figure 3A; we term this activity NSA, for netrin-synergizing activity [see below]). We therefore examined whether the netrins had similar activities on E13 and E11 explants when assayed in the presence of NSA.

NSA had only a minor effect on E13 explants. In the presence of threshold (<31 ng/ml) concentrations of the netrins in the E13 assay, addition of NSA caused a slight increase in the length and number of bundles (Figure 7C and data not shown), although NSA elicited little outgrowth on its own (Figure 7C). Consistent with an increased sensitivity of the explants to the netrins, in the presence of NSA the inhibitory effect of large amounts of netrin-containing

extracts was observed at lower concentrations (Figure 7C; similar results were observed with netrin-2 [data not shown]).

In contrast, a dramatic potentiation of netrin activity by NSA was observed on E11 dorsal spinal cord explants (Figures 7D and 8C–8F; data not shown). Addition of NSA increased the sensitivity of the explants to the netrins by at least a factor of eight (Figure 7D; similar results were observed with netrin-2 [data not shown]). This effect of NSA was detected both with recombinant netrins and with netrins purified from embryonic brain (Figures 7 and 9; data not shown). The axons that grew out in the presence of NSA were TAG-1$^+$ (data not shown). Thus, in the presence of NSA, E11 explants are essentially as sensitive to the netrins as are E13 explants.

To determine whether NSA acted synergistically with the netrins, we identified a concentration of a mix of netrin-1 and netrin-2 that, alone, evoked relatively little outgrowth from E11 explants (2×, Figure 9B), just as twice the standard concentration of NSA alone evoked little outgrowth from these explants (2×, Figure 9C). When E11 explants were cultured with both the netrin mix and NSA at only half these defined concentrations, robust outgrowth was observed (1× each, Figure 9D). Thus, the interaction between NSA and netrins cannot be accounted for by a simple additivity of their effects (Figure 9E) and is a synergy. These results show that, in addition to the netrins, embryonic brain contains biochemically distinct components that may cooperate with the netrins to influence axon growth in vivo.

Discussion

Floor plate cells have been implicated in the guidance of commissural axons in the embryonic spinal cord by providing both long-range chemotropic cues that attract axons to the ventral midline, and contact-mediated cues that operate once the axons reach the midline (Jessell and Dodd, 1992). The long-range influence of the floor plate can be assayed in vitro by the ability of the floor plate to attract commissural axons at a distance and to promote their outgrowth into a three-dimensional collagen matrix (Tessier-Lavigne et al., 1988; Placzek et al., 1990a). As a first step toward identifying the signaling molecules that mediate these activities of the floor plate, we have purified two proteins, netrin-1 and netrin-2, each of which can promote the outgrowth of commissural axons from embryonic dorsal spinal cord explants. Their predicted sequence identifies them as vertebrate homologs of UNC-6, a C. elegans protein that regulates the dorsal and ventral circumferential migrations of mesodermal cells and axons (Hedgecock et al., 1990; Ishii et al., 1992). The netrins and UNC-6 are related to the ECM molecule laminin, which is itself a potent promoter of neurite outgrowth for many different classes of neurons (reviewed by Nurcombe, 1992), but which does not promote commissural axon outgrowth into collagen gels (Tessier-Lavigne et al., 1988; unpublished data).

The Netrins and UNC-6: A Structural Homology

The homology of the netrins to the C. elegans UNC-6 protein defines a family of axon growth–modulating proteins that have been highly conserved thoughout evolution. The netrins and UNC-6, like laminins (Beck et al., 1990), appear to be modular proteins. Domain V is highly conserved among the netrins and UNC-6 and contains three repeats of eight characteristically spaced cysteine residues with a spacing seen in EGF-like repeats in a variety of ECM molecules (Engel, 1989). The array of three repeats in domain V is predicted to confer a rigid rodlike structure on this region of the polypeptide (Engel, 1989; Beck et al., 1990).

In laminin, domain VI appears to mediate the calcium-dependent polymerization of preassembled laminin heterotrimers (Schittny and Yurchenco, 1990; Yurchenco and Cheng, 1993). Given the homology in this region of the netrins to the corresponding region of the laminins, it is possible that domain VI of the netrins mediates oligomerization of netrins or interactions between netrins and laminin. If heterooligomerization of netrins does occur, it must be reversed at high salt and low calcium concentrations, since in the last step of chromatographic purification, netrin-1 can be separated from netrin-2 under these ionic conditions.

Domain C of the netrins is rich in basic residues, which may mediate the avid binding of these proteins to heparin. This region might therefore also mediate binding of the netrins to sulfated proteoglycans or glycolipids on the surface of neural cells or in the ECM and account for the observed association of the two netrins with membranes. Domain C may also have signaling functions, since both netrins contain a conserved RGD sequence, which in other proteins can mediate interactions with integrin receptors (Hynes, 1992).

The molecular characterization of *unc-6* mutant alleles has provided evidence that the variety of cellular behaviors dependent on UNC-6 function (the ventral and dorsal migration of mesodermal cells and axons) are dependent on different domains of the protein. Mutations that delete the second EGF-like repeat of domain V impair only dorsal migrations (W. Wadsworth and E. Hedgecock, personal communication). Other genetically separable mutations of *unc-6* can result in the selective disruption of ventral but not dorsal migrations, or in the defective migrations of mesodermal cells but not axons (Hedgecock et al., 1990). Taken together, these genetic studies raise the possibility that the netrins may have biological activities other than those revealed by the present assays and that such activities reside in distinct domains of the netrin proteins.

Netrin Function Is Modulated by NSA, a Synergizing Activity

Netrins exhibit a markedly greater potency in promoting commissural axon outgrowth from E13 than from E11 dorsal spinal cord explants, whereas a differential response of E11 and E13 explants to high salt extracts of embryonic brain membranes is not observed. This observation led

to the characterization of a synergizing activity (NSA) that potentiates the effects of each netrin on E11 explants, conferring netrins with activity that is essentially equivalent to that observed on E13 explants. Like the netrins, NSA is membrane associated and salt extractable; unlike the netrins, it does not bind heparin in high salt (see Experimental Procedures).

The basis of the differential response of commissural axons in E11 and E13 explants to purified or recombinant netrins is unknown. E11 and E13 explants both contain newly developing commissural axons, but only E13 explants contain a significant number of regenerating axons (which are severed during preparation of the explants; see Figure 1B) (Altman and Bayer, 1984; Dodd et al., 1988). It is possible that the differential response of E11 and E13 explants results from a higher sensitivity of regenerating axons to the netrins. The differential response could also be explained if the dorsal spinal cord explants themselves express NSA, with E13 explants expressing enhanced levels of the activity. Since it is also possible that E11 explants synthesize NSA at low levels, we cannot exclude the possibility that the netrins have an obligate requirement for NSA to produce their axon outgrowth-promoting effects.

The biochemical nature and mechanism of action of NSA are unknown. NSA could interact physically with the netrins or netrin receptors, enabling the interaction between netrins and their receptors or increasing its affinity. NSA could also have a less direct effect, such as interacting with distinct NSA receptors on commissural axons that enhance the effects of the netrins at the level of signal transduction.

Specificity of Netrin Action

Like the floor plate, recombinant netrins promote the outgrowth of TAG-1$^+$ commissural axons but not the TAG-1$^-$ association axons (Dodd et al., 1988) in the E11 dorsal spinal cord explants. Moreover, netrins do not promote the outgrowth into collagen gels of motor axons from E11 rat ventral spinal cord explants or of sensory axons from E13 rat dorsal root ganglia (unpublished data). At high concentrations, netrin-containing extracts inhibit the outgrowth of axons from dorsal spinal cord explants (Figure 7). It is possible that this inhibition is due not to the netrins but rather to a factor(s) made by COS cells that is present in the extracts, although a similar inhibition was observed with embryonic brain extracts (Figure 2). Inhibition of axon outgrowth from tissue explants is observed for NGF-responsive axons in the presence of high concentrations of NGF (Levi-Montalcini et al., 1972; Ebendal, 1989).

The presence of netrins in the embryonic brain suggests that other classes of central nervous system neurons respond to these proteins. The decussation (contralateral projection) of axons is a highly conserved organizational feature of the vertebrate brain, and many crossing fiber tracts traverse the midline ventrally, through the floor plate or other specialized midline cell groups. With the availability of netrins in pure form it should now be possible to determine their neuronal specificity, and in particular whether all decussating axons in the central nervous system respond to these proteins.

The ability of netrins to promote the outgrowth of spinal commissural axons into collagen gels is not mimicked by any of a large number of growth or survival factors or neurite outgrowth-promoting molecules, including the structurally related protein laminin (Tessier-Lavigne et al., 1988; Placzek et al., 1990b; unpublished data). Thus, the netrins are the only factors identified to date that can mimic the commissural axon outgrowth-promoting activity of the floor plate. In the following article (Kennedy et al., 1994), we examine the possible roles of the netrins in mediating the outgrowth-promoting and chemotropic actions of the floor plate on commissural axons.

Experimental Procedures

Spinal Cord Explant Culture and Immunohistochemistry

Assays using E11 (E0 is the day of vaginal plug) rat dorsal spinal cord were as described (Tessier-Lavigne et al., 1988), except that enzymatic digestion was performed for 30 min on ice in 150 mM NaCl containing 5 × Pancreatine (GIBCO), 1.25 mg/ml trypsin, 0.26 mM EDTA. For E13 assays, spinal cords were isolated from E13 embryos by dissection in L15 medium (GIBCO), opened at the roof plate, and flattened down on the dissection dish in an open book configuration (Bovolenta and Dodd, 1990). Tungsten needles were used to dissect ~50 μm × 50 μm square pieces of dorsal spinal cord (see Figure 1B) in L15 containing 5% heat-inactivated horse serum (HIHS). These dorsal spinal cord explants were embedded in collagen gels and cultured as described (Tessier-Lavigne et al., 1988), with four explants in each gel. Immunohistochemical analysis of TAG-1 expression was performed using monoclonal antibody 4D7 as described (Dodd et al., 1988).

Screen of Tissue Homogenates and Quantification of Outgrowth Activity

For preparation of salt extracts of membranes, adult and embryonic tissues (~0.2–2.0 g, used fresh or snap frozen and stored at −80°C) were homogenized in a dounce homogenizer in 10 vol of 320 mM sucrose, 10 mM Tris (pH 7.5), 1 mM PMSF on ice. Each crude homogenate was centrifuged at 1000 × g for 10 min at 4°C, and the supernatant was centrifuged at 100,000 × g for 1 hr at 4°C. The high speed membrane pellet was resuspended in 1 vol of 1 M NaCl by homogenization in a dounce homogenizer, gently mixed for 1 hr at 4°C, recentrifuged at 100,000 × g for 1 hr at 4°C, and the supernatant saved. For assay, salt extracts were either dialyzed as described below or concentrated ~20-fold on a Centricon C-30 (Amicon) microconcentrator and added directly to culture medium (addition of extracts increased the NaCl concentration by less than 30 mM). Serial dilutions of extracts were tested in the E13 assay, with a starting concentration of added protein of 0.5–3.0 mg/ml. Outgrowth activity was detected in brain and spinal cord of E13–P3 rats and E5–E13 chicks, but not in P10 rat brain, brain and spinal cord from bovine fetuses at 2–5 months of gestation, adult rat brain and spinal cord, E14 and adult rat liver, E14 rat heart, or adult rat kidney.

A semiquantitative measure of activity in different samples was obtained by preparing serial 2-fold dilutions of each sample, examining the responses of at least four E13 explants to each dilution, and comparing these responses with a standard series (Figure 2). A unit of activity was defined as the amount that, when assayed in 0.4 ml of medium, gives outgrowth comparable to that evoked by 20 μg of a standard preparation of high salt extract (i.e., 50 μg/ml in Figure 2). The total length of axon bundles (the sum of individual bundle lengths) per explant was a more sensitive measure of activity than was the average length of individual bundles, because the total length also reflects the increase in the number of axon bundles evoked by higher concentrations of extract. Although characteristic dose–response curves were obtained in these experiments, making it possible to provide a rough estimate of the specific activity of samples, the large variance in bundle length and number made a more precise quantita-

tion of activity impractical. The data presented in Figure 2 do not illustrate the increase in bundle thickness that was observed with increasing concentrations of extract. In fact, the morphology of the bundles was a good predictor of the specific activity of the extracts and was sometimes used in semiquantitative measures of specific activities.

Purification of the Netrins and Netrin Synergizing Activity from Embryonic Chick Brain

The procedure was developed using ~20,000 E10 chick brains. Each purification run used ~2000 brains (processed to membranes in two batches of ~1000).

Buffers

The buffers used in the purification were as follows: HB1 (320 mM sucrose, 10 mM HEPES–NaOH [pH 7.5], *1 × protease inhibitors [1 mM EDTA ,pH 8.0], 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A]), HB2 (HB1 plus *1 mM PMSF), RB (10 mM HEPES–NaOH [pH 7.5], *2 × protease inhibitors), SB1 (1.5 M NaCl, 10 mM HEPES–NaOH [pH 7.5]), SB2 (1.1 M NaCl, 10 mM HEPES–NaOH [pH 7.5], *1 × protease inhibitors), A1 (900 mM NaCl, 10 mM HEPES–NaOH [pH 7.5]), B1 (2 M NaCl, 10 mM HEPES–NaOH [pH 7.5]), A2 (500 mM NaCl, 10 mM HEPES–NaOH [pH 7.5], 100 μM $CaCl_2$, 10 μM $MnCl_2$), B2 (A2 plus 700 mM N-acetylglucosamine), DB (500 mM N-acetylglucosamine, 20 mM Tris–HCl [pH 8.0]), A3 (20 mM Tris–HCl [pH 8.0]), B3 (A3 plus 2 M NaCl), A4 (20 mM NaP, [pH 7.5] [dilute Na_2HPO_4, adjust pH with o-phosphoric acid], 1.5 M NaCl), B4 (20 mM NaP, [pH 3.0] [dilute NaH_2PO_4, adjust pH with o-phosphoric acid], 1.5 M NaCl). Buffer pH was adjusted at ambient temperature; components indicated with an asterisk were added directly before use. All buffers were used ice-cold except for A1, B1, A3, B3, A4, and B4, which were used at 4°C.

Subcellular Fractionation

For each preparation, ~1000 E10 chick brains were dissected into 1 liter L15 medium on ice over ~2 hr. Brains were homogenized in batches of ~125 brains with 25 ml of buffer HB1 and 0.5 ml of 100 mM PMSF (in 2-propanol) in a 55 ml Potter–Elvehjem homogenizer on ice, using five strokes followed by a 1 min pause, followed by five additional strokes. The crude homogenate (~360 ml) was centrifuged at 2700 rpm (1000 × g) in an SA-600 rotor (Du Pont/Sorvall) for 10 min at 4°C. The supernatants (~200 ml) were pooled on ice. The pellets were reextracted with one stroke in a total of 135 ml of buffer HB2, and the supernatants (~120 ml) were pooled with those obtained previously. This low speed supernatant (LSS) was centrifuged at 8300 rpm (10,000 × g) in an SA-600 rotor for 10 min at 4°C, and the supernatants (~240 ml) were pooled on ice. The pellets were reextracted with one stroke in a total of 200 ml of buffer HB2, and the supernatants (~210 ml) were pooled with those obtained previously at this stage. This medium speed supernatant (MSS) was centrifuged at 50,000 rpm (230,000 × g) in a 50.2 Ti rotor (Beckman) for 35 min at 4°C. After discarding the supernatants, 4 ml of buffer RB was added to each tube per 26 ml of MSS originally present, the buffer and pellet transferred to a 50 ml conical tube on ice, the centrifuge tube rinsed with 1 ml of buffer RB, and this rinse pooled with the previously transferred material. The pooled material was homogenized with a dounce homogenizer using 10 up-and-down strokes of an A pestle, frozen in liquid nitrogen, and stored at −80°C. A total of ~120 ml of high speed pellet (HSP) homogenate was obtained.

Salt Extraction

The HSP homogenate derived from 2000 embryonic chick brains was thawed at 37°C, pooled in a 2 liter beaker on ice, and stirred at medium speed. A low salt wash was performed by adding buffer SB1 at the rate of ~1.5 ml/min until the conductance of a 1/100 dilution of the homogenate was ~520 μS/cm (so that the homogenate was ~500 mM NaCl). The homogenate was stirred for 1 hr at a low setting, then centrifuged at 35,000 rpm (100,000 × g) in a 45 Ti rotor (Beckman) for 2 hr at 4°C, and the supernatants were discarded. For high salt extraction, 25 ml of buffer SB2 was added to each centrifuge tube, and the buffer and pellet transferred into a 40 ml dounce homogenizer. The tube was rinsed with 15 ml of buffer SB2 and this rinse added to the homogenizer. The pellet was homogenized with 10 up-and-down strokes of an A pestle and stirred on ice for 1 hr at a low setting (setting 4). The homogenate (~240 ml) was centrifuged at 35,000 rpm in a 45 Ti rotor for 2 hr at 4°C. The supernatant was stored at 4°C overnight, recentrifuged as before, and collected, yielding ~200 ml of high salt extract (HSE).

Heparin Affinity Chromatography (I)

The ionic strength of the HSE was lowered slightly (to ~900 mM NaCl) by stirring the HSE on ice at medium speed and adding dropwise ice-cold 10 mM HEPES–NaOH (pH 7.5) until the conductivity of a 1/100 dilution of the HSE was ~1000 μS/cm. The diluted HSE was loaded at a flow rate of 1.5 ml/min onto a HS CL-6B column (Bio-Rad Econo-Column with flow adapter, 2.5 cm × 20 cm, packed with Pharmacia heparin–Sepharose CL-6B), previously equilibrated with 375 ml of buffer A1. The flowthrough was collected beginning 80 min after the start of loading and ending ~40 min after the last diluted HSE had been loaded onto the column. This flowthrough fraction contained NSA. The flowthrough fraction was frozen in 40 ml aliquots in liquid nitrogen and stored at −80°C. The column was washed with a total of 300 ml of buffer A1 at a flow rate of 1.5 ml/min. The bound protein was eluted with buffer B1 at a rate of 1.5 ml/min. The peak of eluted protein was collected manually in a volume of ~30 ml using absorbance at 280 nm to monitor the column efflux for the beginning of the eluate peak. To the eluate fraction was added 30 μl of 1 mg/ml pepstatin A (in DMSO) and 60 μl of a solution of 1 mg/ml aprotinin and 1 mg/ml leupeptin, before storage at 4°C overnight.

Lectin Affinity Chromatography

The HS CL-6B eluate was concentrated to a final volume of less than 1.5 ml in a 50 ml Amicon ultrafiltration cell at 55 psi nitrogen using a YM30 membrane. The cell and membrane were washed with 0.5 ml of buffer A2, which was pooled with the concentrate. The concentrate was loaded onto a WGA–Agarose column (Bio-Rad Poly-Prep Column, 1 ml bed volume, packed with Vector Laboratories WGA–Agarose, and preequilibrated with buffer A2) in 0.7 ml (maximum volume) batches, with 30 min periods between additional loadings. The column was then washed with two 1 ml vol of buffer A2, followed by 20 ml of buffer A2. The column elution was begun with 0.7 ml of buffer B2, and the eluate was discarded. Elution was continued with an additional 0.3 ml of buffer B2, and the eluate was saved. After 1 hr, the elution was continued with 1 ml and then 0.7 ml of buffer B2, pooling with the previous eluate for a total WGA–Agarose eluate of 2 ml.

Heparin Affinity Chromatography (II)

The WGA–Agarose eluate was diluted with 1.3 ml of buffer DB with gentle vortexing and loaded onto a heparin–Sepharose high performance (HSHP) column (Pharmacia HR 5/10 column, 5 mm × 10 cm, packed with Pharmacia heparin–Sepharose high performance), previously equilibrated with 20 ml of 85% buffer A3/15% buffer B3, at a flow rate of 0.1 ml/min. The column was washed with 85% buffer A3/15% buffer B3 at this flow rate for a total of 90 min for the load and the wash combined. The column was then eluted with a linear gradient from 50% buffer A3/50% buffer B3 to 25% buffer A3/75% buffer B3 over a period of 200 min at 0.1 ml/min, collecting 0.5 ml fractions into 1.5 ml siliconized polypropylene tubes.

IMAC

NaH_2PO_4 (0.2 M, 22.6 μl) was added to each of the six or seven fractions containing most of the protein eluting in the peak centered at ~1.35 M NaCl (the conductivity of a 1/100 dilution of the fraction was ~1.5 mS/cm; see Figure 4). These HSHP column fractions were pooled and loaded at 0.5 ml/min onto an IMAC column (Pharmacia HR 5/2 column, 5 mm × 2.5 cm, packed with Pharmacia chelating Sepharose high performance), previously charged with Zn^{2+} and equilibrated at 0.5 ml/min as follows: first, 5 ml of 75% buffer A4/25% buffer B4; second, 0.5 ml of 0.1 M $ZnSO_4$; third, 5 ml of 75% buffer A4/25% buffer B4; fourth, 5 ml of 25% buffer A4/75% buffer B4; and fifth, 10 ml of 75% buffer A4/25% buffer B4. After loading and then washing for an additional 1 min, the column was eluted with a linear gradient from 75% buffer A4/25% buffer B4 to 25% buffer A4/75% buffer B4 over 20 min at 0.5 ml/min, with a 20 min wash at the end conditions thereafter. Fractions of 1 ml were collected into 1.5 ml siliconized polypropylene tubes. Netrin-2 eluted isocratically (at a pH of ~6.5) after the flowthrough; netrin-1 eluted in a peak centered on pH ~6.1.

Preparation of Fractions for Assay

To assay fractions, 1 ml volumes were adjusted to at least 1 M NaCl using 4 M NaCl as required. Fractions obtained at steps following the crude homogenate stage (which itself was not assayed) through the HSE fractions were mixed at 4°C for 1 hr, and salt-stripped membranes

in the fractions were removed by centrifugation for 100 min at 40,000 rpm (70,000 × g) in a RP100-AT4 rotor (Du Pont/Sorvall). Fractions from the HSE step through the end of the purification were adjusted to 1 mg/ml ovomucoid (which binds WGA) to stabilize the activity during freeze–thaw and dilution and to prevent WGA that had leached off the WGA–Agarose column from either rebinding the active protein during dialysis or inhibiting outgrowth from the explants by itself (ovomucoid alone has no effect in the E11 or E13 assays).

Samples were prepared in one of two fashions. The first is as follows: routinely, volumes (between 0.1 and 0.2 ml) of fractions or dilutions of fractions to be assayed for activity were prepared by dialysis against F12 medium at a flow rate of 1 ml/min for at least 3 hr in a GIBCO BRL microdialyzer using Spectrapor 2 (Spectrum) dialysis membrane. Samples were brought up to a final volume of 0.4 ml with F12 medium (and 0.2 ml of HS CL-6B flowthrough [containing NSA] dialyzed against F12, if the fractions were from a stage in the purification beyond the HS CL-6B column), and other components required to complete the medium (Tessier-Lavigne et al., 1988). Samples were warmed to 37°C before addition to the explant cultures. The cultures were incubated in complete medium for up to 8 hr before addition of the warmed samples. The second method is as follows: in cases where the activity was highly concentrated (e.g., heparin affinity chromatography [II] eluate), a small volume of the sample was added directly into complete culture medium (increasing the NaCl concentration by less than ~40 mM) and warmed prior to assay. Routinely, the specific activity of fractions prepared in this way was found to be 3- to 4-fold higher than when fractions were prepared by dialysis.

Preparation of Synergizing Activity for Assay

The HS CL-6B column flowthrough containing NSA contains a component(s) that is toxic for E11 explants. This component(s) was removed either by concentrating the fraction on a Centricon C-10 (Amicon) microconcentrator and then restoring it to half the starting volume using buffer A1, or by extensive (>6 hr) dialysis. Both methods gave similar results. NSA was used at a final protein concentration of 50–60 μg/ml in the assays.

Protein Analysis

Trichloroacetic acid (TCA) precipitation of proteins was performed essentially as described (Serafini et al., 1991), except that precipitates were routinely washed with 6% TCA (prior to the acetone wash) to remove salt. Protein concentration measurements were performed using either Peterson (Peterson, 1977) or protein gold assays (Integrated Separation Systems), using equine IgG (Pierce) as a standard. The ECL system (Amersham) was used as a detection method for immunoblotting according to the recommendations of the manufacturer.

Protein Microsequencing

TCA Precipitation

A total of 11.75 ml from IMAC fractions (from ~5000 brains, processed in three separate purification runs) containing netrin-1 was TCA precipitated sequentially in two tubes; after the final precipitation, the precipitate was rinsed with 6% TCA, recentrifuged, and washed with acetone. To increase the amount of netrin-2 available for microsequencing, fractions from two HSHP chromatography runs containing predominantly netrin-2 (i.e., the three fractions in each HSHP run collected just before those pooled for the last purification step) were pooled, adjusted with 0.2 M NaH_2PO_4, and chromatographed on the IMAC column as previously described. The netrin-2 present in a total of 14.9 ml from IMAC fractions from three purification runs was TCA precipitated as described for netrin-1.

CNBr Proteolysis and Peptide Isolation

To obtain internal amino acid sequence from netrin-1 and netrin-2, peptides were generated by cyanogen bromide (CNBr) cleavage. The pellets of TCA-precipitated protein were resuspended in 50 μl of a solution of 50 mg/ml CNBr in 70% formic acid and incubated overnight in the dark at ambient temperature. The formic acid was removed by evaporation in a SpeedVac (Savant) followed by resolubilizing in 50 μl deionized H_2O and reevaporation to dryness. The CNBr-generated peptides were separated by SDS–PAGE, blotted to a ProBlott (ABI Biotechnology) membrane and visualized with Coomassie blue as described (Kennedy et al., 1988). Individual bands were excised and directly sequenced from the membrane.

Protein Sequencing

Peptide sequencing was performed on a Porton Instruments automated gas phase sequencer (model PI 2020G) equipped with an online analyzer for phenylthiohydantoin (PTH)-derivatized amino acids.

N-Terminal Protein Sequencing

A portion of the netrin-2-containing pooled IMAC fractions (0.6 ml, adjusted to 1 ml with water) and 1 ml netrin-1-containing IMAC fractions were separately TCA precipitated. The precipitated proteins (~1.2 μg of netrin-1 and ~0.5 μg of netrin-2) were dissolved in 15 μl of 70% formic acid and applied 3 μl at a time to 2 mm × 5 mm pieces of ProBlott membrane. The tubes were washed with an additional 15 μl of 70% formic acid, which was also applied to the membranes. N-terminal sequencing was performed directly on these membrane pieces. Sequencing was also performed on impure netrin-1/netrin-2 subjected to SDS–PAGE and electroblotted as above, with the netrin-1 and netrin-2 bands excised separately. This latter material was sequenced commercially by the Biomolecular Resource Center at the University of California, San Francisco.

Unambiguous Sequences

The following sequences were considered reliable enough to use as a basis for the design of oligonucleotide primers for gene cloning purposes (the letter M in brackets at the beginning of most sequences indicates the methionine residue implied by the generation of the peptide by CNBr). From netrin-1: N-terminus, ??????MFAVQT; 1, [M]ELYKLSGRKSGGVXLNXRH; 2, [M]ELYKLSGQKSGGV; 3, [M]DYGKTWVPFQFYS; and 4, [M]YNKPSRAAITKQNEQEAI. From netrin-2: N-terminus, ANPFVAQQTP; 1, [M]ELYKLSGRKSGGVXLNXRH; and 2, [M]DYGKTWVPYQYYS. X denotes the absence of any PTH derivative observed during that cycle of sequencing, which is usually diagnostic of the presence of a cysteine residue (C). Question mark denotes an ambiguous result for that cycle of sequencing, i.e., no assignment could be made.

Nucleic Acid Experimental Procedures

Standard methods were used for manipulation of nucleic acids (Sambrook et al., 1989; Ausubel et al., 1990).

RNA Isolation

E10 chick brains were frozen in liquid nitrogen and homogenized using a Polytron (Brinkmann) to isolate total cellular RNA as described (Auffray and Rougeon, 1980). Poly(A)$^+$ RNA was isolated from total cellular RNA using oligo(dT)–cellulose (Collaborative Research) (Ausubel et al., 1990), with a yield of ~5%.

Oligonucleotide Synthesis

Oligonucleotides were synthesized using a Cyclone Plus DNA synthesizer (Millipore) and gel purified.

Isolation of a Fragment of the cDNA Encoding Netrin-1

E10 chick brain poly(A)$^+$ RNA was reverse transcribed using MoMLV (GIBCO BRL). To obtain a fragment of a cDNA encoding netrin-1, various combinations of degenerate oligonucleotides designed from unambiguous sequences were used in two-stage nested PCR. A fragment was obtained as follows. In the first-stage reaction, the sense primer sequence was TAYGGNAARACNTGGGT (from the amino acid sequence YGKTWV), and the antisense primer sequence was GCYTCYTGYTCRTTYTG (QNEQEA). In the second-stage reaction, the sense primer sequence was TGGGTNCCNTTYCARTT (WVPFQF), and the antisense primer was that used in the first reaction. A 1:10 dilution of the first reaction provided the template for the second-stage reaction. All PCR (30 cycles, 37°C annealing temperature) was hot started. A single second-stage product of ~100 bp was observed and cloned into the pCRII vector using a TA cloning kit (Invitrogen). This fragment contained 62 bp of unique sequence, including sequence-encoding portions of peptides 3 and 4 of netrin-1 not used for any of the three primers.

Isolation of a Fragment of the cDNA Encoding Netrin-2

Subsequent cloning (see below) showed that netrin-1 was homologous to a portion of the B2 chain of laminin, and this information was used in isolating a fragment of the cDNA encoding netrin-2, again using two-stage nested PCR. The first stage consisted of reactions having as sense primer the same oligonucleotide pool that was used in the first stage of cloning the *netrin-1* fragment (since that pool was designed from a peptide sequence shared between netrin-1 and netrin-2), and having as the antisense primer one of four different pools of degen-

erate oligonucleotides corresponding to the amino acid sequence VCLNCR (also shared between the two proteins): 1, CKRCARTTNAGRCARAC; 2, CKRCARTTNAGRCAYAC; 3, CKRCARTTYAARCARAC; and 4, CKRCARTTYAARCAYAC. PCR (35 cycles) was performed with annealing varying from 35°C to 60°C. A product of ~630 bp appeared at higher annealing temperatures using each of the antisense pools, but most abundantly with pools 1 and 2. Second-stage reactions were performed using the 630 bp products as templates with a sense primer pool having the sequence TGGGTNCCNTAYCARTAYTA (WVPYQYY) and an antisense primer pool having the sequence GCRTGNCCRTTRCAYTTRCA. This sequence corresponds to the amino acid sequence CKCNGHA, which is conserved between netrin-1 and laminin B2 and was assumed to be conserved in netrin-2. A single product of ~380 bp was amplified, isolated, and digested with SacI (which recognizes a site at this point in the *netrin-1* sequence). The uncut portion was isolated, reamplified, redigested with SacI, and cloned into pCRII. Two clones contained 377 bp inserts yielding 341 bp of unique sequence for *netrin-2*.

Library Construction

An oligo(dT)-primed E10 chick brain cDNA library was constructed using the ZAP cDNA Gigapack II Gold cloning kit (Stratagene) as directed, except that cDNAs were size selected using a BRL size selection column (catalog number 8092SA). For the isolation of 3′ coding sequence of *netrin-1*, a plasmid library was constructed using a sequence-specific first-strand synthesis primer (with attached 5′ Xho site AGAGAGAGAGAGAACTAGTCTCGAGCTTCCATCCCTCAATACGAG) designed from sequence 3′ of the translational stop codon that was obtained by 3′ RACE (see below). cDNAs generated using this primer were cloned directly into EcoRI–XhoI-digested pBluescript SK(+) and transformed into the Escherichia coli SURE strain (Stratagene) by electroporation.

3′ RACE

Sequence corresponding to the 3′ end of the *netrin-1* cDNA was amplified and cloned using the RACE protocol as described by Frohman and Martin (1989). Vent DNA polymerase (New England Biolabs) was used for RACE reactions using a final $MgSO_4$ concentration of 4 mM.

Library Screening

Libraries were screened with probes labeled with [α-^{32}P]dCTP either by random priming or by incorporation during synthesis of the probe by PCR. For *netrin-1*, an initial screen of 10^6 clones of the E10 chick brain cDNA library using a 61 bp *netrin-1* probe identified a single internally primed 1 kb *netrin-1* cDNA. A probe designed from this clone was used to isolate two additional partial *netrin-1* cDNAs by screening an additional 10^6 clones of the E10 chick brain library and 2 × 10^6 clones of an E2.5 chick spinal cord library (in λZAP; Basler et al., 1993). As these cDNAs had primed internally, the 3′ coding sequence was obtained by 3′ RACE followed by the generation and screening of a primer extension library (see above), yielding a cDNA containing the full coding sequence of *netrin-1*. For *netrin-2*, eleven overlapping cDNAs were isolated by screening 3 × 10^6 clones of the E10 chick brain cDNA library using a probe derived from the initial *netrin-2* cDNA fragment or with additional probes designed from cDNAs that were isolated as the screening proceeded. Phage were isolated and inserts excised in vivo and recircularized into pBluescript II SK(−) as recommended by Stratagene.

DNA Sequencing and Sequence Analysis

Nested deletions for sequencing were generated using ExoIII digestion as described (Ausubel et al., 1990). Sequence was also obtained in some instances by subcloning small fragments or by using specific internal oligonucleotide primers. Dideoxy sequencing was performed using the Sequenase kit (United States Biochemical Corporation). Sequence compressions were resolved by a combination of dITP sequencing (Sequenase; United States Biochemical Corporation) and Exo(−)PFU Cyclist DNA sequencing (Stratagene). Searches of the National Center for Biotechnology Information databases were performed using BLAST (Altschul et al., 1990) and the BLAST server service (HyperBLAST). Sequence alignments and analysis were performed using GeneWorks software (Intelligenetics). Isoelectric point determinations were performed using Genetics Computer Group software (Genetics Computer Group, 1992).

The relationship among the sequences of domains VI and V of laminin, UNC-6, and the netrins was determined using both parsimony (phylogenetic analysis using parsimony [PAUP]; Swofford, 1991) and unweighted pair group with arithmetic mean algorithms (UPGMA). UPGMA analysis was performed using Geneworks software (Intelligenetics). Multiple sequence alignments for parsimony analysis were generated using PIMA (Smith and Smith, 1992). Similar tree structures were obtained with both algorithms.

Genomic DNA Isolation and Southern Blot Analysis

Genomic DNA was isolated from seven E11 chick brains essentially as described (Ausubel et al., 1990; Laird et al., 1991). DNA (10 μg) was digested overnight with EcoRI, PstI, or XbaI, and the resulting fragments were separated on a 0.7% agarose gel and blotted to Hybond-N (Amersham). Probes corresponding to the full coding regions of *netrin-1* and *netrin-2* were generated by random priming, and hybridization and washing were performed as described (Sambrook et al., 1989).

Generation of Recombinant Proteins

Generation of Netrin-1 and Netrin-2 Expression Constructs

Patch PCR (Squinto et al., 1990) was used to introduce at the C-terminus of netrin-1 the sequence GGEQKLISEEDL, which encodes a glycine bridge and an epitope from the c-Myc protein recognized by monoclonal antibody 9E10 (Evan et al., 1985; Munro and Pelham, 1987). The modified *netrin-1* cDNA was cloned into pMT21 (COS cell expression vector; gift of G. Wong, Genetics Institute) to yield pGNET1^{myc}. Patch PCR was used to introduce the same sequence at the C-terminus of netrin-2. Because sequence encoding the full netrin-2 signal sequence was lacking, kinased, annealed oligonucleotides encoding the netrin-1 signal sequence were ligated together with a fragment encoding mature tagged *netrin-2* and cloned into pMT21 to yield pGNET2^{myc}.

Transfections and Preparation of Netrin-Containing Extracts

Transfections of pMT21, pGNET1^{myc}, and pGNET2^{myc} into COS1 cells (under 20 passages) were performed using LipofectAMINE (GIBCO BRL) as directed. Approximately 4.5 × 10^6 cells were seeded in 150 mm dishes (five each for netrin-1, netrin-2, and control) and transfected with 20 μg of DNA using 100 μl of LipofectAMINE in a 16.7 ml volume. Transfections were stopped after 5 hr by adding an equal volume of medium containing 20% heat-inactivated fetal bovine serum. Cells were washed twice at 24 hr posttransfection with PBS and then incubated with 30 ml of OptiMEM I medium supplemented with GlutaMAX I (GIBCO BRL), 100 μg/ml $CaCl_2$, and antibiotics, for an additional 3 days. To prepare cell extracts, cell monolayers were incubated after removal of media with 10 ml of Extraction Buffer (1 M NaCl, 10 mM HEPES–NaOH [pH 7.5], 1× protease inhibitors) at 37°C for 5 min. Extracts were centrifuged 15 min at 4°C at 3000 rpm (GH-3.7 rotor, Beckman) to remove debris and then concentrated ~25-fold using a YM30 ultrafiltration membrane (as described above for the HS CL-6B eluate). After centrifugation in a microcentrifuge at 4°C for 15 min, supernatants were adjusted by the addition of 1/9 vol of 10 mg/ml ovomucoid, 1 M NaCl, 10 mM HEPES–NaOH (pH 7.5) and were snap frozen in liquid nitrogen in 100 μl aliquots.

Quantification of Netrin-Containing Extracts

To measure the netrin concentration in extracts, a netrin-1^{myc} standard for immunoblot analysis was prepared. Extracts from the transfected cell monolayers of ten 150 mm plates were centrifuged at 3000 rpm (as above), then at 8300 rpm (as above to prepare MSS), adjusted to 1% ethylene glycol (Fluka), and loaded at 0.25 ml/min onto an HSHP column (as above) equilibrated with 50% buffer A3 (with 1% ethylene glycol)/50% buffer B3 (with 1% ethylene glycol). After washing with 6 ml of 50% buffer B3 (with 1% ethylene glycol), netrin-1^{myc} was eluted with a 20 ml linear gradient from 50% to 75% buffer B3 (with 1% ethylene glycol) at 0.1 ml/min, collecting 0.5 ml fractions. The nearly homogeneous netrin-1^{myc} (as assessed by silver staining and immunoblotting with monoclonal antibody 9E10) was TCA precipitated from half of the seven peak fractions and resolubilized in 60 μl of SDS-PAGE sample buffer. Of this standard, 15 μl was subjected to SDS-PAGE in a 7.5% polyacrylamide gel and electroblotted to ProBlott membrane. The netrin-1^{myc} band was excised and subjected to amino acid analysis by the Howard Hughes Medical Institute/Columbia Protein Chemistry Core Facility, yielding 0.35 ± 0.02 μg of netrin-1^{myc} (on the basis of an analysis of 14 amino acids). Dilutions of the standard were used in immunoblotting and densitometry to determine the netrin-1^{myc} and netrin-2^{myc} concentrations in the extracts.

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Chapter III

Preliminary characterization of the diffusion properties of the netrin-1 protein

Abstract

Netrin-1 acts as a long range guidance cue in the developing spinal cord but as a short range cue in the developing eye. To investigate how this difference may arise, we studied how netrin-1 diffuses in a cellular environment. Despite of netrin-1's role as a chemoattractant, the majority of netrin-1 protein associates tightly with tissue culture cells and with cells of the ventral spinal cord. Free gycosaminoglycans can extract netrin-1 protein off of cell membranes, suggesting that cell binding is mediated by cell associated proteoglycans. Furthermore, soluble and cell associated netrin-1 protein from tissue culture cells have identical biochemical properties and specific activity profiles, suggesting that netrin-1 continuously rebinds to cells as it diffuses. Cell binding may seem like an unfavorable feature for a protein whose function depends on its ability to diffuse; in fact, it may be important for facilitating the establishment of a far ranging netrin-1 gradient.

Introduction

Each of the 10 billion neurons in the adult human brain makes connections with roughly a thousand target cells. The remarkable feat of generating these connections occurs during embryonic development as neurons extend axonal processes to their targets. Axons are guided to their targets by both positive and negative, short range and long range environmental guidance cues (Tessier-Lavigne and Goodman, 1996). Despite these categorizations, many guidance cues are actually multi-functional signals and are able to function differently in different settings. For example netrin-1, a protein phylogenetically conserved from nematodes to mammals, can function both as an attractive and repulsive cue (Hedgecock et al., 1990; Wadsworth and Hedgecock, 1992; Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997), it can function permissively as well as instructively (Kennedy et al., 1994; Serafini et al., 1994; de la Torre et al., 1997), and functions in both axon guidance and target recognition (Mitchell et al., 1996). In the case of attraction and repulsion, differences in response to netrin-1 are in part due to differences in the types of netrin-1 receptors expressed on axons. In *C. elegans*, the UNC-5 receptor is required for axons which grow away from the nematode netrin, UNC-6, whereas the UNC-40 receptor is predominantly required for axons attracted to UNC-6 (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Hamelin et al., 1993; Chan et al., 1996; Keino-Masu et al., 1996).

The range of the netrin-1 activity also varies within different tissues. For instance, in the spinal cord of netrin-1 -/- mice, commissural axons are misdirected up to 250 μm away from the floor plate, where netrin-1 expression is high, suggesting that netrin-1 functions at a distance (Serafini et al., 1996). In contrast, retinal ganglion axons of mutant animals grow all the way to the optic nerve, where netrin-1 is expressed, then fail to enter it. This suggests that in the eye, netrin-1 functions as a short range cue (Deiner et al., 1997). One possible explanation for this difference is that netrin-1 diffuses differently in these tissues. Indeed, while netrin-1 is expressed only in the floor plate of the chick spinal

cord, netrin-1 protein extends as a gradient hundreds of micrometers dorsally (Kennedy et al., 1996). In contrast, a netrin-1 gradient could not be detected emanating from the optic nerve (Deiner et al., 1997).

To determine how netrin-1 gradients are established and if the shape of the netrin-1 gradient differs within different tissues, it will be necessary to characterize how netrin-1 diffuses in a cellular environment. When expressed in COS cells, a fraction of the netrin-1 protein collects in the conditioned medium, but the majority remains associated with cell surfaces and can be extracted with 1 M NaCl (Kennedy et al., 1994). Like netrin-1, many growth factors have both cell associated and soluble forms. For example, TGF-α is produced on the cell surface as a membrane anchored receptor, proTGF-α, then cleaved extracellularly into a diffusible form (for review see Massague and Pandiella, 1993). Extracellular cleavage is thought to regulate the range of TGF-α's action by converting one active form into another. This is in contrast to other molecules, such as sonic hedgehog, which require proteolytic cleavage for proper tethering. Heparin binding growth factors such as heparin binding EGF (hbEGF) and basic FGF (bFGF), also exist in both cell-associated and soluble forms (Vlodavsky et al., 1987; Friedl et al., 1997), but these factors do not appear to be post-translationally modified. Instead, they are produced in a secreted form that can bind proteoglycans on the cell surface and therefore, exist in equilibrium between cell-bound and soluble states. Cell binding has generally been thought to limit the range of growth factors by immobilizing them close to the site of their expression (Ruoslahti, 1989; Flaumenhaft et al., 1990; Vlodavsky et al., 1996).

The results presented in this chapter suggest that netrin-1's diffusion properties are similar to those of the heparin binding growth factors. Netrin-1 probably associates with cells by binding to cell surface proteoglycans, and as it diffuses, continues to bind to cells. Yet, netrin-1 is clearly not immobilized to the site of expression. *In vitro*, it can diffuse from COS cells and through cells of the dorsal spinal cord to elicit turning of commissural axons at a distance of ~250 μm (Kennedy et al., 1994). *In ovo*, netrin-1 protein, injected

into the central canal of developing chick embryos, diffuses through the dorsal spinal cord to alter the trajection of commissural axons ~200μm away (E. Stoeckli, personal communication). Cell binding may seem like an unfavorable feature for a chemoattractant whose function depends on its ability to diffuse over long distances. However, while cell binding will decrease netrin-1's diffusion rate, a slow diffusion rate may in fact be an important parameter to facilitate the establishment of a far ranging gradient. Our results for netrin-1 suggest that other heparin binding factors may not necessarily be limited to their site of expression.

Results

Production and characterization of the 293 $^{netrin\text{-}1}$ stable cell line

To determine how netrin-1 diffuses in a cellular environment, we first generated a stable cell line producing high levels of netrin-1 protein. We overexpressed netrin-1^{myc} protein off of an extrachromasomal plasmid replicated by the Ebstein Barr Virus Nuclear Antigen (EBNA), pCEP4 (Invitrogen). When expressed in COS cells, the myc epitope at the C-terminus of netrin-1 does not interfere with function *in vitro* (Serafini et al., 1994). One copy of the EBNA gene (Ebstein Barr Virus Nuclear Antigen) is present on the pCEP4 vector but the number of extrachromosomal plasmids is limited by the concentration of EBNA protein made. Thus, by transfecting into cells containing a second copy of the EBNA gene, greater numbers of the netrin-1 plasmid were maintained. 293-EBNA $^{netrin\text{-}1myc}$ cells were generally grown in selective medium to maintain the pCEP4 $^{netrin\text{-}1}$ plasmid, but could be co-cultured with primary embryonic explants in the absence of selection. Indirect immunofluorescence of the stable cells using 9E10 monoclonal antibodies directed to the myc epitope verified that all cells express netrin-1 protein (figure 1a).

This stable cell line has several advantages over transiently transfected cells. First, it minimizes variation in protein production, allowing us to readily make meaningful comparisons between experiments. Second, it provides a source from which we can purify netrin-1 protein, allowing us to use a more defined and concentrated preparation of netrin-1 in our experiments. Approximately 30 fold higher levels of netrin-1 are obtained from the 293 $^{netrin1\text{-}myc}$ stables than from transiently transfected COS cells (figure 1b). Higher expression is partially due to the fact that 293 cells are approximately 1/10th the diameter of COS cells, allowing more cells to be cultured per plate. Moreover, unlike transiently transfected cells (which die within 3-4 days), 293 stable cells can be conditioned over the course of 7-10 days allowing netrin-1 protein to accumulate in the medium without degradation. The success of the 293 cell line followed two earlier attempts to overexpress

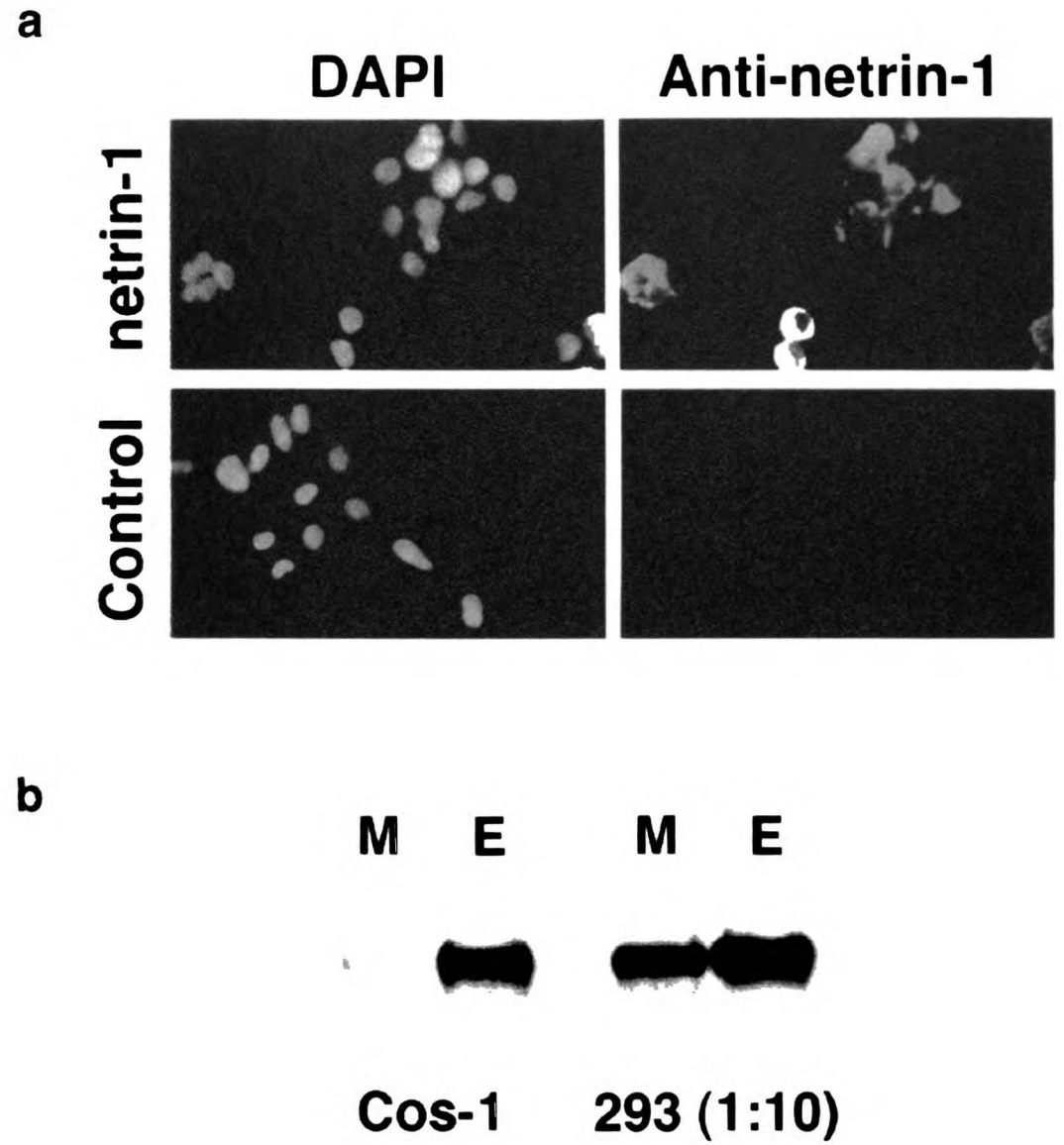


Figure 1. Expression of recombinant netrin-1 protein from stably transfected 239 cells. (a) Indirect immunofluorescence of 293-EBNA cells (control) and 293-EBNA cells stably transfected with netrin-1 myc (netrin-1) using the 9E10 monoclonal antibody directed against the myc epitope. The nuclei of these cells were stained with DAPI and are shown on the left. (b) Conditioned medium and (M) and 1M NaCl extract (E) were collected from transiently transfected COS cells expressing netrin-1 and from 293 cells stably expressing netrin-1, subjected to high speed centrifugation, TCA precipitated, resuspended in equal volumes of sample buffer, subjected to SDS-polyacrylamide electrophoresis, and immunoblotted using the 9E10 monoclonal antibody. A 10-fold lower volume of each of the samples from the 293 cells was loaded onto the gel. The total amount of netrin-1 as well as the fraction of netrin-1 present in the medium is greater from 293 cells than from COS cells.

netrin-1, first in SF-9 cells using the baculovirus system (L. Hinck, personal communication) and next, in CHO cells by genomic integration of the netrin-1 gene (C. Mirzayan, not shown). Both produced only ~ 100 picograms/ml netrin-1 protein, which is comparable to levels of expression of transiently transfected COS cells.

Approximately 40% of netrin-1 secreted by 293-EBNA $^{\text{netrin-1 cells}}$ accumulates in the conditioned medium (see figure 1b). This conditioned media contains far fewer other heparin binding proteins than the 1 M NaCl extract, allowing for the production of nearly pure netrin-1 protein (10-20% purity with only three contaminated bands) after a heparin affinity chromatography step. Netrin-1 elutes at 1.2 M NaCl (20mM NaPi [pH 7.5]) and comprises 10-30% of the total protein in this eluate. In general, 1mg of netrin-1 protein is obtained from 1 L of conditioned medium. This eluate was generally used directly in most experiments, but when necessary netrin-1 was purified to homogeneity by ion exchange chromatography on mono-S (personal communication, A. Faynboym). The concentration of netrin-1 in the impure heparin eluate was determined by immunoblotting with anti-myc antibodies and comparing the intensity of the signal to standards of pure netrin-1 protein.

In order to reliably use recombinant netrin-1 protein produced by 293 cells, we had to verify that pure protein could elicit outgrowth of commissural axons into collagen and that aggregates of 293-EBNA $^{\text{netrin-1myc}}$ cells could elicit turning of E11 axons within the dorsal spinal cord (Kennedy et al., 1994; Serafini et al., 1994). To measure outgrowth activity, dorsal spinal cords from E13 embryos were microdissected and cultured *in vitro* in the presence of pure netrin-1 protein. 30ng/ml pure netrin-1 protein elicits robust commissural axon outgrowth from E13 dorsal spinal cord explants into collagen (figure 2a). Moreover, aggregates of 293-EBNA $^{\text{netrin-1myc}}$ cells elicit axon turning, indicating that netrin-1 from 293 cells is also active as a chemoattractant (figure 2b). Axon turning, as assessed by measuring the distance of turning and the degree of axon fasciculation, looked best if transfected 293 cells were diluted two times with untransfected cells prior to

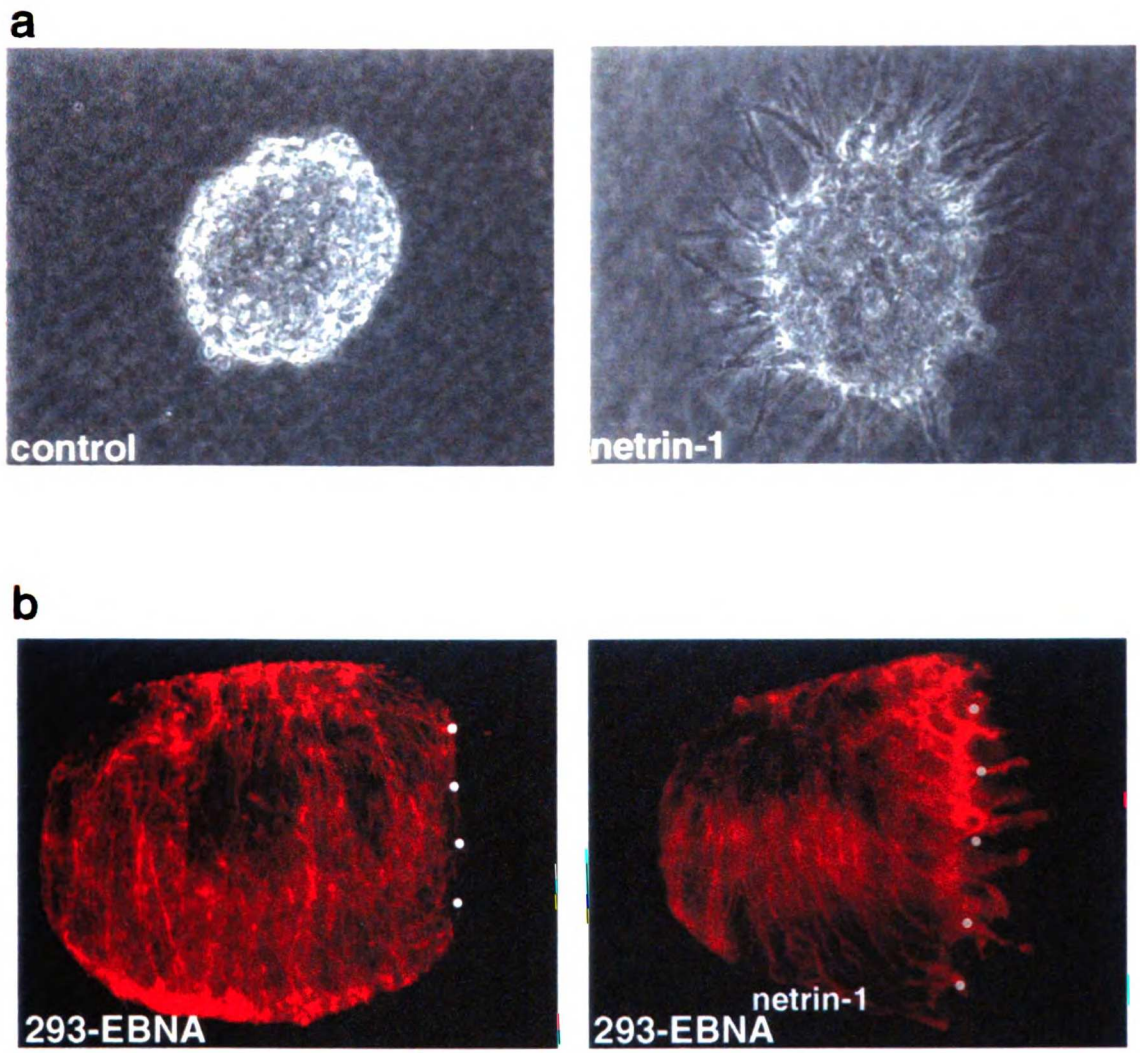


Figure 2. The 293-EBNA netrin-1 stable cell line produces netrin-1 protein which is active in both the outgrowth and turning assays. (a) E13 dorsal spinal cord explants were cultured for 16 hours in a collagen matrix alone (left) or in the presence of 30ng/ml recombinant netrin-1myc protein, purified from the conditioned medium of the 293-EBNA netrin-1 stables (right). (b) E11 dorsal spinal cord explants were co-cultured with aggregates of 293-EBNA cells (left) or with aggregates of 293-EBNA cells stably transfected with netrin-1 but diluted 1:2 with untransfected cells (right). Commissural axons were visualized by indirect immunofluorescence using antibodies to TAG-1. The white dots mark the positions of the cell aggregates.

aggregation, indicating that the large amount of netrin-1 produced by 293-EBNA $^{netrin1\text{-}myc}$ aggregates may be slightly inhibitory for turning.

Cell binding properties of netrin-1

Netrin-1 was purified from 1 M NaCl extracts of membranes from embryonic chick brains (Serafini et al., 1994), indicating that it associates tightly with cell membranes. Since our primary interest is to understand netrin-1's activity in the developing spinal cord, we asked if netrin-1 expressed by floor plate also associates with cell membranes. Indeed, 1M NaCl extracts of floor plate cells contain axon outgrowth activity (Serafini et al., 1994), suggesting that netrin-1 produced by the floor plate probably associates with the cell membranes. Here, we have determined the localization of netrin-1 directly using polyclonal antibodies made to domains VI-V of the recombinant netrin-1 protein (Kennedy et al., 1996). Floor plate and ventral spinal cord were dissected from 40 E13 embryos and conditioned in the absence of serum for two days. The conditioned medium and 1M NaCl extract were then collected, subjected to high speed spin at 100,000 x g to remove membrane associated proteins and analyzed by immunoblotting using anti-netrin-1(VI-V) antibodies. As shown in figure 3, some netrin-1 is in the conditioned medium, but the majority is in the 1 M NaCl extract, indicating that endogenously produced netrin-1 binds strongly to the membranes of floor plate and ventral spinal cord cells.

Netrin-1's strong cell association properties seem, at face value, difficult to reconcile with its chemoattractant activity, and therefore, warranted further study. Since it would be difficult to study the cell binding properties of netrin-1 using floor plate cells given the small amount of floor plate tissue obtained from each dissection, we chose to study netrin-1's binding to cultured cell lines, like COS cells and 293 cells. As with floor plate cells, netrin-1 associates with membranes of COS (Kennedy et al., 1994) and of 293 cells but can be extracted off of cells with 1 M NaCl. While only ~60% of netrin-1 produced in stably transfected 293-EBNA cells associates with membranes, ~90% netrin-1

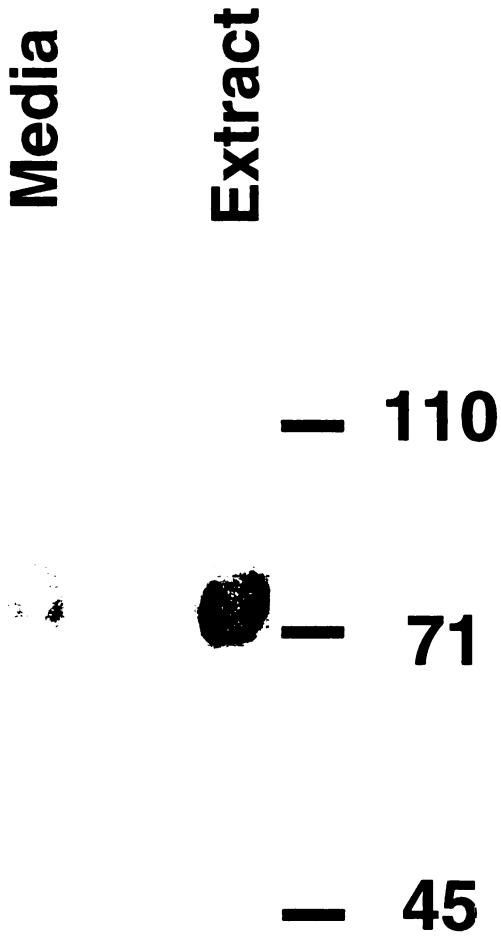


Figure 3. The majority of netrin-1 protein produced by the floor plate and ventral spinal cord associates with the cell membranes. Floor plate and ventral spinal cords were dissected from 40 E13 rat embryos, and cultured for 3 days in vitro. The conditioned medium and 1M NaCl extract were collected, TCA precipitated, and resuspended in SDS sample buffer. 1/10 of the samples were separated on a 6% polyacrylamide gel and subjected to immunoblot analysis using rabbit polyclonal antibodies to netrin-1(VI-V).

associates with membranes of floor plate cells (figure 3) and COS cells (figure 1b). This difference reflects the binding properties of these cell membranes and is unlikely to be due to differences in the concentration or modification of the netrin-1 proteins because netrin-1 protein from 293 cells binds to untransfected COS cells more than to 293 cells (Keino-Masu et al., 1996). Despite this quantitative difference in the binding of netrin-1 to COS and 293 cells, netrin-1 can in all cases by eluted with 1 M NaCl, suggesting that it binds to floor plate cell, COS cells and 293 cells through the same general mechanism.

Netrin-1 from 293 cells exists in equilibrium between cell bound and soluble forms

Despite its strong binding to cell membranes, netrin-1 must be able to diffuse from the cells that express it to function as a chemoattractant. How then is the solubility of netrin-1 regulated? Has soluble netrin-1 protein been post-translationally modified or does one pool of netrin-1 exist in equilibrium between cell associated and soluble forms? To determine whether netrin-1 found in the conditioned medium and cell associated fractions represent different populations of protein, we tested the ability of netrin-1 from the conditioned medium and salt extract of COS cells to associate with membranes of untransfected cells. To do this, both samples were first centrifuged at high speed to remove insoluble proteins, dialyzed to OptiMEM medium, then transferred to tissue culture wells containing equal numbers of untransfected 293 cells. After an overnight incubation, the medium and salt extracts from each well was collected and analyzed. As shown in figure 4, the majority of netrin-1 from both pools remains in the medium (lane 1a and 2a). However, the percentage of netrin-1 which rebinds to cells is the same for netrin-1 from the conditioned medium and netrin-1 from the 1M NaCl extract (the ratio of lanes 1b and 2b is identical to the ratio of lanes 1 and 2). This suggests that netrin-1 in the conditioned medium is not a modified soluble version of cell associated netrin-1. Rather, netrin-1 appears to be biochemically homogeneous and in equilibrium between cell associated and

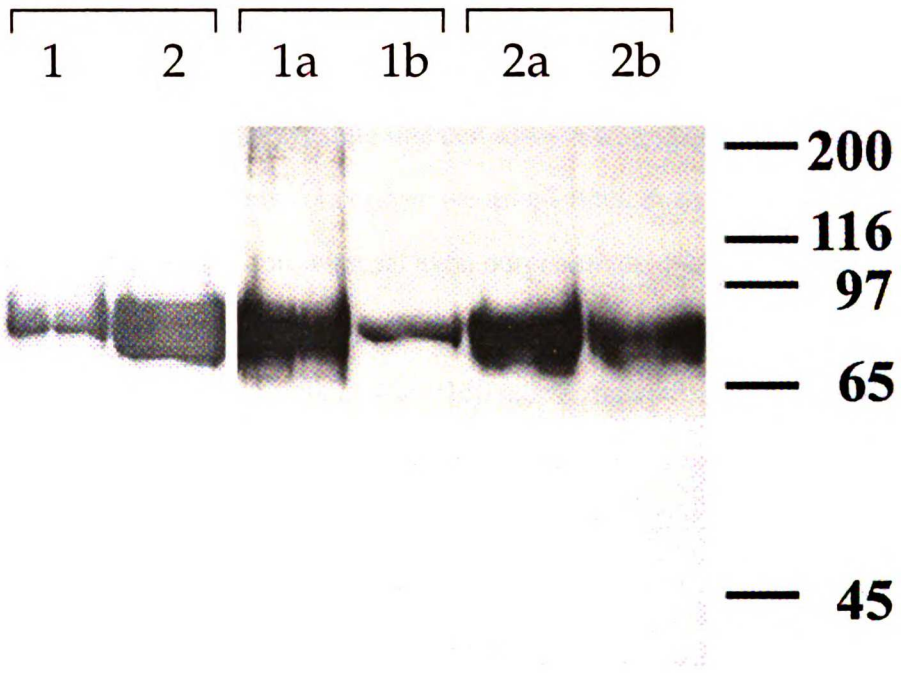


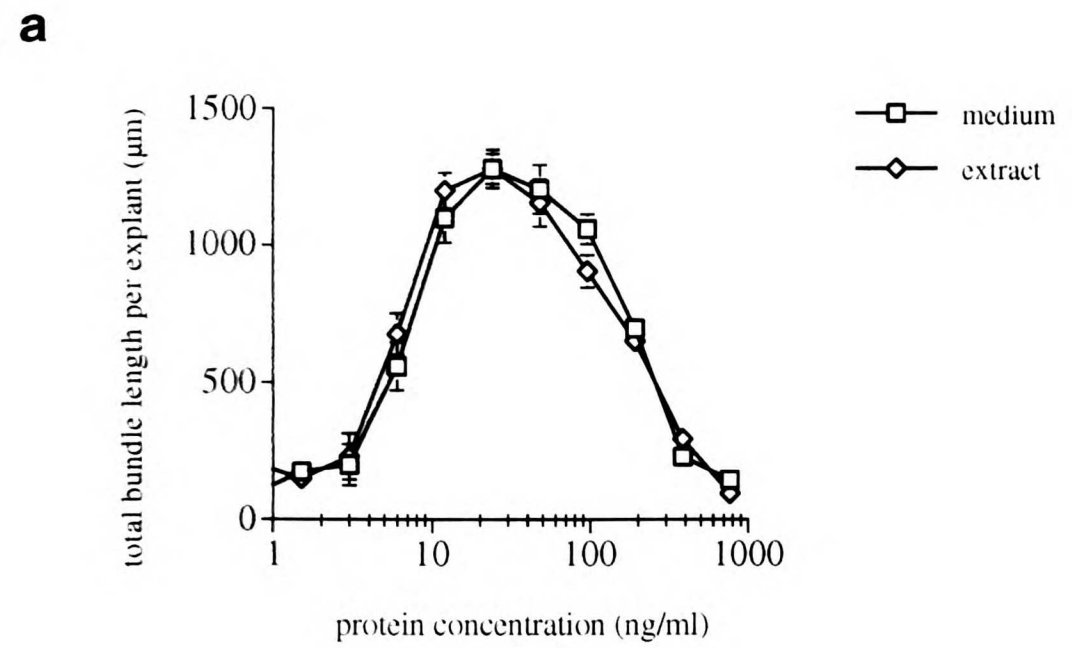
Figure 4. Netrin-1 in the conditioned medium and 1M NaCl extract of transfected COS-1 cells rebinds to untrasfected cells with equivalent efficiency. The conditioned medium and 1M NaCl extract were collected from COS-1 cells transfected with the netrin-1myc gene. Half of these were subjected to high speed centrifugation to generate soluble protein fractions, TCA precipitated, separated on a 7.5% SDS-polyacrylamide gel and subjected to immunoblot analysis using antibodies directed to the myc epitope (the conditioned medium is shown in lane 1, the extract is shown in lane 2). The remaining halves were dialyzed to OptiMEM medium and incubated with untrasfected cells overnight. The supernatants and 1 M extracts from these were collected and processed as described above. Lanes 1a and 1b are the supernatant and extract fractions of lane 1 (the conditioned medium). Lanes 2a and 2b are the supernatant and extract fractions lane 2 (the 1 M NaCl). A shorter exposure of the gel is shown for lanes 1 and 2. The ratio of signal in lanes 1 and 2 and 1b and 2b were quantified using NIH image software.

unassociated states. This conclusion is consistent with there being no size differences between the soluble and cell associated netrin-1 proteins (figure 1b).

The specific activities of cell associated and soluble netrin-1 proteins are identical

The cell associated and soluble forms of netrin-1 do not differ in size or in their ability to associate with cells, suggesting that cell associated and soluble netrin-1 exist in equilibrium. To corroborate this conclusion, we asked whether these cell associated and soluble fractions have similar commissural axon outgrowth promoting activities from E13 dorsal spinal cord explants. The two specific activity curves closely match for these two fractions with the peak of outgrowth at 40ng-160 ng/ml (figure 5a). As shown in figure 5b, 3 ng/ml netrin-1 from the soluble and cell associated fractions of floor plate and ventral spinal cord cells elicit comparable outgrowth from E13 dorsal spinal cord explants, suggesting that the soluble and cell associated fractions of netrin-1 endogenously made in the spinal cord also behave identically. Moreover, since the specific activity curve of recombinant netrin-1 from the conditioned medium was generated using pure netrin-1 protein, we are now certain that the lack of outgrowth at high netrin-1 concentrations (> 1 μg/ml) reflects the actual activity profile of the netrin-1 protein, and is not instead due to an inhibitor present in the conditioned media and extract fractions (Serafini et al., 1994).

Interestingly, while 30 ng/ml recombinant netrin-1 protein is required for maximal outgrowth from E13 dorsal spinal cord explants, robust outgrowth is obtained from the salt extract of floor plate containing 10 ng/ml netrin-1 protein, indicating that floor plate conditioned medium and extract have 3-fold higher specific activities in the E13 outgrowth assay than does recombinant netrin-1 protein. This may reflect differences in activity between the recombinant netrin-1 protein and the endogenous netrin-1 protein. Alternatively, there may be another factor in both the conditioned medium and the extract fractions of floor plate which increase netrin-1's potency. The netrin synergizing activity



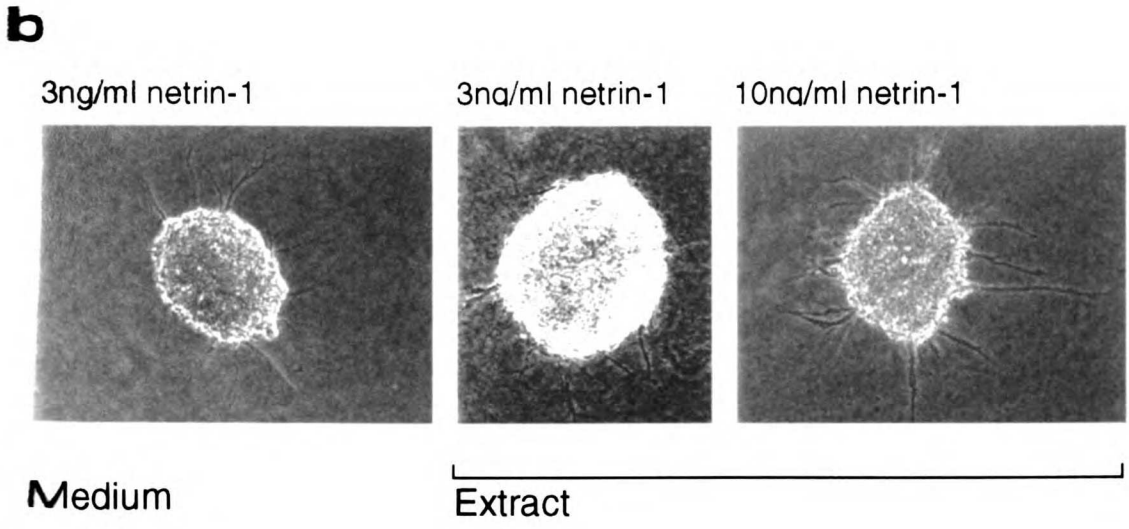


Figure 5. Netrin-1 proteins from the conditioned medium and 1M NaCl extract of stably transfected 293 cells or embyonic floor plate/ventral spinal cord cells have the same specific activity in their ability to elicit axon outgrowth. (a) E13 dorsal spinal cord explants were grown either in the presence of netrin-1 protein purified from the conditioned medium (medium) or in the presence of netrin-1 protein from 1 M NaCl extracts of stably transfected 293 cells (extract). The total length of axon bundles was measured from eight explants per condition. The average outgrowth and standard errors for each are shown. (b) Floor plate and ventral spinal cords were dissected from 40 E13 rat embryos, and cultured for 3 days in vitro. The conditioned medium and 1M NaCl extract were collected, dialyzed separately into F12 medium and assayed in the E13 outgrowth assay at three fold dilutions from 0 .006 µl-200µl. The concentration of netrin-1 present in the samples was determined by immunoblotting with anti-myc antibodies and comparing the intensity of the signal to standards of pure recombinant netrin-1 protein.

(NSA) from embryonic chick brain extracts potentiates netrin-1 elicited outgrowth from E11, but not E13, dorsal spinal cords (Serafini et al., 1994). Thus, if netrin-1 elicited outgrowth in the E13 outgrowth assay is enhanced by a floor plate derived factor, this factor must be different from NSA.

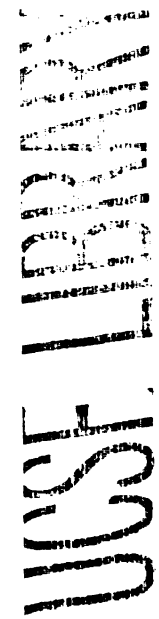
Netrin-1 protein can be extracted from cells by soluble glycosaminoglycans

Netrin-1 protein associates with all cell types tested, suggesting that it binds receptors expressed ubiquitously on cell membranes. It also binds tightly to heparin (Serafini et al., 1994), suggesting that netrin-1 may be associating with proteoglycans on the cell surface. As a first test of this model, we have asked if soluble glycosominoglycans can extract netrin-1 protein bound to the cell surface. Equal numbers of stably transfected 293 netrin-1 cells were grown in 6 well dishes. Upon removing the conditioned medium, the cells were incubated with PBS, 1 M NaCl, 1 μg/ml or 100 μg/ml fucoidin, a heavily sulfated polymer of the sugar fucose, or PBS containing 1 μg/ml or 100 μg/ml of each of the following glycosaminoglycans: heparin, heparan sulfate, chondroitin sulfate A, chondroitin sulfate B (dermatan sulfate), and chondroitin sulfate C. After 30 minutes, extracts were collected and analyzed by immunoblotting with antibodies to the myc epitope. At 100 μg/ml, heparin, heparan sulfate, dermatan sulfate and fucoidin can extract netrin-1 from the cells (figure 6; compare glycosaminoglycan extraction lanes with 1M NaCl lane). However at 1 μg /ml, only heparin (lanes 1) extracts netrin-1 protein associated with the cell membrane of 293 cells. The ability of soluble glycosaminoglycans to extract netrin-1 from cell surfaces suggests that netrin-1 is binding to cells by binding cell-associated proteoglycans. However, we cannot rule out the possibility that netrin-1 binds to receptors other than cell associated proteoglycans, and that glycosaminoglycans inhibit this binding.

Earlier in this chapter, we showed that soluble and cell associated netrin-1 protein do not show differences in specific activity or cell binding. In light of our new finding, we

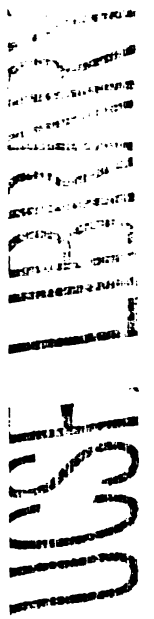
Figure 6. Netrin-1 can be extracted off of 293 cells with soluble glycosaminoglycans. 293-EBNA cells stably transfected with netrin-1myc were grown to near confluence in 35mM dishes. Upon removal of the conditioned medium, the cells were washed with PBS, then incubated for half an hour at 37° C with PBS, 1 M NaCl or with PBS containing 1μg/ml or 100μg/ml heparin (lanes 1), fucoidin sulfate (lanes 2), heparin sulfate (lanes 3), condroitin sulfate B (lane 4), condroitin sulfate A (lane 5) or condroitin sulfate C (lane 5). The mixtures were collected, subjected to high speed centrifugation to remove insoluble protein, TCA precipitated and resuspended in sample buffer. 1/60 of each sample was then separated on an 8% SDS-acrylamide gel and subjected to immunoblot analysis using 9E10 culture supernatants.

compared the heparin affinity of netrin-1 in the conditioned medium with the heparin affinity of netrin-1 from the 1M NaCl extract. Consistent with earlier experiments, netrin-1 from both fractions binds similarly to heparin agarose, eluting roughly at a NaCl concentration of 1.2 M NaCl (personal communication, A. Faynboym). Likewise, netrin-1 in these fractions co-elute on Mono-S at roughly 950 mM NaCl (personal communication, A. Faynboym).



Discussion

Netrin-1 expressed in the ventral half of the spinal cord is required for guiding commissural axons, born in the dorsal spinal cord, to the ventral midline. It does so in two ways. First, commissural axons of netrin-1 -/- mice have difficulty invading the ventral spinal cord, suggesting that netrin-1 is a permissive cue for commissural axons. Second, axons which enter the ventral spinal cord fail to reach the floor plate, suggesting that netrin-1, expressed at high levels in the floor plate, diffuses to form a gradient which can attract axons to the floor plate (Serafini et al., 1996). Netrin-1's role as a chemoattractant for commissural axons is supported by two experiments. First, in the spinal cord of chick embryos, netrin-1 is expressed exclusively in the floor plate. However, in chick, netrin-1 protein extend hundreds of micrometers dorsally, indicating that it does form a gradient within the developing spinal cord (Kennedy et al., 1994; Kennedy et al., 1996). Second, netrin-1 cell aggregates have been shown to elicit turning of commissural axons up to 250 µm away within dorsal spinal cord explants, indicating that netrin-1 can diffuse through the spinal cord to attract chemoattractant in culture (Kennedy et al., 1994).



To determine how netrin-1 gradients are established, we sought to understand how netrin-1 diffuses through a cellular environment. Netrin-1 protein is secreted into the conditioned medium of cultured floor plate cells. However, paradoxically, most netrin-1 protein made endogenously by the floor plate associates tightly with membranes of floor plate and ventral spinal cord cells. On tissue culture cells, netrin-1 seems to exist in an equilibrium between cell associated and soluble forms, suggesting that in the spinal epithelium, netrin-1 protein may continuously rebind to cells as it moves away from the floor plate. This may seem like an unfavorable feature for a protein whose function depends on its ability to diffuse but as we will describe later, cell binding may be important for facilitating the establishment of a far ranging netrin-1 gradient.

Netrin-1 exists in equilibrium between cell associated and soluble states

The difficulty of isolating significant quantities of spinal cord tissue precluded a direct examination of netrin-1 cell binding in the spinal cord. However, netrin-1 binding to COS cells and 293 cells closely mimics netrin-1 binding to floor plate cells. Thus, we studied the diffusion properties of netrin-1 on tissue culture cells with the belief that this may reflect how netrin-1 interacts with cells within the spinal cord.

Netrin-1 in the cell associated and soluble fractions of transiently transfected COS cells bind equally to untransfected cells, suggesting that netrin-1 in the conditioned medium is not a distinct diffusible form. Consistent with this conclusion, cell associated and soluble netrin-1 proteins have similar biochemical properties. Their size on SDS-polyacrylamide gels is the same and they elute off of heparin sepharose and Mono S columns at the same salt concentration. In addition, cell associated and soluble netrin-1 proteins, from both recombinant cells and floor plate cells, have identical specific activities in the E13 assay (i.e. with equal concentrations of each eliciting equal levels of outgrowth). Thus, there seem to be no inherent differences between the soluble and cell associated netrin-1 proteins, suggesting that netrin-1 continually rebinds to cells as it diffuses.

The biological implications of cell binding

These results imply that netrin-1 secreted from the floor plate may continuously reattach to cells in the neural epithelium and slowly inch its way dorsally. While this may seem like an unfavorable feature for a chemoattractant, this type of a buffered diffusion may be one way of generating a far ranging gradient. To illustrate this point, we have used the diffusion equation solved for a continually producing source and an infinite sink[1] to predict the concentration profiles of molecules with different rates of diffusion (Crank, 1975). At steady state (i.e. equilibrium), the diffusion equation predicts that the steepness of the gradient, defined as the percentage of concentration change along a given length, is independent of the diffusion coefficient. However, protein concentration is highly

[1] $C = q / (4\pi Dr)$ where C is protein concentration, D is the diffusion coefficient, q is the rate of protein production and r is distance from the source

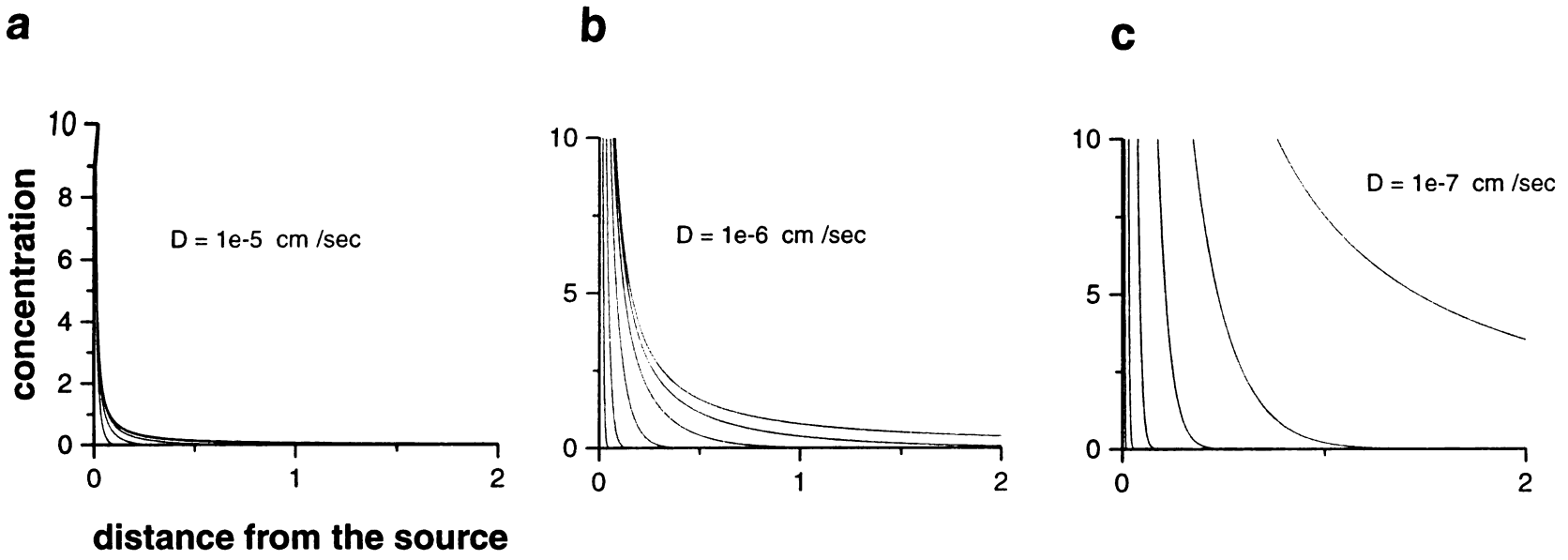


Figure 7. Molecules with small diffusion coefficients (i.e. less diffusible molecules) actually extend greater distances than molecules with large diffusion coefficients once they reach equilibrium. Using the diffusion equation solved for a continually producing source and an infinite sink (Crank, 1975; C= q / [4pDr] where C is protein concentration, D is the diffusion coefficient, q is the rate of protein production and r is distance from the source) we have graphed the concentration profiles of three molecules with different rates of diffusion. The y-axis is the concentration of the molecule and the x-axis is distance of the molecule from the source. The intermediate graphs show the concentrations of each molecule before equilibrium is reached. The molecule of graph (a) has a large diffusion coefficient and can reach equilibrium rapidly. This means that its concentration changes sharply over small distances preventing it from extending far from the source (y-axis). At the other extreme is molecule (c) which has a small diffusion coefficient, and takes a long time to reach equilibrium, but once it does, extends substantially from the source. Molecule b has an intermediate diffusion coefficient.

dependent on the diffusion coefficient. Molecules with large diffusion coefficients change in concentration rapidly over a small distance (figure 7a) and would be unable to function from a far distance, whereas molecules with small diffusion coefficients could ultimately extend far (figure 7c). Obviously, this does not mean that guidance cues which are least soluble generate the most far ranging gradients, since such slow diffusing molecules would also take a longer time to reach steady state.

Therefore, a chemoattractant must diffuse at a rate which can balance these two opposing effects: diffusion must be slow enough so that the gradient extends far but also fast enough, so that the gradient forms within a reasonable time frame. Cell binding may provide the appropriate balance for a far ranging netrin-1 gradient to form rapidly. Furthermore, differences in the number or affinity of cell binding receptors in different tissues may modulate the range of the netrin-1 protein. For example, unlike the long-range effects of netrin-1 in the spinal cord, netrin-1 seems to act as a short range guidance cue in the developing eye because retinal ganglion axons in netrin-1 -/- mice grow near to the optic nerve, where netrin-1 is expressed, even though they fail to enter it (Deiner et al., 1997). While retinal ganglion axons turn towards gradients of netrin-1 *in vitro*, a netrin-1 gradient emanating from the optic nerve is probably short ranged because Deiner et al. (1997) were unable to visualize such a gradient. It has been suggested that the failure of a long range gradient of netrin-1 to form in the eye is due to strong netrin-1 binding to cells of the optic nerve which prevent netrin-1 from diffusing (Deiner et al., 1997). We suggest an alternative possibility: the failure of a long range gradient of netrin-1 to form may be due to the *absence* of cell binding receptors. Without cell binding to buffer its diffusion, netrin-1 would diffuse too quickly and the netrin-1 gradient would be unable to extend far. In chapter IV, we describe a mutant of netrin-1, netrin-1(VI-V), which is active in the turning assay but has been deleted of its tight cell binding domain, C. As predicted by our model, netrin-1 (VI-V) elicits turning at a shorter distance than does the full length netrin-1 protein.

Proteoglycans may mediate netrin-1 binding to cells

Clearly, understanding how cell binding affects diffusion of netrin-1 within the spinal cord, requires the identification of the low affinity cell surface receptors which bind to netrin-1. As a first step, we have shown that free gycosaminoglycans can extract netrin-1 protein off of cell membranes. Of the glycosaminoglycans tested, heparan sulfate and dermatan sulfate but not chondroitin sulfate A and C, can extract netrin-1 at 100 μg/ml. However, at 1 μg/ml, only heparin is able to extract the netrin-1 protein. Heparin differs from heparan sulfate in its extent of sulfation, suggesting that an increased charge density may facilitate netrin-1 extraction. However, fucoidin, a highly sulfated sugar, failed to extract netrin-1 off of cells more efficiently than heparin, indicating that charge alone does not determine the efficacy of extraction. Because heparin extracts netrin-1 protein most efficiently from cell membranes, it is possible that netrin-1 binds to heparan sulfate proteoglycans. To extend these studies, it will be important to test whether netrin-1 can associate with cells deficient in proteoglycan synthesis. These cells have been used successfully to verify that FGF, amongst other proteins, binds to cell associated proteoglycans (Spivak-Kroizman et al., 1994).

The fact that proteoglycans are present ubiquitously on the surface of cells may explain why netrin-1 associates with so many cell types. Furthermore, it is possible that differences in the affinity of netrin-1 for 293 cells and COS cells may be due to differences in the numbers and types of proteoglycans expressed. This conclusion is consistent with studies by Lindsay Hinck and David Leonardo in our laboratory, that identified a cDNA encoding a glucosaminyl N-deacetylase/N sulfotransferase (GNDNS) that, when transfected into COS cells, increases netrin-1 binding to these cells. GNDNS in 293 cells is known to increase the level of sulfation on cell surface heparin sulfate proteoglycans (Cheung et al., 1996), suggesting that the increased binding seen in the GNDNS transfected cells is due to increased sulfation levels of endogenous netrin-1 binding proteoglycans.

Proteoglycan binding: a general mechanism for modulating the diffusion rate of long range signals

Proteoglycan mediated diffusion as a means to establish long range gradients may not be used exclusively by netrin-1. Heparin binding is a common property of all long range axon guidance proteins identified thus far. Hepatocyte Growth Factor (HGF) which is expressed in the developing limb bud and functions chemotropically to attract spinal motor axons, is also a strong heparin binding protein (elutes off of heparin column at 1.1M NaCl) (Ebens et al., 1996). Semaphorin III, which functions as a chemorepellent for NGF responsive sensory axons, also binds tightly to heparin (eluting at 1.1 M NaCl) (Luo et al., 1993). This commonality suggests that proteoglycans may play a general role in defining the diffusion rate of chemotropic cues and provides one reason why proteoglycans have been implicated in axon guidance (Brittis et al., 1992; Wang and Denburg, 1992; Walz et al., 1997). Furthermore, these results suggest that small heparin binding proteins, such as KAL1, implicated in guiding olfactory axons may also be capable of functioning at a distance (Franco et al., 1991; Legouis et al., 1991; Soussi-Yanicostas et al., 1996).

Aside from a role in axon guidance, proteoglycans may also be involved in establishing stable long range gradients of other inductive developmental signals. For example, branchless, a member of the heparin binding FGF family, apparently forms a gradient to specify the position of tracheal branch formation in the developing *Drosophila* lung (Sutherland et al., 1996). Wingless, a member of the wnt family of diffusible morphogens involved in axis formation binds tightly to heparin (Reichsman et al., 1996). Finally, two TGFβ family members, activin which induces formation of dorsal mesoderm, and DPP which causes ventralization, form long range gradients and bind to heparin (Jones et al., 1996). These observations suggest a novel role for proteoglycans during development. Proteoglycan binding has, in general, been thought to limit a protein's ability to diffuse. Our results with netrin-1 open the possibility that by decreasing the diffusion

rate of proteins, proteoglycans may actually facilitate the establishment of far ranging gradients.

Methods and Materials

Spinal cord explant culture and Immunohistochemistry

Outgrowth assays using E13 rat dorsal spinal cord (E0 is the day of vaginal plug) were done as described in (Serafini et al., 1994). Embryonic explants were cultured in 64% OptiMEM-1 (GIBCO BRL), 25% F12, 40 mM glucose, 2 mM Glutamax-1, 100 µg/ml streptomycin sulfate and 100 µg/ml penicillin G.

Production of the 293-EBNA $^{netrin\text{-}1myc}$ stables

Netrin-1^{myc} from pGNET^{myc} (Serafini et al., 1994) was first transferred to pcDNA3 (Invitrogen) by digestion with EcoRI and XhoI, then to pCEP4 (Invitrogen) by digestion with HindIII and XhoI. This construct was introduced into 293-EBNA cells (Invitrogen) using LipofectAMINE (GIBCO BRL) as directed and grown under selection (250 µg/ml Geneticin, 200µg/ml hygromycin B). To prevent loss of the extrachromosomal episomes, the transfected cells were continually maintained in selective media.

Production of netrin-1^{myc} in tissue culture cells

A COS-1 cell monolayer was transiently transfected in 35mm wells with pGNET^{myc} as described previously (Kennedy et al., 1994). Upon removal of the transfection media, the monolayer was washed with PBS, then cultured for 3 days in 1.5 ml of OptiMEM I supplemented with GlutaMAX I (GIBCO BRL) and antibiotics. In parallel, netrin-1 stably transfected 293-EBNA cells were grown to confluence in 35mm wells, washed with PBS, then cultured for 3 days with 1.5 ml of OptiMEM I, GlutaMAX I, and antibiotics. After harvesting the conditioned media from both cell types to which 1 x protease inhibitors were added (1 µg/ml each of aprotinin, leupeptin and pepstatin), the cells were rinsed with PBS, then incubated for 30 min with 1.5 ml of extraction buffer (1

M NaCl, 20mM NaPi [pH7.5], 1x protease inhibitors). Media and extracts were centrifuged at 2000 x g for 10 minutes, then at 60,000 x g for 100 min. The supernatants were TCA precipitated as described (Kennedy et al., 1994), then resolubilized in SDS sample buffer. Half of the protein from transiently transfected COS-1 cells expressing netrin-1 and 1/20 of the protein from 293-EBNA cells stably expressing netrin-1 were subjected to western blot analysis using 9E10 culture supernatant.

Cell binding of netrin-1 from the conditioned medium and 1 M NaCl extract

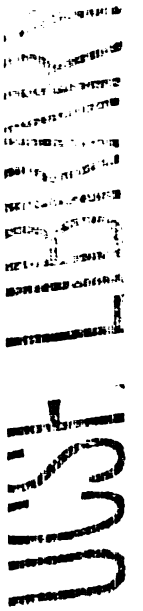
Conditioned medium and extract (2 mls each) from COS cells transfected with pGNET^{myc} were collected and centrifuged as described above, then dialyzed into F12 medium. 1 ml of each fraction was DOC-TCA precipitated and resuspended in sample buffer. The NaCl concentration of the remaining 1 ml of the medium was increased to 1M, and together with the extract fraction, was dialyzed to F12. Following dialysis, the medium and extract fractions were incubated overnight with untransfected COS cells which had been plated at equal densities in 35 mm dishes (2 x 10^5cells/well). The conditioned medium and extract from each of these wells were then collected, centrifuged as before, TCA precipitated, then resuspended in sample buffer. Equal volumes of each sample were separated on a 7.5% SDS-acrylamide gel and subjected to Western blot analysis using 9E10 antibodies directed against the myc epitope.

Production of netrin-1 protein in floor plate cells

Floor plate and ventral spinal cord tissue were dissected from 40 E13 embryos in L15 medium, and cultured for 3 days in 1 ml OptiMEM-I containing 40 mM glucose, GlutaMAX-I and antibiotics. After harvesting the conditioned medium (to which 1 x Complete protease inhibitors cocktail [Boehringer Mannheim] was added), the floor plate and ventral spinal cord tissues were washed with 1 ml PBS, then incubated for 30 minutes in 1 ml extraction buffer (1.1M NaCl in 20mM NaPi pH7.5 + 1x "Complete protease

inhibitor cocktail"). The medium and extract were then centrifuged at 2000 x g for 10 minutes, then at 60,000 x g for 100 min.

0.5ml of the conditioned medium and extract were DOC-TCA precipitated (Serafini et al., 1994) and resuspended in SDS sample buffer. 1/5 of the samples were separated on a 6% SDS-acrylamide gel and subjected to Western analysis using rabbit anti-netrin-1 antibodies (1:1000) (Kennedy et al., 1996). The netrin-1 concentration in these samples was determined by comparing the intensity of these signals with standards of pure netrin-1 protein (ranging from 0.1ng/ml to 20 ng/ml at two fold dilutions). The remainder of the conditioned medium and extract were dialyzed into F12 medium (GIBCO BRL) and assayed at three fold dilutions from 0.006 µl-200µl.



Large scale production of the netrin-myc protein in 239-EBNA$^{netrin\text{-}1myc}$ cells

293-EBNA $^{net\text{-}myc}$ cells were grown to confluence in fifty 150mm plates. Each plate was washed twice with 10 mls of PBS and conditioned for 10 days in 18 mls of OptiMEM medium (GIBCO BRL) containing GlutaMAX-1 (GIBCO BRL) and antibiotics. Conditioned medium and extract were collected and centrifuged as described above. The extract (1 L) was concentrated 10-fold over a centricon-30 concentrator (Amicon), aliquoted and quick frozen in liquid nitrogen. The medium was used as a source for purification of the netrin-1 ^{myc} protein (see below).

Purification of netrin-myc from the conditioned medium of 239-EBNA$^{netrin\text{-}1myc}$ cells

One liter of netrin-myc conditioned medium was filtered through a 0.2µm filtration unit (Nalgene) and loaded at a rate of 0.5ml/minute onto a 1 ml heparin Hytrap column (Pharmacia), previously equilibrated in buffer A (20mM NaPi [pH7.5], 150mM NaCl). The column was washed first with 20% Buffer B (20mM NaPi [pH 7.5], 2M NaCl), then

with 40% buffer B, until UV absorption leveled (~30 mls for each). Buffer B was then raised to 45% over 6 column volumes. The column was eluted with a linear gradient from 45% B to 65% B over 15 column volumes, and 1ml fractions were collected into 1.5 ml siliconized polypropylene tubes (PGC Scientifics). 10μL of each fraction was subjected to western-blot analysis (Serafini et al., 1994) using the 9E10 antibody. Netrin-1 was pooled into peak and side fractions (~7 mls each), then concentrated 10-20 fold over a centriprep-30 filtration apparatus (Amicon), aliquoted and snap frozen in liquid nitrogen. This eluate could also be stored at -20 for up to a year in netrin-1 storage buffer (50% glycerol, 1 M NaCl, 20mM NaPi [pH7.5]).

To purify netrin-1 to homogeneity, the netrin-1 eluate from the heparin column was diluted 2 fold in buffer C (20mM NaPi [pH7.7]), then loaded at a rate of 0.5ml/min onto a mono-S column (HR 5/5; Pharmacia), pre-equilibrated with buffer C. The column was first washed with 30% buffer D (20mM NaPi [pH7.7], 2M NaCl), then with 35% D until the UV absorption leveled. Finally, the column was eluted with a linear gradient from 35% D to 60% D over 15 column volumes. Netrin-1 protein eluted roughly at 950mM NaCl.

Extraction of netrin-1 bound to 293 cells with glycosaminoglycans

293-EBNA ^{netrin1myc} cells were grown in 35mm dishes to near confluence (8 x 10^5 cells). The conditioned medium was removed, the cells were washed with PBS, then incubated for half an hour at 37°C with 1 ml of each of the following: 1 M NaCl, PBS, or PBS containing heparin (Calbiochem), heparin sulfate, chondroitin sulfate A, B, C or fucoidin (at 1μg/ml and 100 μg/ml). All glycosaminoglycans, except for heparin, were purchased from Sigma. The mixtures were removed, protease inhibitors were added to 1x, centrifuged as described previously, DOC-TCA precipitated and resuspended in sample buffer. 1/60 of each sample was then separated on a 8% SDS-acrylamide gel and subjected to Western blot analysis using 9E10 culture supernatants (recognizing the myc epitope).

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Chapter IV

Structure-function analysis of netrin-1 as a means to probe the mechanism of commissural axon outgrowth activity

Abstract

Netrin-1 functions as an instructive and permissive cue for commissural axons of the developing spinal cord. To study how netrin-1 functions permissively, we turned to the *in vitro* "outgrowth" assay, where netrin-1 elicits growth of commissural axons from dorsal spinal cord explants into collagen. Using deletion mutants of netrin-1, we investigated the importance of DCC and heparin binding for outgrowth. Our results show that domain C is netrin-1's tightest heparin binding domain and that domain v3 is required neither for DCC binding or for outgrowth. One mutant, netrin-1(VI-V), binds tightly to DCC but does not elicit axon outgrowth when presented uniformly in the medium; dimers of netrin-1(VI-V) do elicit outgrowth. These results indicate that DCC binding is not sufficient for outgrowth and suggest that domain C may itself be a dimerization domain. While domain C does not seem to dimerizes netrin-1 directly, we show that the heparin binding domain of semaphorin III can partially replace netrin-1(C) suggesting that domain C may mediate dimerization indirectly by first binding to HSPGs.

Introduction

Netrins are a multifunctional guidance cue involved in directing the growth of vertebrate retinal ganglion axons, motor axons, and commissural axons during development (Mitchell et al., 1996; Serafini et al., 1996; Deiner et al., 1997). A netrin was originally identified in *C. elegans* as a secreted laminin related factor (Ishii et al., 1992) limited in expression to the ventral midline (Wadsworth et al., 1996) but required for both dorsal and ventral circumferential migrations (Hedgecock et al., 1990). Independently, netrin-1 and netrin-2 were isolated in vertebrates. Using *in vitro* assays, netrin-1 was shown to function as a bifunctional chemotropic guidance cue, capable of attracting ventrally directed spinal commissural axons while repelling dorsally directed trochlear motor axons (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995). Whether axons are attracted or repelled by the netrins seems to be due, in part, to the types of receptors they express.

Two classes of netrin-1 receptors have been identified, homologues of the UNC-40 gene, a member of the Ig superfamily, and homologues of the nematode UNC-5 gene, comprised of two immunoglobulin like domains and two thrombospondin type-1 repeats. Genetic studies in *C. elegans* suggest that UNC-5 is a repellent receptor because UNC-5 is required for cells migrating away from the netrin UNC-6 and because ectopic expression of UNC-5 in longitudinally migrating touch neurons causes them to change trajectory away from UNC-6 (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Hamelin et al., 1993). In contrast, UNC-40 is predominantly required in cells growing towards UNC-6, but does not seem to act solely as a receptor for attraction because UNC-40 on its own is not sufficient for attraction (i.e. it is expressed in netrin unresponsive cells) and is sometimes required in cells which migrate away from UNC-6 (Hedgecock et al., 1990; Keino-Masu et al., 1996). In vertebrates, the functions of the UNC-5 homologues are still poorly understood and it is unclear whether UNC-5 proteins mediate the repulsion of trochlear motor axons from netrin-1. In contrast, the function of the vertebrate homologue

to the UNC-40 gene, DCC, is better understood and seems to be intimately linked to the functions of netrin-1. We known this because mice homozygous mutant for the DCC gene exhibit defects which are strikingly similar to those of netrin-1 -/- mice (Serafini et al., 1996; Fazeli et al., 1997).

One such defect is within the developing spinal cord. Commissural neurons, which express the DCC receptor, normally project axons from the dorsal spinal cord to floor plate cells at the ventral midline. However, in DCC and netrin-1 null mice, most commissural axons fail to enter the ventral half of the spinal cord. In netrin-1 null mice, where this inhibitory phenotype is slightly less penetrant, a few axons do grow into the ventral spinal cord but are misdirected and have difficulty reaching the floor plate. These results suggest that netrin-1 acts as a permissive cue, by allowing commissural axons entry into the ventral spinal cord, and as an instructive cue by directing commissural axons to the floor plate. Furthermore, the similarities of the netrin-1 and DCC null embryos suggest that DCC is the receptor or a component of a receptor complex required for a response to netrin-1's permissive and perhaps instructive signals (Serafini et al., 1996; Fazeli et al., 1997).

It has been possible to study a permissive activity of netrin-1 for commissural axons *in vitro*. This assay, known as the "outgrowth" assay, has allowed us to probe the molecular mechanism by which commissural axons may respond to netrin-1 *in vivo*. In this assay E11 or E13 dorsal spinal cord explants containing commissural neurons are dissected and cultured within a collagen matrix. Commissural axons (which at these stages express the cellular marker TAG-1) grow normally within the explant but do not enter the collagen. However, netrin-1, present solubly in the culture media allows axons to grow profusely into the collagen (Serafini et al., 1994). What is known about netrin-1 elicited outgrowth? First, commissural axons from E13 dorsal spinal cord explants respond with approximately 10-fold higher sensitivity than axons from E11 explants, but the sensitivity of E11 axons can be enhanced by a second activity from extracts of embryonic chick brains, suggesting that the netrin-1 outgrowth activity can be modulated by other factors.

Second, outgrowth is inhibited by antibodies against DCC, suggesting that DCC receptor activation is required for the response of commissural axons (Keino-Masu et al., 1996). Finally, heparin potentiates netrin-1 elicited axon outgrowth, suggesting that heparan sulfate receptors on commissural axons may be involved in the transduction of the netrin-1 signal (M. Galko, personal communication). This would not be surprising since many heparin binding growth factors, such as FGF, HGF and heparin binding EGF (hbEGF), associate with proteoglycans to bind and activate their high affinity receptors (Aviezer and Yayon, 1994; Spivak-Kroizman et al., 1994; Herr et al., 1997; Sakata et al.,).

We have used a structure-function approach to probe the role of DCC and heparin in the commissural axon outgrowth response. Our aim has been to identify mutations within netrin-1 which selectively disrupt DCC and heparin binding and ask if these correlate with defects in axon outgrowth. The amino acid sequence of netrin-1 suggests that it is comprised of five separate domains: domain VI, three EGF domains (domain V) and a C terminal basic domain. Domains VI and V are homologous to the N-terminus of the laminin B2 chain which form a globular structure and rigid rod like structure by rotary shadow electron microscopy (Beck et al., 1990). Domain C of netrin-1 is not found in laminin but shares strong homology with other netrins and with the C terminus of Frzb, an antagonist of the wnt receptor (Leyns et al., 1997; Wang et al., 1997). Modular structures within proteins often represent functional units. Thus, we began our structure-function studies by making deletions of netrin-1's domains and were optimistic that these netrin-1 mutants would exhibit specific defects since an in-frame deletion of domain v2 in UNC-6 specifically disrupts the migration of dorsally directed cells in nematodes.

The mutants generated here should be useful not only in studying commissural neurons, but also in exploring netrin-1's affects in other neurons, such as retinal ganglion cells and trochlear motor neurons. Moreover, as more netrin-1 receptors are identified, the deletion mutants may provide a means to probe the interaction of these receptors with one-another and with the DCC receptor.

Results

Construction of the netrin-1 deletions

Netrin-1 is comprised of five structural domains, an N-terminal domain VI followed by three EGF repeats in domain V, and a C terminal basic domain. To generate deletions within the netrin-1 gene for structure-function studies, each domain (VI, V, C) was amplified by PCR and cloned into an expression vector (pCM39n) flanked 5' with the netrin-1 untranslated sequence, sequence encoding for the netrin-1 signal peptide, and sequence encoding the first 3 amino acids of the mature netrin-1 protein. At the 3' end, each amplified fragment was flanked by the myc epitope followed by a translational stop codon. The individual EGF repeats were also amplified by PCR but were cloned into a vector already containing domains VI and C (pCM54).

To facilitate subcloning of the domains, synthetic restriction sites were added to the primers (Xho I at the 5' primer and Sal I at the 3' primer), and cloned into the Sal I site of pCM39n and pCM54 (see methods and materials). This was possible because digestion with Sal I generates sticky ends compatible with both Xho I and Sal I digested DNA. Once inserted, a Sal/Xho fusion was generated at the 5' end of the fragment and could no longer be digested with either enzyme, but a new Sal I site was generated at the 3' end, allowing a second PCR fragment to be added. Thus, domains could be linked sequentially in any order and number desired.

One drawback of this approach is that the addition of restriction sites at the junction of each domain introduced changes into the netrin-1 sequence. We minimized changes which could interfere with netrin-1's activities by designing the PCR junctions in nonconserved areas of the netrin-1 gene. This meant that the amplified fragment did not exactly match the domains predicted by netrin-1's amino acid sequence (see figure 1). However, in all but one case, the junctions of the amplified domains were within 5 amino acids of the actual junctions. To determine if the mutations we introduced affected the bioactivities of netrin-1, we reconstituted the full length netrin-1 gene by sequentially

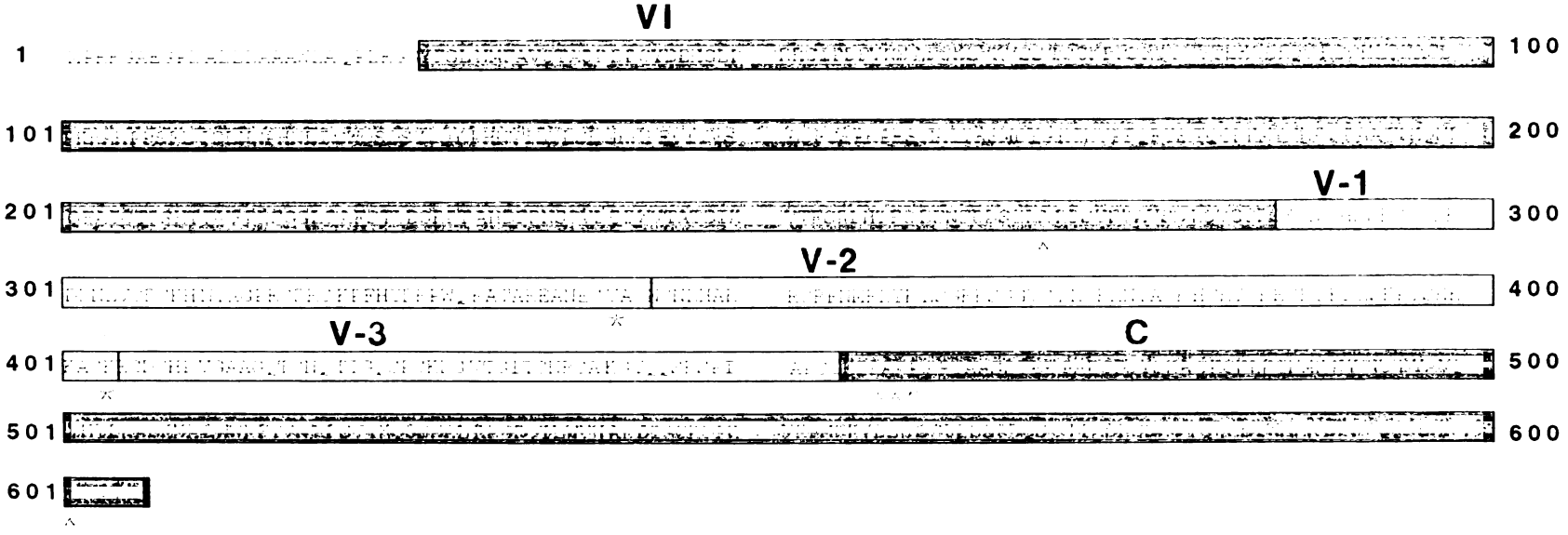

Figure 1. The amplified domains of netrin-1 match approximately with netrin-1's actual domains. The predicted domains within netrin-1's amino acid sequence are boxed (domain VI, green; domain V, orange; domain C, blue). The amino acid junctions of the domains we amplified by PCR are shown in red, as are the mutations introduced into the netrin-1 sequence (marked by *).

linking domains VI, V and C. This reconstituted netrin-1 gene was expressed as efficiently as the wild type netrin-1 gene and was indistinguishable from wild type netrin-1 in its ability to elicit axon outgrowth and to function as a chemoattractant (figure 2). This control simplified our interpretations. For example, we could be certain that the defects in domains VI-V (described later in this chapter and in chapter V) are due to the absence of domain C, and not due to the mutations at the junction of domain VI and domain V. Unfortunately, we have not yet linked all three EGF domains to reconstitute the netrin-1 protein, and thus had to be more cautious in interpreting these experiments.

Expression of the netrin-1 deletion constructs

Prior to characterizing the activities of the netrin-1 deletions, we had to verify that sufficient levels of each protein were produced. Each of the constructs was transiently transfected into COS-1 cells and conditioned media and 1 M NaCl extracts were analyzed by immunoblot analysis (figure 3). Using recombinant netrin-1 ^{myc} protein of known concentration as a standard, the total expression levels of each domain was measured and is shown below the gels. Except for netrin-1(Δv-1), all constructs are expressed at a concentration greater than 30 ng/ml. However, expression levels vary with domains VI, VI-V and VI-C expressed the most (3-10 fold greater than netrin-1). The concentration of the low expressing clones was enhanced ~3 fold by generating extrachromosomal stable cell lines in 293-EBNA cells but netrin-1(Δv-1) expression was still undetectable. Thus, using a combination of these two techniques, transient expression in COS-1 cells and stable expression in 293-EBNA cells, all but one of the deletion constructs were expressed at high enough concentration to be tested in binding studies and in the E13 outgrowth assay.

Domain C is netrin-1's cell binding domain

As discussed in chapter III, netrin-1 binds tightly to the surface of cells, but binding can be disrupted with 1 M NaCl (Kennedy et al., 1994). To identify the domain within

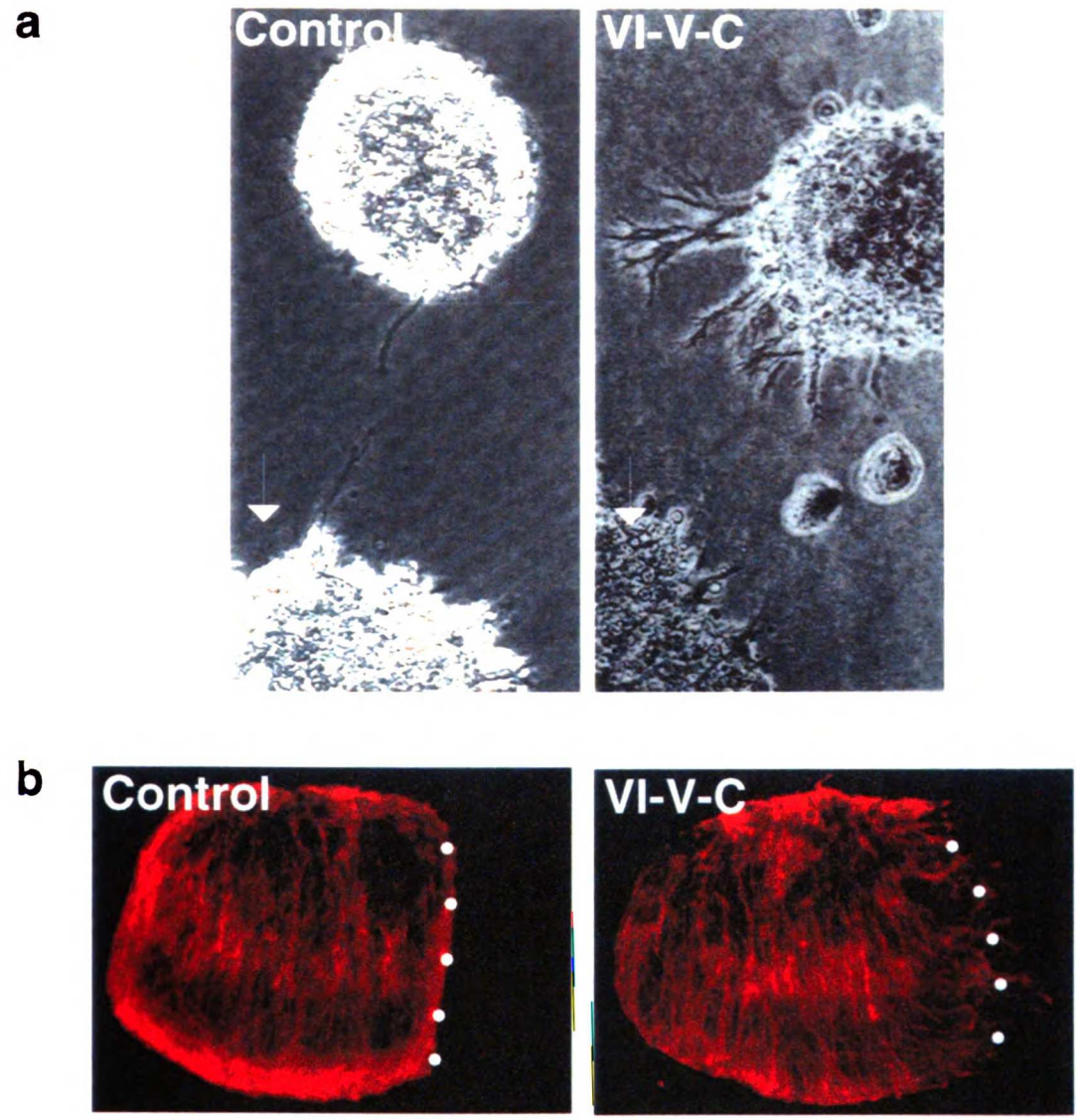

Figure 2. Reconstituted netrin-1 protein elicits commissural axon outgrowth and turning. COS-1 cells were transfected with the parental expression plasmid pCM39n or with pCM39n containing netrin-1 domains VI, V, and C which have been amplified by PCR and linked together as described in the text. In panel (a), the transfected cells were aggregated (arrow), and co-cultured with E13 dorsal spinal cord explants within collagen gels. Cells transfected with the control plasmid elicit little axon outgrowth, whereas the cells transfected with the reconstituted netrin-1 gene elicit outgrowth of fasciculated axon bundles into collagen. In panel (b), the trasfected cell aggregates were cocultured along the cut edge (white dots) of E11 dorsal spinal cord explants. The trajectory of the commissural axons within the explant was visualized by indirect immunofluorescence using antibodies directed to TAG-1. Aggregates of control cells have no affect on the growth of commissural axons. In contrast, aggregates of cells transfected with the reconstituted netrin-1 gene cause commissural axons to reorient.

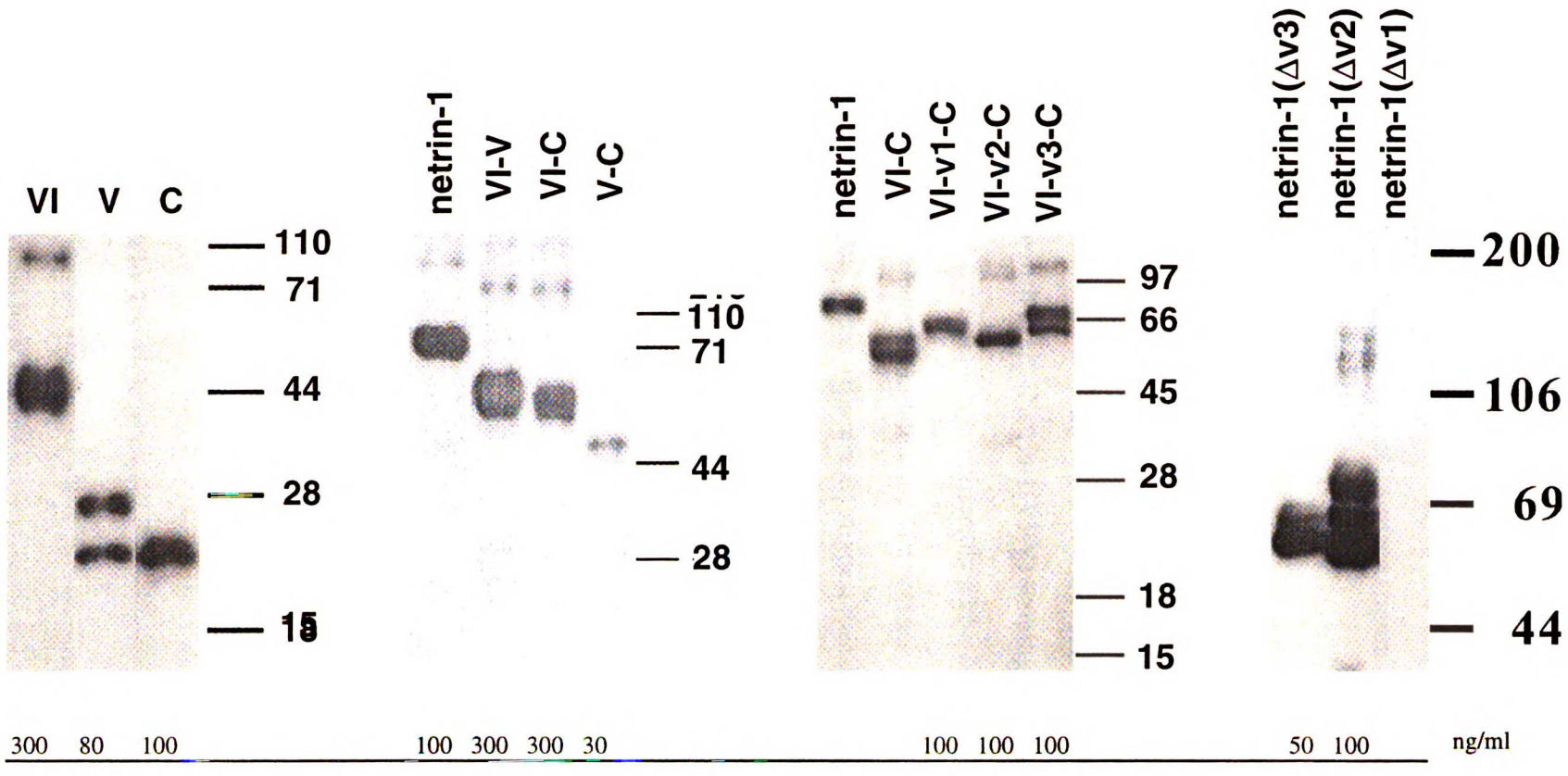

Figure 3. Protein expression of the netrin-1 deletion constructs. The netrin-1 deletion constructs were trasfected into COS-1 cells and conditioned in OptiMEM medium for 3 days. After the media was collected, the cells were extracted with 1 M NaCl. These were TCA precipitated, resuspended in SDS sample buffer, separated on SDS polyacrylamide gels, and subjected to immunoblot analysis using 9E10 monoclonal antibodies directed to the myc epitope. The extract fractions are shown for all the samples except domain VI (here, the medium fraction is shown). Different volumes of each sample were loaded onto the gels, so that each would be within the range of detection. In parallel experiments, we quantitated the total level of protein production by comparing the intensity of the signal to recombinant netrin-1 protein of known concentration (below the gels).

netrin-1 which mediates cell binding, domains VI, V, and C were transiently expressed in COS-1 cells, and protein distribution in the conditioned media and 1 M NaCl extracts was determined by immunoblot analysis. Domain VI is exclusively in the conditioned medium, indicating that it does not bind to the cell surface (figure 4a). In contrast, domains V and C partially partition in the 1 M NaCl extract. Of these, only domain C localizes to the cell membrane by indirect immunofluorescence (figure 4b). In contrast, the distribution of domain V looks "artificial" and is present even in areas of the dish containing no cells (compare domain V staining with DAPI staining), indicating that it binds to something other than cell membranes, possibly to a component of serum used to block the tissue culture dish or to the dish itself. Netrin-1(VI-V) staining also looks artificial (not shown), even though this deletion mutant is conditionally active in outgrowth (as we shall see later in this chapter and in chapter V), indicating that domain V is not behaving anomalously when expressed alone. These experiments suggest that domain C is netrin-1's cell binding domain. Furthermore, VI-C binds to the cell surface, indicating that domain C is able to tether a soluble domain, VI, to cells (figure 4a).

As one would expect from these results, domain C's cell binding properties are similar to those of the full length netrin-1 protein. Specifically, the ratio of domain C in the conditioned medium and in the 1 M NaCl extract is identical to the ratio of netrin-1 in these fractions (figure 4a for domain C). In addition, as shown in the indirect immunofluorescence panels (figure 4b), full length netrin-1 and netrin-1(C) proteins associate with the surface of nearly all the cells present, even though only 10-20% of these have been transiently transfected (preliminary experiments, not shown), suggesting that netrin-1(C), like netrin-1, is able to diffuse from one cell onto another.

Domain C is netrin-1's tightest heparin binding domain

Heparin modulates the function of many growth factors and extracellular matrix proteins. To understand how heparin is involved in the outgrowth response, we identified

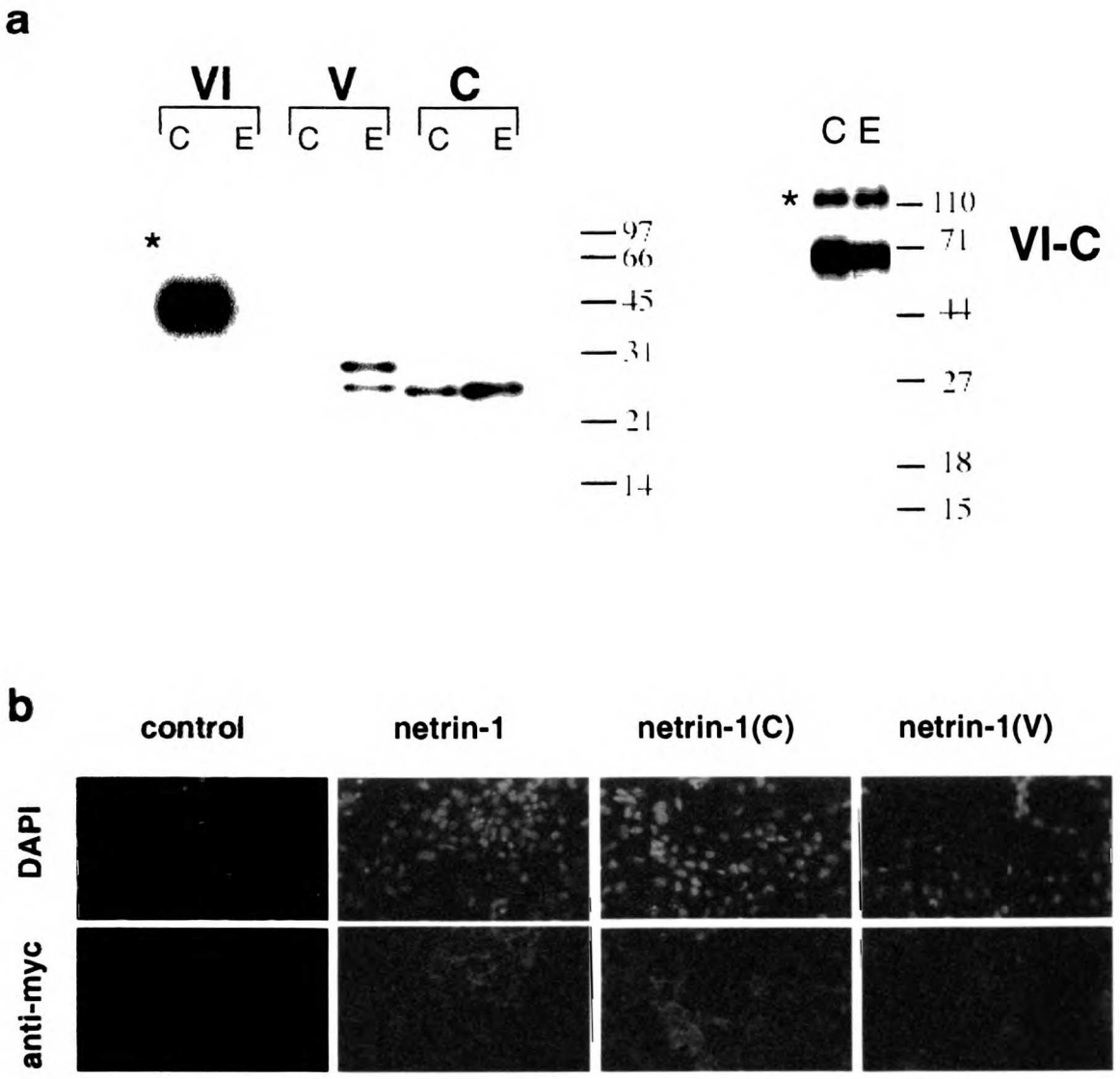

Figure 4. The cell binding properties of the netrin-1 domains. (a) An immunoblot of the conditioned medium (C) and 1 M NaCl extract (E) of COS cells transfected with domains VI, V, C or VI-C. Primary antibodies were directed to the myc epitope at the C-terminus of each construct. Netrin-1 deletions containing domain VI migrate predominantly as monomers, but doublets exist and have been marked with an astrix. (b) Indirect immunofluorescence of untransfected COS cells or COS cells transfected with netrin-1, netrin-1(C), or netrin-1(V) using antibodies directed to the myc epitope (mab 9E10). The cells were incubated with mab 9E10 prior to fixation so that only secreted netrin-1 protein would become labeled. To mark the positions of the cells, nuclei were stained with DAPI and are shown in the top panels. Only 10-20% of the cells become transfected (not shown) whereas netrin-1 and domain C bind to the surface of almost all the cells.

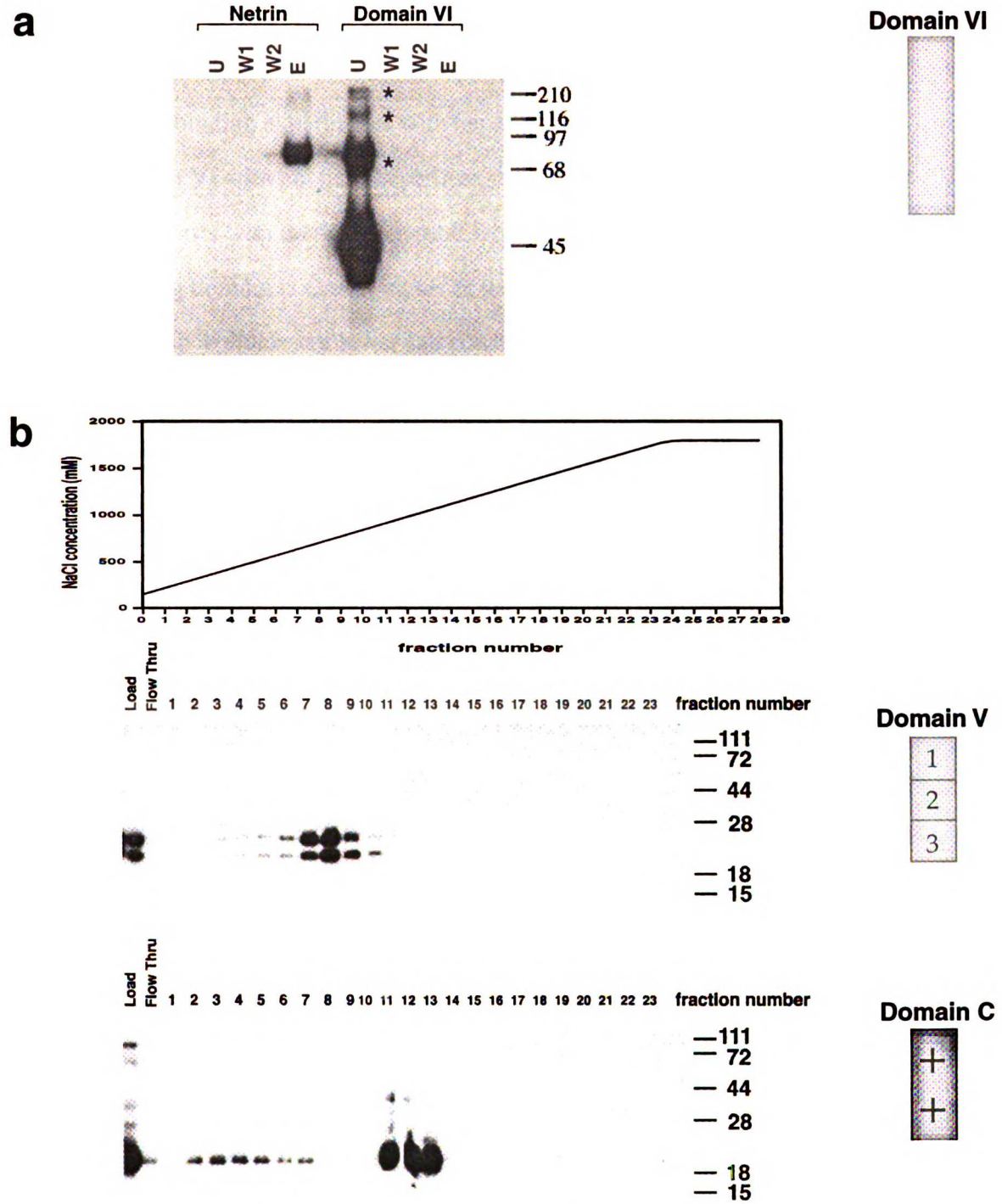

Figure 5. Domain C is netrin's tightest heparin binding domain. (a) Heparin batch binding experiments were conducted for the netrin-1 and netrin-1(VI) in 300mM NaCl. The unbound material (U), the two washes in binding buffer (W1 and W2) and the 1.5M NaCl eluate (E) were separated on a 10% SDS polyacrylamide gel and subjected to immunoblot analysis using antibodies to the myc epitope. We could detect a domain VI doublet and higher order oligomers (see astrices) because high concentrations of domain VI protein were loaded onto the gel. (b) Using the 9E10 monoclonal antibody directed to the myc epitope, an immunoblot of the elution profiles of netrin-1 domains V and C off of a heparin affinity column. The NaCl concentration in each fraction is indicated in the corresponding graph.

the heparin binding domain(s) within netrin-1. Conditioned media and extracts from COS cells transfected with each domain VI, V or C were combined, dialyzed into low salt, and tested in batch binding experiments with heparin-agarose beads. Unlike full length netrin-1 protein, domain VI does not bind heparin at salt concentrations greater than 300 mM (figure 5a). This contrasts with domains V and C which can bind to heparin. Of these, the tightest heparin binding domain is C, eluting off of a heparin column at 1.1 M NaCl (figure 5b, lower). Domain V elutes at a lower salt concentration of 800 mM (figure 5b, upper). Neither of these domains binds heparin as tightly as full length netrin-1 suggesting that some cooperativity in binding occurs between domains V and C. Nonetheless, our finding that domain C is both netrin-1's cell binding domain and netrin-1's tightest heparin binding domain is consistent with earlier work suggesting that netrin-1 binds to cells by binding proteoglycans on the cell surface (Chapter III).

Domains v3 and C are not required to bind DCC

DCC is required for netrin-1 elicited outgrowth of commissural axons from dorsal spinal cord explants because antibodies to DCC block axon outgrowth (Keino-Masu et al., 1996). To determine how DCC functions in the outgrowth assay, we identified the domains of netrin-1 which mediate DCC binding. Binding to DCC transfected 293 cells was performed in the presence of 2 μg/ml heparin to eliminate background binding to the cells and was visualized by indirect immunofluorescence using antibodies directed to the myc epitope (figure 6). As shown previously, 300 ng/ml netrin-1 protein binds tightly to DCC transfected 293 cells but not to untransfected 293 cells (Keino-Masu et al., 1996). As shown in figure 6, Netrin-1(VI-V) also binds to DCC. However, neither domains VI or V fused to domain C or expressed alone bind the DCC receptor.

To test if the netrin-1 mutants deleted of EGF repeats bind to DCC, 293-EBNA cells stably transfected with each EGF deletion construct were incubated with the extracellular domain of DCC fused with alkaline phosphatase (made by Yimin Zou). This

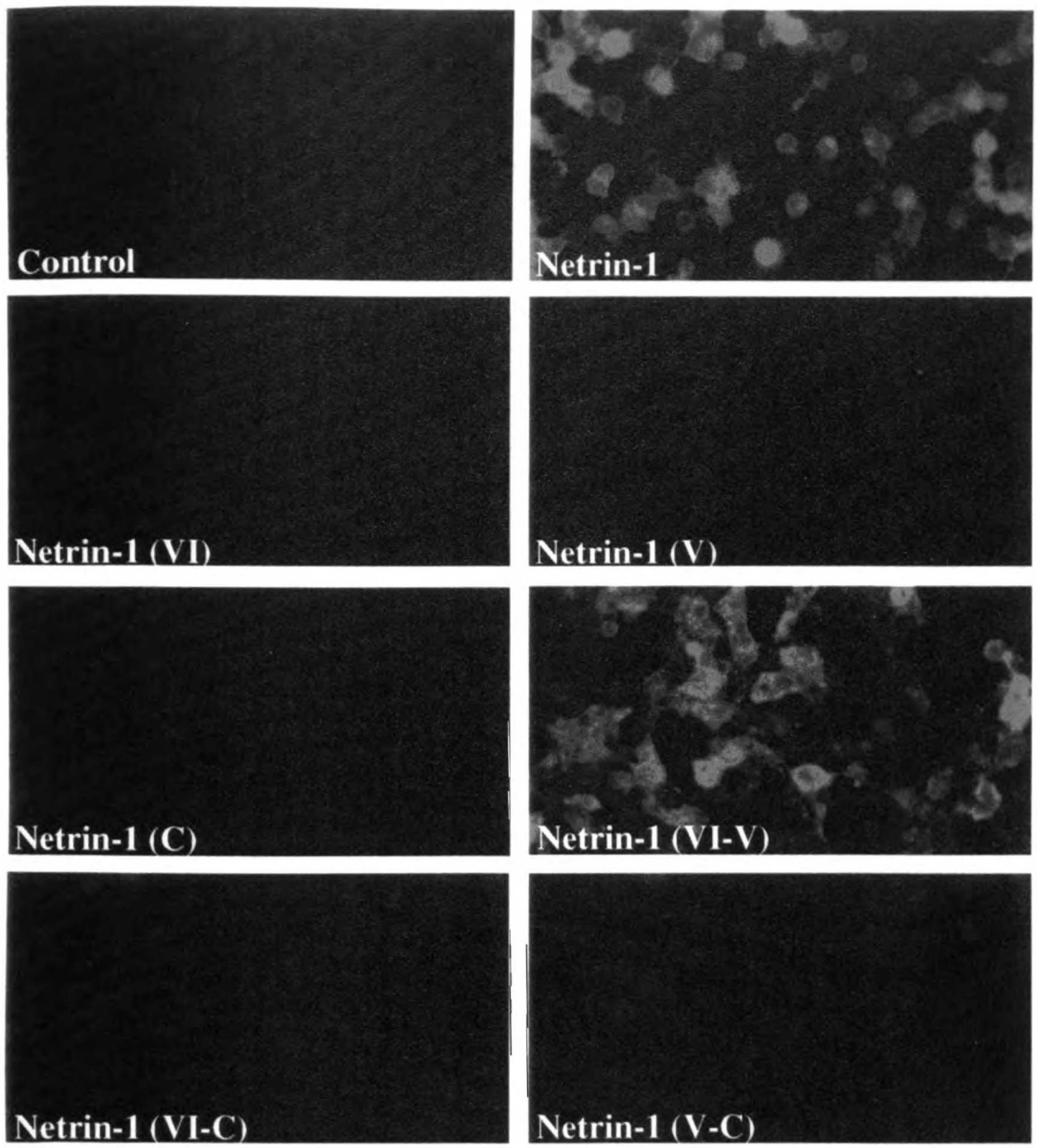

Figure 6. Binding of the netrin-1 mutants to DCC transfected 293 cells. Conditioned media and 1M NaCl extracts from COS cells transfected with nothing (control), netrin-1, or with each of the indicated mutations were combined and dialyzed to low salt. This figure shows binding of each of these with 293-EBNA cells stably expressing the DCC receptor. Binding was visualized by indirect immunofluorescence using antibodies directed to the myc epitope.

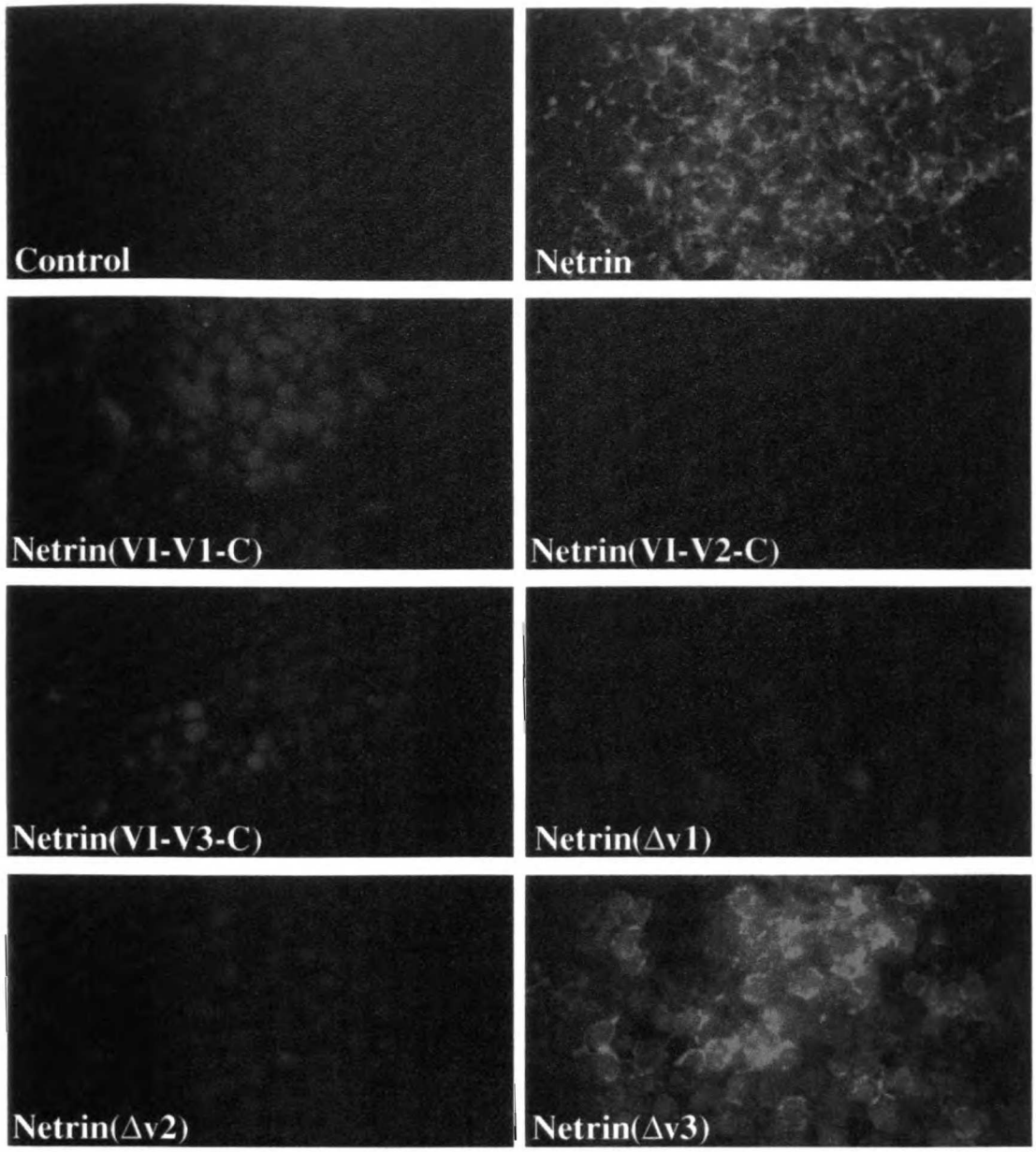

Figure 7. DCC binding to the netrin-1 deletion mutants lacking one or two EGF repeats within domain V. Untransfected 293-EBNA cells (control) or 293-EBNA cells stably transfected with netrin-1 or with the indicated netrin-1 mutants were grown to confluence. All of the expressed proteins contain domain C and remained attached to the cell surface after secretion (not shown). These cell lines were incubated with 293 cell supernatants containing the extracellular domain of DCC fused with alkaline phosphatase (DCC-AP), washed, then fixed. Binding of DCC-AP binding was visualized by indirect immunofluorescence using monoclonal antibodies directed to DCC.

was possible because all the deletion constructs remain bound to the cell surface presumably due to the presence of domain C (not shown). As shown in figure 7, our control, the netrin-1 expressing 293 cells, and the netrin-1(Δv3) expressing 293 cells bind to DCC-AP, indicating that domain v-3 is not required for DCC binding. In contrast, domain v-2 is required to bind to DCC, suggesting that the sequence of the EGF repeats and not merely the number of repeats present is important. These experiments indicate that domains C and domain v3 are not required for DCC binding.

Although binding to DCC is required for outgrowth, it is insufficient for outgrowth

Once the binding properties of the mutants were characterized, we asked if any elicited commissural axon outgrowth. The conditioned medium and extract fractions of each deletion construct, at netrin-1 protein concentrations spanning the active range of the full length netrin-1 protein, were tested for commissural axon outgrowth activity from E13 dorsal spinal cord explants. Netrin-1 elicited outgrowth peaks at 30 ng/ml, and is inhibited at concentrations greater than 1 μg/ml. The results we obtained are consistent with antibody blocking experiments which indicate that DCC is required for axon outgrowth (Keino-Masu et al., 1996): none of the mutants that do not bind DCC are active.

Of the mutants which do bind DCC, netrin(Δv3) elicits robust axon outgrowth, though the axon bundles appear slightly thinner than netrin-1 responding bundles (figure 8b). Like netrin-1, 30 ng/ml netrin(Δv3) elicits outgrowth of axon bundles which add up to 1.2mm in length (per explant) (figure 8c-diamonds). Also, similar to netrin-1, high concentrations of netrin-1(Δv3) (>1 μg/ml) inhibit axon outgrowth. Interestingly, the nematode netrin, UNC-6, also elicits outgrowth of rat commissural axons (figure 8a), even though it shares only 50% identity with the vertebrate netrin proteins. However, we could not measure the binding properties of UNC-6 to DCC or to heparin because we were unable to express sufficient levels of myc-tagged UNC-6 protein.

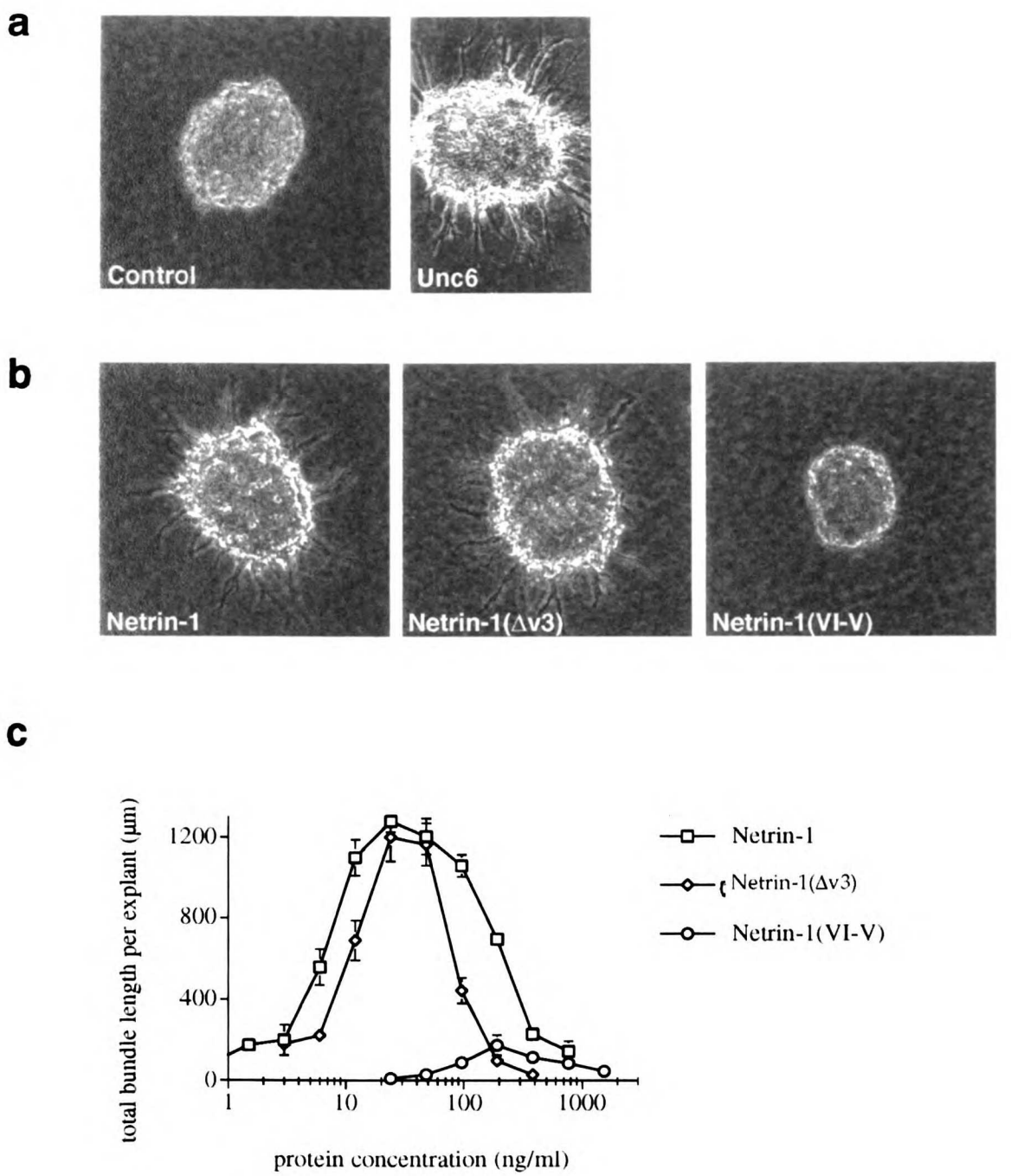

Figure 8. Netrin-1(Δv3) can elicit outgrowth from E13 dorsal spinal cords into collagen (a) E13 dorsal spinal cord explants grown alone (control) or cocultured with aggregated COS cells transfected with UNC-6. (b) E13 dorsal spinal cord explants cultured in the presence of 30ng/ml netrin-1, 30ng/ml netrin-1(Δv3), or 100ng/ml netrin-1(VI-V) (c) E13 dorsal spinal cord explants were grown in the presence of extracts containing 1ng/ml -1μg/ml netrin-1, netrin(Δv3), and netrin(VI-V) protein. The total length of axon bundles was measured from eight explants per condition. The average outgrowth and standard errors are shown for each.

In contrast to netrin-1(Δv3) and UNC-6, netrin-1(VI-V) protein has little commissural axon outgrowth activity. Even though it binds tightly to DCC (figure 6) (Keino-Masu et al., 1996), the axons which do respond to netrin-1(VI-V) are sparse and weakly fasciculated, indicating that DCC binding is not sufficient for axon outgrowth. While netrin-1(VI-V) elicits only weak axon outgrowth, its specific activity curve has the same bell shape as that of netrin-1. Netrin-1(VI-V) activity peaks at ~ 200 ng/ml and is inhibited at high concentrations (>1 μg/ml).

Netrin-1(VI-V) is a potent inhibitor of netrin-1 elicited axon outgrowth

Since netrin-1(VI-V) can bind tightly to DCC but is not a strong agonist for axon outgrowth, we predicted that netrin-1(VI-V) may act as a competitive inhibitor and interfere with netrin-1 elicited commissural axon outgrowth. As shown in figure 9a, netrin-1(VI-V) is indeed an potent inhibitor of netrin-1 elicited outgrowth. Axon outgrowth was elicited with 6ng/ml netrin-1 (arrow), and either additional netrin-1 protein (diamonds) or equal concentrations of pure netrin-1(VI-V) (circles) was added. Both netrin-1 and netrin-1(VI-V) inhibit outgrowth when the total protein concentration is 1 μg/ml.

We also asked if high concentrations of netrin-1(VI-V) could interfere with netrin-1's permissive function within the ventral spinal cord. Normally, when whole E11 spinal cords are cultured *in vitro*, commissural axons extend ventrally and coalesce at the center of the floor plate (figure 9b, arrows). High concentrations of netrin-1 (3 μg/ml) present in the culture medium disrupt ordered growth of commissural axons within the dorsal spinal cord and obstruct their extension into the ventral spinal cord (below the white dots). These defects mimic those within the spinal cord of netrin -/- mice (Serafini et al., 1996), indicating that exogenous netrin-1 can interfere with the function of netrin-1 present endogenously within the spinal cord. This defect can be mimicked with high concentrations of netrin-1(VI-V) protein (3 μg/ml or 10 μg/ml), indicating that netrin-1(VI-

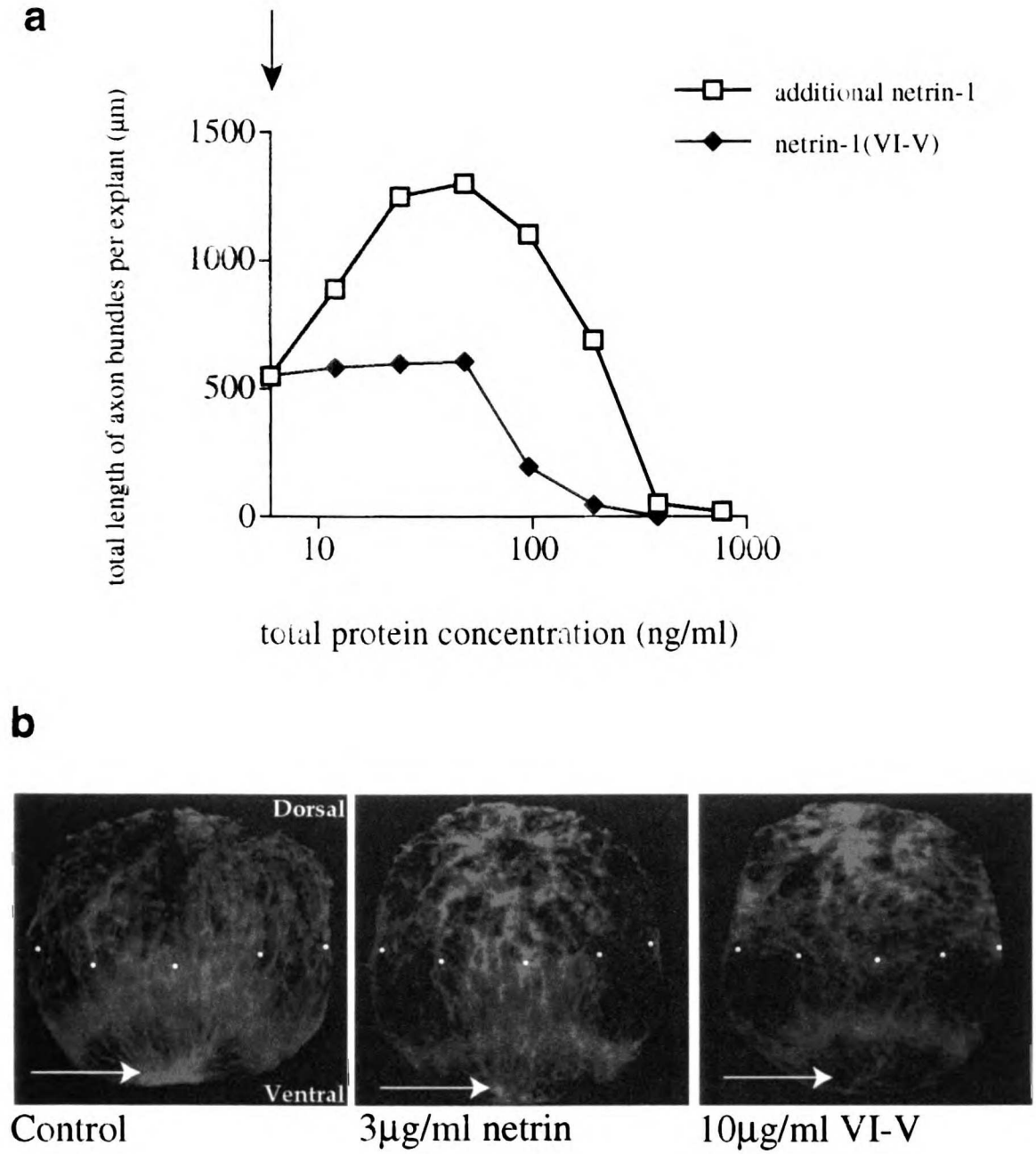

Figure 9. Netrin-1(VI-V) can inhibit commissural axon outgrowth into collagen and into the ventral spinal cord. (a) 6 ng/ml netrin-1 protein was added to E13 dosal spinal cord explants to elicit sub-threshold levels of axon outgrowth (arrow). To these, either additional netrin-1 protein was added or netrin-1(VI-V) protein was added. The x-axis shows the total protein concentration. For each culture condition, the total length of axon bundles was measured from eight explants and the average outgrowth and standard errors of the mean are shown. (b)Whole E11 spinal cords were cultured alone (control), in the presence of 3 μg/ml netrin-1 or 10 μg/ml netrin-1(VI-V). Commissural axons within the explant were visualized by indirect immunoflurescence using a monoclonal antibody directed to TAG-1. In controls, axons cross the floor plate at the center of the cultured spinal cord explant (arrow). In explants grown in the presence of netrin-1 and netrin-1(VI-V) most axons fail to grow into the ventral spinal cord (below the white dots) and do not reach the floor plate (arrow).

V) does not only inhibit netrin-1's collagen outgrowth activity, but can also inhibits netrin-1's permissive activity within the ventral spinal cord.

Only the tight heparin binding fraction of netrin-1(VI-V) can elicit commissural axon outgrowth

The fact that netrin-1(VI-V) can bind tightly to DCC, can inhibit netrin-1 elicited outgrowth but is unable to elicit strong outgrowth itself, suggested that netrin-1(VI-V) retains some of netrin-1's properties and encouraged us to study it in greater depth. We began by purifying netrin-1(VI-V) protein. Netrin-1(VI-V) is produced at high levels in stably transfected 293 cells (3 μg/ml in the conditioned medium), allowing us to obtain ~90% pure protein after a heparin affinity step[2]. Interestingly, when a shallow gradient of salt is used to elute netrin-1(VI-V) off of the heparin column, it elutes at two peaks (figure 10a, arrows): The majority (>95%) elutes at 530 mM NaCl and the remainder binds to heparin more tightly, eluting at 650 mM NaCl. Whereas the low affinity protein is relatively pure, netrin-1(VI-V) in the high affinity peak is impure constituting a small fraction of the total protein.

The discovery that netrin-1(VI-V) is actually comprised of two different species encouraged us to assay each fraction for axon outgrowth activity. Outgrowth elicited by the low affinity peak was minimal, with only a few poorly fasciculated axons invading the collagen (figure 10b and 10c). In contrast, the high affinity netrin-1(VI-V) peak elicited substantial outgrowth of axon bundles which added to 800 μm in length (per explant) at its peak (equivalent to ~ 3/4 of the outgrowth elicited optimal netrin-1). Like netrin-1, axon outgrowth is inhibited at high protein concentrations (>1 μg/ml).

To investigate why the two species of netrin-1(VI-V) differ in their affinity for heparin and in their ability to promote outgrowth, equal concentrations of netrin-1(VI-V) from each fraction were separated by electrophoresis and subjected to immunoblot analysis.

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[2] This purification was performed by S. Faynboym

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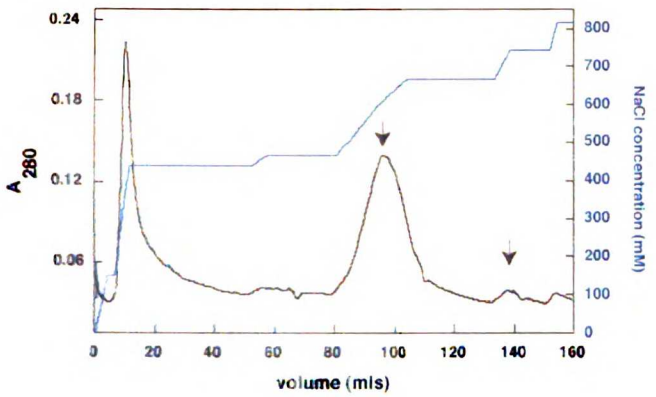

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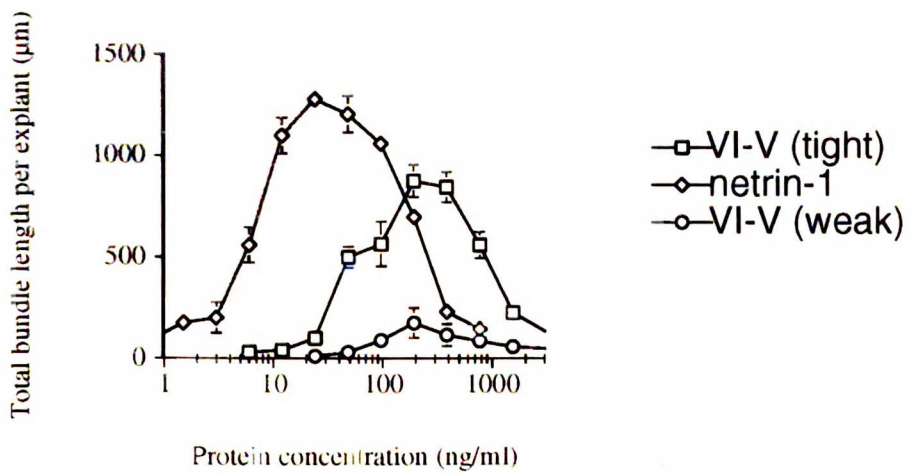

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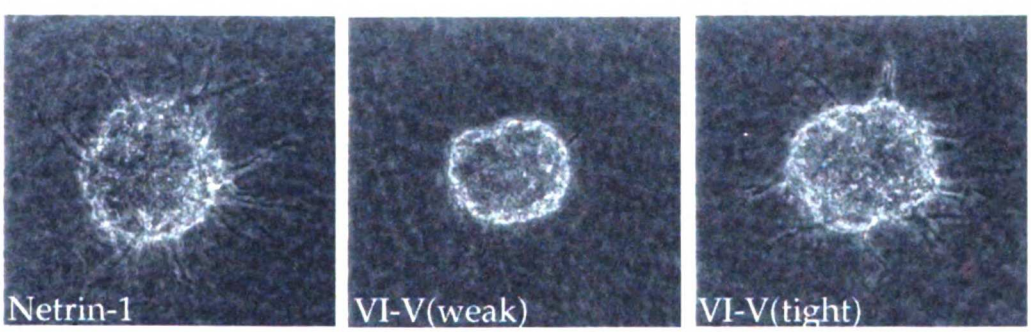


Figure 10. The tight, but not low, heparin binding fraction of netrin-1(VI-V) elicits axon outgrowth. (a) Recombinant netrin-1(VI-V) protein elutes off of a heparin affinity column at two peaks (arrows) : 530mM NaCl (weak binding) and 650mM NaCl (tight binding). (b) The weak and tight heparin binding fractions of netrin-1(VI-V) were tested for their ability to elicit outgrowth from E13 dorsal spinal cord explants at the concentrations indicated. The total length of bundles was measured from eight explants at each condition and the average outgrowth and standard errors of the mean were calculated. The diamonds represent the specific activity of full length netrin-1 protein for comparison. (c) Representative outgrowth elicited by netrin-1 protein and by the the weak and tight heparin binding fractions of netrin-1(VI-V).

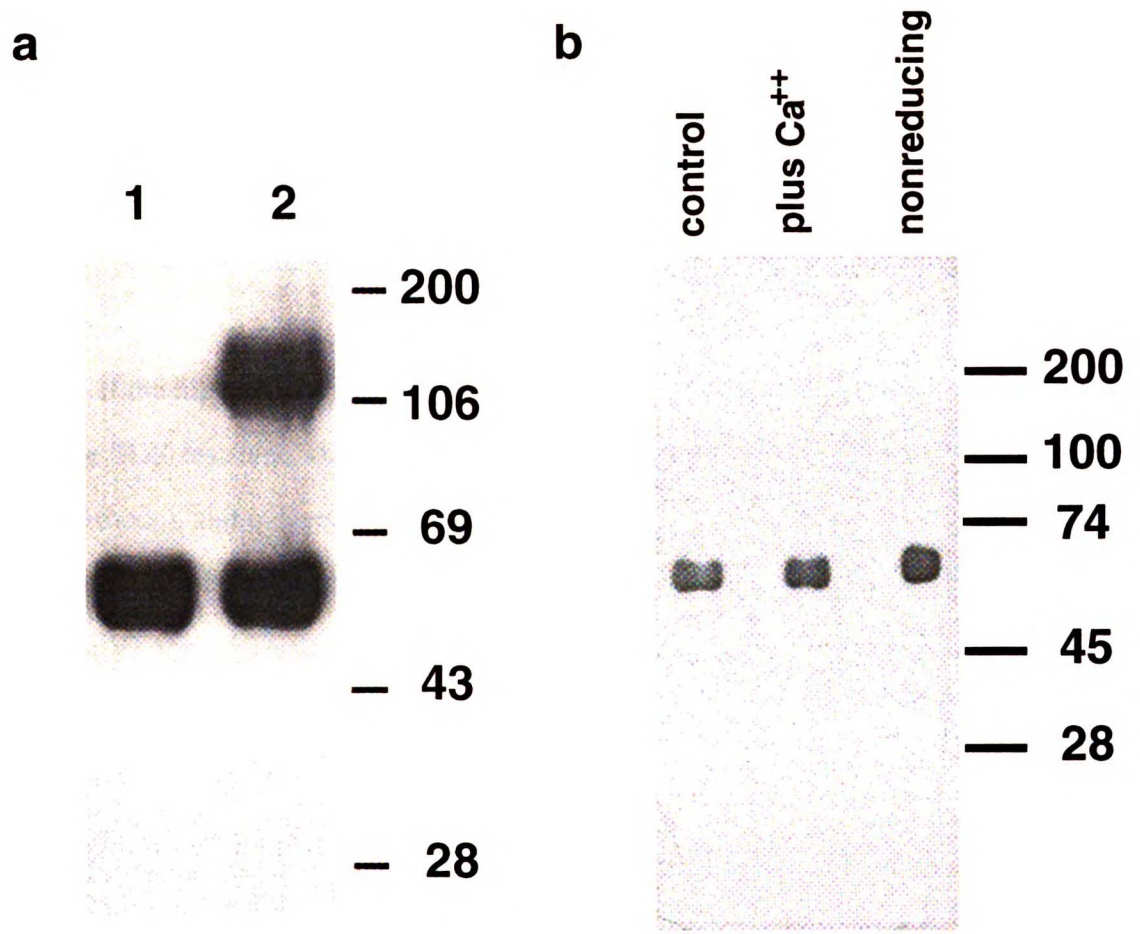


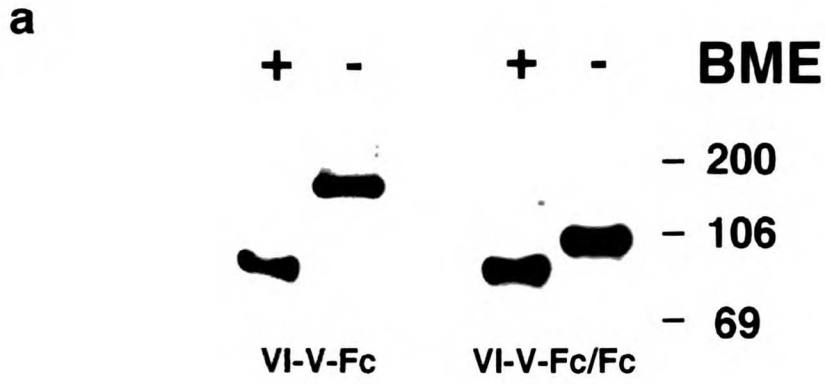
Figure 11. The tight heparin binding fraction of netrin-1(VI-V) protein migrates partially as a doublet on reducing SDS-polyacrylamide gels. (a) Equal concentrations of netrin-1(VI-V) from the 530 mM NaCl heparin eluate (lane 1) and from the 650mM NaCl heparin eluate (lane 2) were separated on an SDS-polyacrylamide gel. Approximately half of the tight binding netrin-1(VI-V) protein migrates as a doublet at ~120kD. (b) Netrin-1(VI-V) protein in the low affinity heparin binding fraction does not migrate as a dimer if β-mercaptoethanl, a reducing agent, is ommitted from the sample buffer (non-reducing) or if netrin-1 (VI-V) protein is preincubated with 1mM Ca++ prior to separation on the gel. 1mM Ca++ elicits polymerization of laminin heterotrimers.

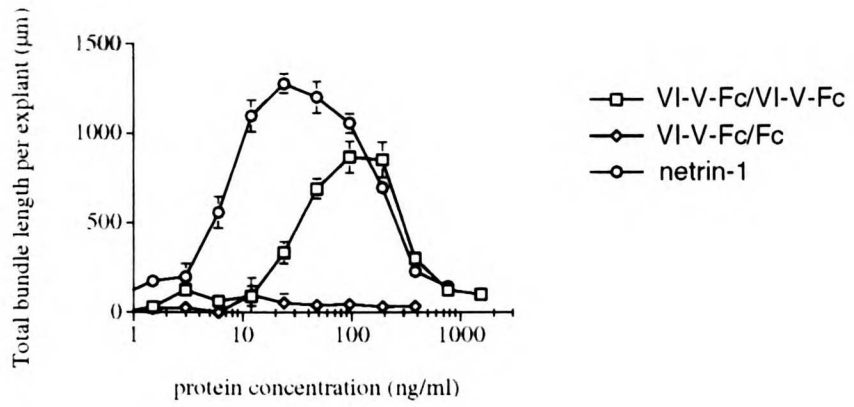
As shown in figure 11, the migration properties of netrin-1(VI-V) in the two fractions differ. In the low affinity fraction, netrin-1(VI-V) migrates as one band at 62 kD whereas netrin-1(VI-V) in the high affinity fraction migrates as two bands, 62 kD and 123 kD. This larger band is exactly double the size of the first, suggesting that the high affinity heparin eluate may contain netrin-1(VI-V) dimers. Differences in the dimerization state of netrin-1(VI-V) in the two fractions could certainly account for differences in their affinity for heparin.

If the high affinity heparin eluate does contain netrin-1(VI-V) dimers, dimerization must be SDS-resistant, stable to storage in 1M NaCl buffer for 2-3 months (see materials and methods), and independent of disulfide linkage (figure 11b). Furthermore, while domain VI of laminin regulates Ca^{++} dependent hetero-oligomerization of laminin trimers, domain VI of netrin-1 does not seem to mediate Ca^{++} dependent dimerization, since preincubating the low heparin affinity netrin-1(VI-V) with 1 mM Ca^{++} does not change its migration profile (figure 11b) and since10 mM EDTA, a Ca^{++} chelator, does not eliminate the netrin-1(VI-V) dimer band (not shown).

Dimerized netrin-1(VI-V) molecules can elicit outgrowth

Thus, while the majority of netrin-1(VI-V) protein binds to heparin at 530 mM NaCl, a small fraction binds to heparin more tightly (650 mM NaCl) and is able to elicit outgrowth of commissural axons into collagen. We suspected that this 650 mM NaCl heparin eluate contains dimers of netrin-1(VI-V) and that dimers but not monomers are active for outgrowth. To test this directly, we turned to a netrin-1(VI-V) derived construct which had been previously made in our laboratory and which was known to have axon outgrowth activity, netrin-1(VI-V) expresses as a fusion with human immunoglobulin Fc (Keino-Masu et al., 1996). Fc subunits dimerize covalently through disulfide bonds which can be severed by reducing agents such as β-mercaptoethanol. VI-V-Fc is a dimer and migrates at 84 kD in the presence of β-mercaptoethanol but as a larger band roughly double





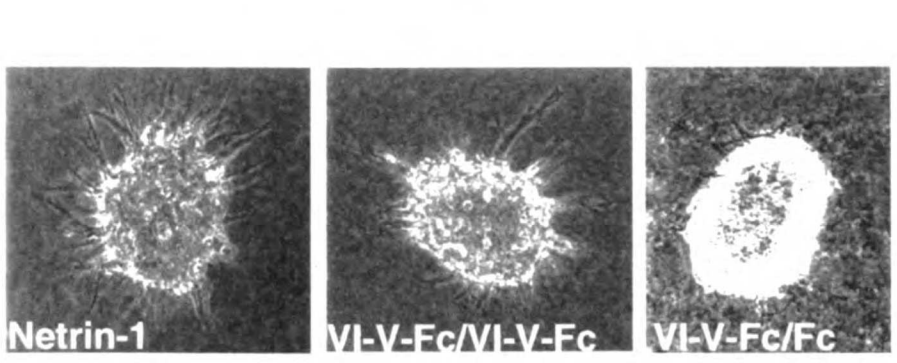


Figure 12. VI-V-Fc homodimers but not VI-V-Fc/Fc heterodimers elicit commissural axon outgrowth from E13 dorsal spinal cord explants. COS cells were transiently transfected with the VI-V-Fc and Fc expression constructs. Under non-reducing conditions, conditioned medium from these cells contains two protein complexes immunoreactive with anti-VI-V antibodies and correspond in size to the VI-V-Fc/Fc and VI-V-Fc/VI-V-Fc complexes. These can be separated by heparin affinity chromatography. An immunoblot of the two major heparin eluate fractions is shown in (a) . We have indicated whenever the reducing agent β-mercaptoethanol was present (BME). (b) Netrin-1, VI-V-Fc homodimers and VI-V-Fc/Fc heterodimers were tested in the E13 outgrowth assay at the concentrations indicated. The total length of bundles was measured from eight explants at each condition. The average outgrowth and standard errors of the mean are shown. (c) Representative explants from the peak outgrowth elicited by netrin-1, VI-V-Fc/VI-V-Fc, and VI-V-Fc/Fc.

in size (~148 kD) in the absence of β-mercaptoethanol (figure 12a, compare VI-V-Fc lanes).

We investigated why Fc fusions of netrin-1(VI-V) can elicit more commissural axon outgrowth than netrin-1(VI-V) alone. One possibility is that Fc restores axon outgrowth activity by dimerizing two netrin-1(VI-V) domains. Alternatively, Fc may stabilize the VI-V protein allowing netrin-1(VI-V) monomers to elicit outgrowth. To differentiate between these possibilities, COS-1 cells were simultaneously transfected with two constructs, VI-V-Fc and Fc. Under nonreducing conditions, two complexes containing VI-V are produced and present in the conditioned medium, homodimers of VI-V-Fc and heterodimers of VI-V-Fc /Fc (not shown). These complexes can be separated by heparin affinity chromatography because VI-V dimers (VI-V-Fc) elute off of heparin at 660 mM NaCl, whereas VI-V monomers (VI-V-Fc/Fc) elute at 490 mM NaCl. As shown under non-reducing conditions, there is no contaminating complexes between the 490 and 660 mM eluates. Under reducing conditions, the subunits of both complexes sever, revealing an immunoreactive band corresponding to the VI-V-Fc subunit (figure 12a, plus BME).

If dimerization of VI-V is important for activity, only the VI-V-Fc homodimer should elicit axon outgrowth. Conversely, if Fc functions to stabilize the VI-V protein, the VI-V-Fc/Fc heterodimer should also be active. As shown in figure 12b, the VI-V-Fc/Fc heterodimer elicits little outgrowth, with few poorly fasciculated axons innervating the collagen. In contrast, the VI-V-Fc homodimer elicits strong outgrowth, with a specific activity curve lower than that of full length netrin-1 but similar to that of the tight heparin binding VI-V protein (figure 10). These results exclude the possibility that Fc stabilizes VI-V monomers, and indicate that dimerization of domains VI-V is partially sufficient for outgrowth.

At low netrin-1 concentrations, domain C does not mediate the direct dimerization of netrin-1 molecules

Because dimerization of netrin-1(VI-V) is partially sufficient for outgrowth, domain C itself may be a dimerization domain. We tested if netrin-1 but not netrin-1(VI-V) exists as dimers in solution. Equivalent concentrations of netrin-1 and netrin-1(VI-V) were incubated with different concentrations of Bis(sulfosuccinimidyl)suberate (BS3), a bifunctional crosslinker which reacts with primary amines. As the concentration of BS3 is increased, the ratio of oligomers to monomers increases, indicating that both the netrin-1 and netrin-1(VI-V) proteins can be adequately crosslinked. However, at equivalent concentrations of BS3, the ratio of oligomers/monomers in each sample is identical (figure 13a), suggesting that netrin-1 and netrin-1(VI-V) have similar stoichiometries in solution.

To confirm this conclusion, we collaborated with Peter Yurchenco (Robert Wood Johnson Medical School) to image netrin-1 and netrin-1(VI-V) by rotary shadow electron microscopy. This procedure has been used successfully to image the B2 arm of laminin which shares sequence homology with domains VI-V of netrin-1 (Beck et al., 1990). Laminin domain VI is globular in structure and domain V is a long rod like structure. As shown in figure 13b, netrin(VI-V) is also comprised of a globular domain linked to a rod-like domain indicating that it is homologous to laminin(VI-V) in structure as well as sequence. The netrin-1 micrographs shows that domain C is globular but slightly smaller than domain VI.

As seen in electron micrographs, the majority of both netrin-1(VI-V) and netrin-1 proteins are monomers and a small percentage of both (<5%) are dimers which seem to be bound through domain VI. Domain C does not seem to enhance the direct dimerization of netrin-1 molecules.

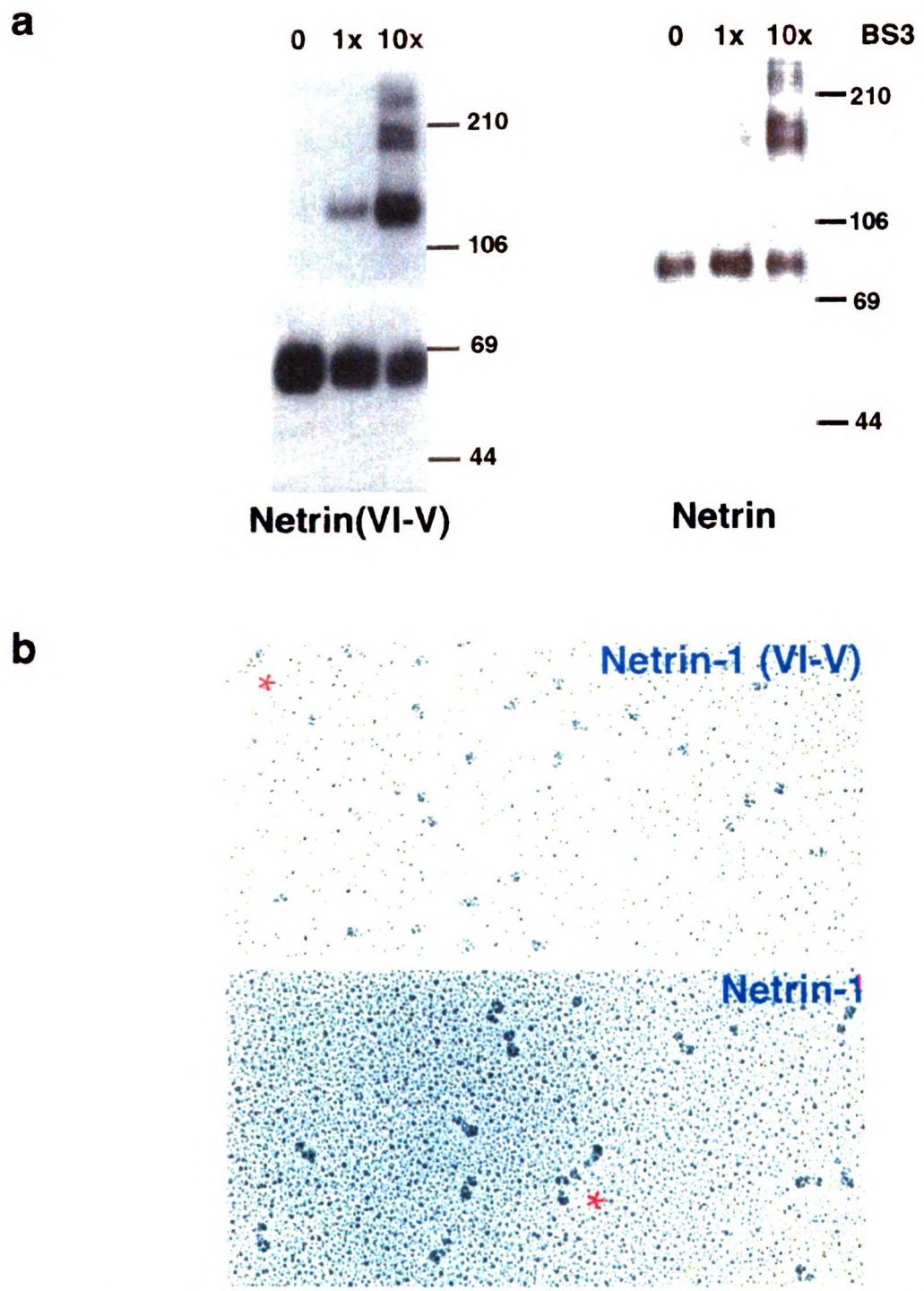


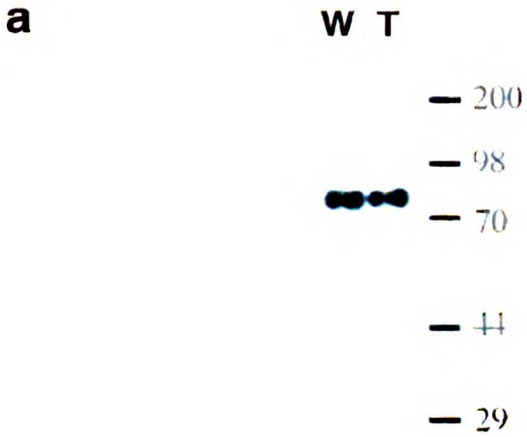
Figure 13. Domain C does not seem to mediate the direct dimerization of netrin-1 molecules. (a) 50 ng/ml netrin-1(VI-V) and netrin-1 proteins were incubated with zero, 3.7µg/ml (1x) or 37µg/ml (10x) of the crosslinker Bis(sulfosuccinimidyl)-suberate (BS3). After 30 minutes at 37°C, the reaction was quenched with 100mM Tris-HCl [pH 7.0], separation on 7.5% SDS acrylamide gels, and subjected to immunoblot. analysis with antibodies directed to netrin-1(VI-V) (b) Rotary shadow electron micrographs of the netrin-1(VI-V) and full length netrin-1proteins. Examples of the netrin-1 and netrin-1 (VI-V) doublets are shown with a red astrix.

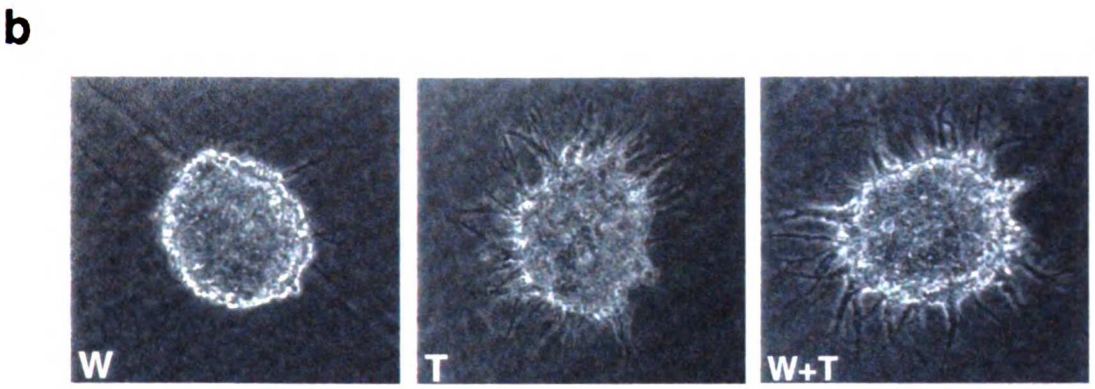
Heparin binding is a primary function of domain C

While domain C does not seem to mediate the direct dimerization of netrin, it may oligomerize netrin-1 indirectly by first binding to a co-factor. To explore this possibility, we defined the region within C required for outgrowth. Deletion studies of domain C have suggested that outgrowth activity becomes progressively worse as domain C is shortened (L. Hinck, unpublished results), indicating that domain C has a general function. Since one general feature of domain C is that it is extremely basic, we investigated if heparin binding was the primary function for C.

First, we isolated netrin-1 protein which binds to heparin weakly and asked if a correlation exists between the affinity for heparin and the ability to elicit outgrowth. The overwhelming majority of netrin-1 elutes off of a heparin column at 1.2 M NaCl, but a small fraction elutes at 670 mM NaCl. Netrin-1 in both fractions are identical in size and have intact domain C's, because they are recognized by antibodies directed against the myc epitope at the C-terminus. However, the high affinity netrin-1 elicits robust axon outgrowth from E13 dorsal spinal cord explants at 30 ng/ml, whereas the low affinity netrin-1 elicits minimal outgrowth at concentrations from 0.1 ng/ml to 320 ng/ml (figure 14b). Since the 670 mM eluate of netrin-1 is impure, we were concerned that it may contain an inhibitor which masks outgrowth activity. However, this seems like an unlikely possibility since the outgrowth elicited by mixtures of the 1.2M and 670mM netrin-1 eluates is equivalent to that elicted by the 1.2M eluate alone (figure 14b; compare T and W+ T).

While netrin-1 protein in the 670 mM NaCl eluate elicits poor axon outgrowth, it binds tightly to DCC transfected 293 cells (figure 14c). Also, similar to netrin-1(VI-V) and the tight heparin binding netrin-1 protein, it inhibits outgrowth elicited by the 1.2M NaCl eluate at high concentrations (not shown). Thus, the weak heparin binding netrin-1





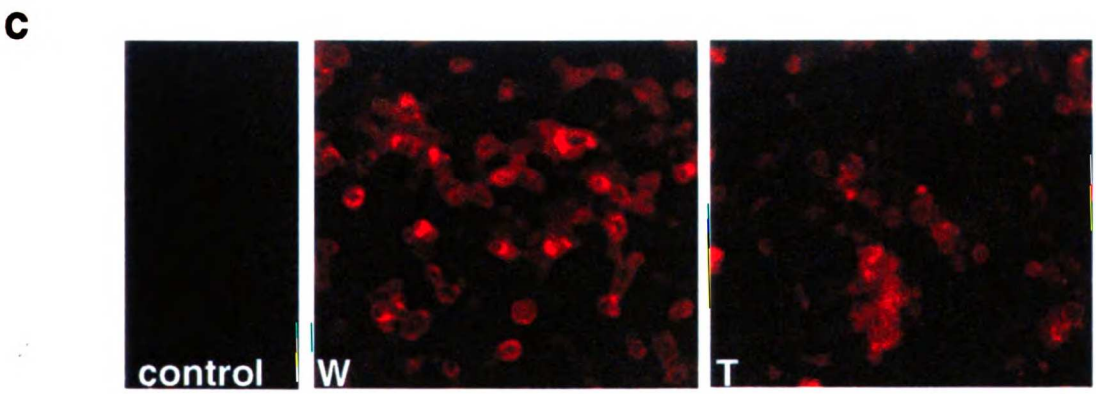
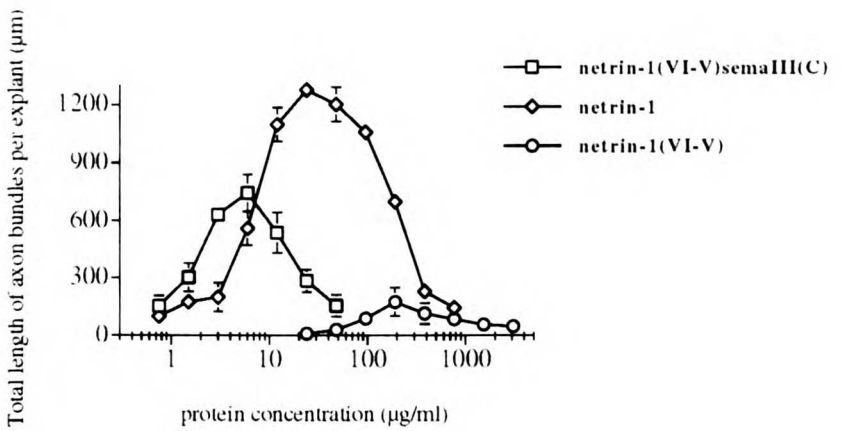


Figure 14. Netrin-1's affinity for heparin correlates with its ability to elicit axon outgrowth. Conditioned medium of 293-netrin cells was loaded onto a heparin column and eluted with a gradient of NaCl. Netrin eluted predominantly at 1.2M NaCl, though some eluted at lower NaCl concentrations. Figure (a) shows an immunoblot of the 670mM (Weak) and 1.2M eluates (Tight) using antibodies directed to the C-terminal myc epitope. (b) Axon outgrowth activities of the weak (W) and tight (T) heparin binding netrin at 30ng/ml netrin-1 protein. Outgrowth was also measured for the combined eluates (W+T) where total netrin concentration was 60ng/ml. (c) At 30ng/ml, the weak (W) and tight (T) heparin binding fractions bind equally to 293 cells transfected with DCC, whereas netrin-1 which elutes earlier off of heparin does not bind to the DCC transfected cells (see control). Binding was assessed by indirect immunofluorescence using antibodies directed to the myc epitope.

fraction is not inactive in all aspects of netrin-1 function. The fact that it does not act as an agonist for axon outgrowth suggests that heparin binding may be required for activity.

To test if heparin binding is a primary function of domain C, we asked whether axon outgrowth could be elicited by a fusion of netrin-1(VI-V) with the heparin binding domain of another molecule which shares no sequence homology with netrin-1. The C-terminal domain of human semaphorin III, a chemorepellent for developing axons of the central and peripheral nervous systems, was used because semaIII and netrin-1 bind to heparin with comparable affinity (Luo et al., 1993). Netrin-1(VI-V)semaIII(C) elutes off of heparin at 1 M NaCl, as compared to full length netrin-1 protein which elutes at 1.2 M NaCl. As shown in figure 15, netrin-1(VIV)-semaIII(C) is active in the outgrowth assay, eliciting thick axon fascicles into the collagen matrix. It's specific activity curve is similar in shape to that of netrin-1, but the total amount of outgrowth is less (~3/4 of netrin-1 elicited outgrowth), indicating that semaIII's heparin binding domain cannot fully restore netrin-1's outgrowth activity. Nonetheless, the fact that it restores 3/4ths of the outgrowth suggests that heparin binding is a primary function of domain C.

a



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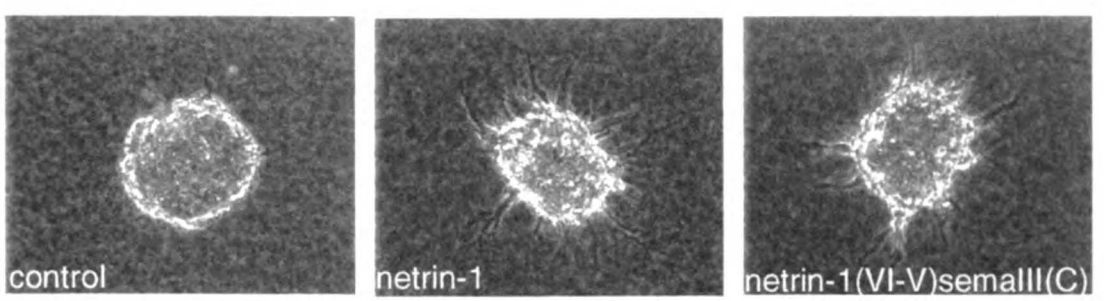

Figure 15. Netrin-1(VI-V) semaIII(C) has axon outgrowth activity. (a) Netrin-1(VI-V) was expressed as a fusion with the C-terminal heparin binding domain of human semaphorin III and tested for its ability to elicit outgrowth from E13 dorsal spinal cord explants at the concentrations indicated. The total length of bundles was measured from four explants at each condition. The average outgrowth and standard errors of the mean are shown. The specific activity of full length netrin-1 and netrin-1(VI-V) proteins are shown for comparison. Panel (b) shows examples of outgrowth elicited in the absence of conditioned medium (control) or in the presence of 293 conditioned medium containing netrin-1 or netrin-1(VI-V)semaIII(C) proteins. Qualitatively, outgrowth looks similar.

Discussion

Structure-function analysis of netrin-1

The amino acid sequence of netrin-1 suggests that it is comprised of five domains, domain VI, three EGF repeats within domain V, and domain C. Domains VI-V of netrin-1 are homologous in sequence (Serafini et al., 1994) and in structure (figure 13; Beck et al., 1990) to the N terminus of the laminin B2 chain. Because structural domains often reflect functional units within proteins, we generated single and double deletions of netrin-1's domains, hoping to define the minimal region responsible for binding to DCC and to heparin. These deletions were then used to determine which properties are required for eliciting outgrowth. Although expression levels varied, expression was high enough to perform binding studies and to test for outgrowth activity from E13 spinal cord explants for all but one construct, netrin-1(Δv1).

Consistent with earlier studies suggesting that netrin-1 binds to cells by binding proteoglycans on the cell surface, we found that heparin binding and cell binding of netrin-1 is mediated by the same domain, C. Domain C binds to heparin with slightly lower affinity than full length netrin. However, similar to netrin-1, domain C partitions between cell bound and soluble fractions, can be extracted with 2 μg/ml soluble heparin (not shown), bind to cells in a punctate pattern and diffuses from the cells expressing it onto neighboring untransfected cells.

To understand the role of DCC in eliciting outgrowth, we identified deletion mutants which bind to the DCC receptor and which can elicit axon outgrowth. Consistent with earlier studies suggesting that DCC is required for axonal outgrowth (Keino-Masu et al., 1996), all of the constructs which elicit outgrowth also bind to DCC. However, we identified a mutant, netrin-1(VI-V), which binds tightly to DCC but does not elicit robust axon outgrowth, indicating that DCC binding is insufficient for activity.

How can dimers of netrin-1(VI-V) elicit outgrowth, when monomers cannot?

Although netrin-1(VI-V) protein cannot elicit axon outgrowth itself, dimers of netrin-1(VI-V), generated by fusion with human immunoglobulin Fc, do elicit axon outgrowth. How can dimers of netrin-1(VI-V) be sufficient for outgrowth, when monomers are not? Since netrin-1(VI-V) binds tightly to DCC, one possibility is that dimers of netrin-1(VI-V) cause DCC to dimerize and this elicits axon outgrowth. Dimerization is required for the activation of receptor protein serine/threonine kinases, such as the TGFβ receptors, and receptor protein tyrosine kinases (RPTK), such as the FGF receptors, the Trk family of neurotrophin receptors, and the Eph receptors, many which are involved in axon guidance. Like most Ig family members, the cytoplasmic domain of DCC does not contain a kinase domain. However, dimerization may allow DCC to associate with nonreceptor protein tyrosine kinases. For example, neuronal immunoglobulin cell adhesion molecule, contactin/F11, is known to complex with Fyn, a member of the src-family of nonreceptor tyrosine kinases (Zisch et al., 1995).

An alternative mechanism by which netrin-1(VI-V) dimers may elicit outgrowth is to heterodimerize DCC with another netrin-1 receptor. Although little is known about the signal transduction mechanisms of Ig receptors, IgCAMs are known to interact with other receptors *in cis*. For example, L1 binds to the Eph receptor, cek5 (Zisch et al., 1997) and NCAM and L1 bind the FGF receptor (Saffell et al., 1997). What types of netrin-1 receptors may DCC possibly interact with *in cis*? A large family of netrin-1 receptors are homologues of the UNC-5 gene in *C.elegans*, comprised of two Ig domains and two thrombospondin type I domains. Three of the four UNC-5 homologues identified in vertebrates bind tightly to netrin-1 but none are expressed on commissural neurons (Ackerman et al., 1997; Leonardo et al., 1997). However, the fact that UNC-40, a DCC homologue in nematodes, functions together with UNC-5 to mediate repulsion of some axons/cells from the UNC-6 signal (Hamelin et al., 1993), opens the possibility that an

UNC 5 homologue, not yet identified but expressed on commissural axons, functions along side DCC to respond to netrin-1.

Domain C as a potential dimerization domain of netrin-1

Our experiments demonstrate that human immunoglobulin Fc, a dimerization domain, can partially substitute the function of netrin-1's domain C. We are certain that the ability of Fc to dimerize netrin-1(VI-V) is important because heterodimers of VI-V-Fc and Fc do not elicit outgrowth. These results suggest that domain C itself may be a dimerization domain. Two lines of evidence support this contention. First, the specific activity profile of the netrin-1 protein is bell shaped, active at low concentrations and inhibitory at high concentrations. This is characteristic of ligands, such as HGF, which activate receptors by dimerization because high concentrations saturate and monomerize the receptors (Ebens et al., 1996). High concentrations of netrin-1(VI-V) would also inhibit outgrowth because netrin-1(VI-V) would compete for receptor binding but would be unable to oligomerize receptors itself. Second, dimerization by domain C would explain the difference in fasciculation of axons responding to netrin-1 and those responding to netrin-1(VI-V). By forming dimers, netrin-1 could possibly bind two receptors on different axons bridging them into a fascicle.

If domain C does mediate netrin-1 oligomerization, it may do so directly, or indirectly by binding first to a co-factor. Our experiments do not support a direct dimerization model because the addition of a bifunctional crosslinker to netrin-1 and to netrin-1(VI-V) generates the same ratio of oligomerized products. Furthermore, rotary shadow electron micrographs of netrin-1 and netrin-1(VI-V) suggest that both are predominantly monomeric.

In order for domain C to dimerize netrin-1 molecules indirectly, domain C's of two netrin-1 molecules would have to simultaneously bind to one co-receptor. FGF1 and FGF2 function in this way. After binding to their high affinity receptors, FGF1 and FGF2

are thought to aggregate on the cell surface by binding heparin sulfate proteoglycans (HSPG) (Ornitz et al., 1992; Spivak-Kroizman et al., 1994; Herr et al., 1997) which then cause the FGF receptors to aggregate and activate. Soluble heparin can also clustering FGF, but only when the heparin molecules are large enough to bind simultaneously to two FGF ligand *in cis* (Moy et al., 1997). We have shown that a primary function of netrin-1(C) is to bind heparin, suggesting that netrin-1 may function like FGF and bind to cell associated proteoglycans to oligomerize. In support of this model, soluble heparin has been shown to enhance netrin-1 elicited axon outgrowth (unpublished results, M. Galko), as it does for FGF mediated functions (Ornitz et al., 1992).

The fact that VI-V-Fc and netrin-1(VI-V)semaIII(C) elicits only 3/4 of the outgrowth elicited by netrin-1 indicates that netrin-1(C) may have an additional function unrelated to oligomerization. Alternatively netrin-1(C) may oligomerize netrin-1 into larger complexes which are more affective in eliciting outgrowth than either of these constructs. This can be tested by measuring the outgrowth activity of VI-V-Fc clustered with anti-Fc antibodies.

Domain C independent dimerization of netrin

Rotary shadow electron micrographs of the netrin-1 and netrin-1(VI-V) proteins suggest that a small fraction of netrin-1 dimerizes directly through domains VI. Moreover, when deletion mutants containing domain VI are separated by electrophoresis, they migrate predominantly as monomers but always contain a minor doublet band. This domain VI mediated dimerization seems extremely stable allowing dimers to be purified from monomers. Netrin-1's domain VI shares 40% sequence identity with domain VI of the laminin B2 chain. In the presence of Ca^{++}, domain VI oligomerizes laminin trimers into a large network which sediments by centrifugation (Yurchenco and Cheng, 1993), suggesting that netrin-1(VI) may regulate Ca^{++} dependent oligomerization of netrin-1. However, we found that Ca^{++} was unable to dimerize monomeric netrin-1(VI-V) proteins.

The biological significance of netrin-1 dimerization by domain VI is unclear, but the modest representation of dimers together with the fact that doublets of netrin-1 have not been detected in extracts of floor plate (Chapter III, figure 1b) suggests that they play a limited role, if any, *in vivo*.

Conclusion

Our results indicate that domain v-3 is not required for DCC receptor binding or for eliciting axon outgrowth. In addition, domain VI-V can bind to DCC but is unable to elicit axon outgrowth, indicating that binding to DCC is not sufficient for outgrowth. However, dimers of VI-V can elicit outgrowth, suggesting that domain C itself may mediate the dimerization of netrin-1. Our results argue that domain C does not form direct dimers but that it may mediate dimerization indirectly by first binding to cell associated heparan sulfate proteoglycans. To extend these studies, it will be important to test whether heparan sulfate proteoglycans expressed on commissural axons are important for netrin-1 mediated axon outgrowth.

Methods and Materials

Construction of the parent expression plasmids, pCM39n and pCM54

Patch PCR (Squinto et al., 1990) was used to amplify a 150 base pair stuffer sequence with a SmaI site and a SalI site at its 5' end, and the myc epitope (with the sequence GGEQKLISEEDL), followed by a stop codon and a XhoI site the 3' end. This fragment was digested with SmaI and Xho I and cloned into the pGNET1^{myc} vector (Serafini et al., 1994) which had also been digested with SmaI and XhoI to excise the netrin-1 coding sequence (except for sequence encoding the signal peptide and the first 3 amino acids of the mature protein) to generate the expression plasmid pCM39n used to express the primary domains of netrin-1 (i.e., domains VI, V, and C).

Domain VI of netrin-1 was amplified (see below), digested with SmaI and SalI and cloned into the SmaI/SalI sites of pCM39n, generating pCM40n. Domain C of netrin-1 was amplified by PCR using primers which would introduce a Sal1 site at its 5' end and a XhoI site at its 3' end. This was then cloned into SalI site of pCM40n to generate pCM54, the expression vector used to express the EGF deletions of netrin-1.

Construction of the netrin-1 deletions

Primers with synthetic restriction sites (Xho1 on the 5' primer end and Sal 1 on the 3' primer) were designed to the three netrin-1 domains, and to each EGF repeat. The fragments were amplified by PCR using Vent polymerase, subcloned into the Bluescript vector, then sequenced to identify PCR errors. Only one error was introduced in at residue 601 (in domain C) and has been marked with an astrix in figure 1.

For transient expression, each domain was subcloned from bluescript into the Sal 1 site of the pCM39n or PCM54 vectors. Domains inserted in the correct orientation generated a Sal/Xho fusion at their 5' end and a Sal 1 site at their 3' end. A second PCR fragment could thus be inserted into the newly generated Sal 1 site.

To generate the netrin-1(VI-V)semaIII(C) fusion, the C terminal 134 amino acids of human semaphorin III were amplified by PCR utilizing the same strategy described above and placed into a pCM39n expression construct already containing netrin-1 domains VI and V.

Expression of the netrin-1 deletions

Each deletion construct was transfected into COS-1 cells (in 35mM wells) using LipofectAMINE as described (Kennedy et al., 1994). Upon removal of the transfection media, the monolayer was washed with PBS, then incubated for 3 days with 1.5 mls of OptimMEM I supplemented with GlutaMAX1 (GIBCO BRL) and antibiotics. After harvesting the conditioned medium, to which 1x protease inhibitors (1 µg/ml of each leupeptin, aprotinin, pepstatin) were added, the cells were rinsed with PBS, then incubated for 30 minutes with 1.5 ml extraction buffer (1 M NaCl, 20 mM NaPi [pH 7.5], 1x protease inhibitors). Media and extracts were centrifuged at 2000 x g for 10 min, then at 60,000xg for 100 min. These fractions were then TCA precipitated as described (Kennedy et al., 1994) then resolubilizing in SDS sample buffer. Half of the protein was subjected to immunoblot analysis using 9E10 supernatant directed to the myc epitope. Purified netrin-1 protein was diluted and used as a standard for immunoblotting to determine the concentration of proteins produced in each transfection.

Protein expression of the EGF deletions, and of domains V, C and V-C, was boosted by generating Ebstein Barr Virus extrachromosomal stables in 293 EBNA cells. Each construct was digested with EcoR1, treated with Klenow, then digested with Xho1 prior to subcloning into the PvuII and Xho I sites of PCEP4 (Invitrogen). These were each transfected into 293-EBNA cells using LipofectAMINE (GIBCO BRL) as directed and grown under selection (250 µg/ml Geneticin, 200 µg/ml Hygromycin B). To prevent loss of the extrachromosomal episomes, the transfected cells were continually maintained in selective media. To determine the levels of protein production, each cell line was grown in

35 mM wells, conditioned and processed exactly like COS-1 transfectants, except only 1/10- 1/60th of the samples were subjected to immunoblot analysis.

Immunofluorescence on transfected COS-1 cells

A COS-1 cell monolayer was transiently transfected in 35mm wells with either pGNET^{myc}, PCM41$^{v\text{-}myc}$, or pCM42$^{c\text{-}myc}$, then trypsinized and plated on in 8 well chamber slides (Lab-Tek) at a density of 1 x 104 cells/well overnight. In order to label secreted protein only, the cells were incubated with 9E10 culture supernatant for 1 hour, rinsed twice with PBS, prior fixation with 4% paraformaldehyde in PBS for 15 minutes at RT. These were then blocked with PHT solution (PBS, 1% heat-inactivated goat serum, 0.1% Triton X-100), washed 3 times (5'each) with PHT, incubated with a secondary antibody, CY3- conjugated goat anti-mouse IgG (Jackson ImmunoResearch; 1:350 in PHT solution), for 1 hr at RT, washed as before prior to mounting with Fluoremount G (Fisher).

Heparin Binding of the netrin-1 domains

COS-1 cell monolayers were transiently transfected in 100mm dishes with full length netrin-1 protein or with netrin-1, domains, VI, V, or C, and conditioned in serum free OptiMEM medium (GIBCO BRL) for 3 days. Upon harvesting the conditioned medium (to which 1x protease inhibitors were added), the cells were incubated with 5mls extraction buffer (1 M NaCl, 10 mM Hepes [pH 7.5]) for 30 minutes. Medium and extracts were pooled, centrifuged at 2000 xg for 10 min, then at 60,000 xg for 100 min and dialyzed extensively into binding buffer (10 mM Hepes [pH7.5], 300 mM NaCl).

4.5 mls of each protein mixture was then incubated with 0.4 ml of 50% heparin agarose beads (binding buffer) at 4°C for 3 hrs. After centrifugation of the heparin beads, the unbound supernatant was removed and TCA precipitated as described (Kennedy et al., 1994). The beads were then washed twice with binding buffer, and eluted with 1.5 M

NaCl in 10 mM Hepes [pH 7,5]. These too were TCA precipitates. All samples were resuspended in SDS running buffer and 1/3 of each was separated on a SDS polyacrylamide gel and subjected to immunoblot analysis with 9E10 antibodies.

Condition media and extracts for domains V and C were prepared as described above but this time dialyzed into 250 mM NaCl. 10 mls of each sample was loaded at a rate of 0.5 ml/min onto a 1 ml heparin Hytrap column (Pharmacia) preequilibrated with 10% buffer B (where buffer A, 10 mM Hepes [pH7.5], 150 mM NaCl; buffer B, 10 mM Hepes [pH 7.5], 2 M NaCl). The column was then washed with 10% buffer B for 10 minutes (at 0.5 ml/min), then eluted with a linear gradient from 10% B to 100% B over 20 column volumes, collecting 1 ml fractions into 1.5 ml siliconized polypropyline tubes. The salt concentration of each fraction was measured directly with a conductivity meter. The fractions were then TCA precipitated as described (Kennedy et al., 1994) and resolubilized in SDS lysis buffer. Half of the protein was loaded onto polyacrylamide gels and subjected to immunoblot analysis using the 9E10 supernatant.

Binding to DCC transfected 293 cells

Conditioned media and extracts for the deletions of the primary domains were prepared as described above, dialyzed extensively into PBS and tested for binding to DCC transfected 293 cells in the presence of 2μg/ml heparin as described previously (Keino-Masu et al., 1996).

To determine binding of the EGF domain deletions to DCC, the 293 cells stably transfected with each of the EGF deletion constructs were grown in 8 well chamber slides overnight. These cells were then incubated with 293 cell supernatants containing the extracellular domain of DCC fused with alkaline phosphatase (made by Yimin Zou in our laboratory). The cells were then rinsed rapidly with PBS and fixed with 4% paraformaldehyde for 15 minutes. Binding was detected using monoclonal antibodies to the DCC receptor. The cells were incubated in PHT buffer (PBS, 1% heat-inactivated goat

serum, 0.1%Triton X-100) containing 1μg/ml mab Ab-1 (Oncogene sciences). Cells were then washed 3 times (5 minutes each) with PHT, incubated with a Cy-2 conjugated secondary antibody to mouse IgG (1:350 in PHT), washed as before prior to mounting with Fluoremount G (Fisher).

E13 spinal cord explant culture in the outgrowth assay

Spinal cord explants were dissected and cultured as described previously (Tessier-Lavigne et al., 1988; Serafini et al., 1994). Conditioned medium and extracts from COS cells/293 cells transfected with each of the mutant constructs were pooled, and centrifuged as described above. Prior to assaying, these were either dialyzed into F12 medium (GIBCO BRL) or concentrated ~10-fold on a centricon-30 (Amicon) microconcentrator and added directly to the culture medium (the NaCl concentration was never made higher by more than 180 mM NaCl). Each domain was tested for outgrowth activity at concentrations ranging approximately from 0.1 ng/ml to 300 ng/ml.

For inhibition studies, basal levels of pure netrin-1 were present in the culture medium (6 ng/ml) but was supplemented with additional netrin-1 protein or netrin-1(VI-V) at the indicated concentrations. For inhibition within E11 spinal cord explants, spinal cords were dissected as described (Tessier-Lavigne et al., 1988) except the dorsal and ventral spinal cord were not separated and cultured in the presence of 3 μg/ml netrin-1 or 3-10 μg/ml netrin-(VI-V). Higher concentrations of netrin-1 could not be used because netrin-1 precipitate at high concentrations in physiological salt. After 40 hours, explants were fixed with 4% paraformaldehyde in PBS for 1 hr at room temperature and commissural axons were stained with mab 4D7 recognizing the TAG-1 protein (Dodd and Jessell, 1988) as described (Kennedy et al., 1994).

Expression and purification of netrin-1(VI-V) protein

Netrin-1(VI-V) DNA was transferred into the PCEP4 vector (Invitrogen) by digestion of PCM43$^{VI\text{-}V\ myc}$ with EcoR1, treating with Klenow, then digesting with XhoI prior to subcloning into the PvuII and XhoI sites of PCEP4 . The generated plasmid, PCEP4$^{VI\text{-}V\text{-}myc}$, was transfected into 293-EBNA cells, selected and maintained in medium containing 250 µg/ml Geneticin and 200 µg/ml Hygromycin. These cells were grown to confluence on fifty 15 cm plates, rinsed in PBS, then conditioned for 7 days in serum free OptiMEM medium (GIBCO-BRL). Upon harvesting the medium, 1 x protease inhibitors were added, the medium was centrifuged at 2000xg for 10 min, then at 60,000 xg for 100 min, filtered though a .2 mm filtration unit (Nalgene) and loaded onto a 2 heparin column (4 cm in length) pre-equilibrated with buffer A (20 mM NaPi [pH7.5], 150 mM NaCL). The column was washed first with 10% buffer B (20 mM NaPi [pH 7.5], 2 M NaCL), then with 17% buffer B, until UV absorption leveled. 1 ml fractions were collected as buffer B was raised to 28% over 20 column volumes. The column was then washed with at 28% B for 10 column volumes. 1 ml fractions were collected as we exposed the column to a second gradient: buffer B was raised to 32% over 10 column volumes. The conductivity of each fraction was measured directly using a conductivity meter. Fractions containing domains VI-V were identified by dot blot analysis using 9E10 supernatants, and were combined into two pools (containing 530 mM and 650 mM NaCl) and concentrated ~10-fold on a centricon-30 (Amicon) microconcentrator. ~1ml of each was analyzed by silver staining and subjected to immunoblot analysis using 9E10 supernatant. As estimated by silverstained gels, VI-V protein comprised ~90% of the total protein in the 530 mM heparin eluate.

For bioactivity studies, both pools were directly added into the culture medium at VI-V concentrations from 1 ng/ml- 10 µg/ml. The remainder was stored over a two month period at -20 C in storage buffer (50% glycerol, 1.3M NaCl, 20mM phosphate pH 7.5). To test if the VI-V doublet was sensitive to Ca^{++}, netrin-1(VI-V) protein was incubated at 2 µg/ml in 20mM TrisCl [pH 7.0], 150mM NaCl in the presence or absence of 1mM Ca^{++}

for 1 hour at 37°C. Loading buffer was added, the samples were boiled for 3 min, prior to separation on a 7.5% acrylamide gel and to performing immunoblot analysis using 9E10 culture supernatants.

Purification of VI-V-Fc/VI-V-Fc from VI-V-Fc/Fc

1 x 10^6 COS-1 cells were plated on each of five 10 cm dishes, transfected simultaneously with the VI-V-Fc and Fc constructs (Keino-Masu et al., 1996), then conditioned for three days with OptiMEM medium without serum (GIBCO BRL). Upon collection of the conditioned medium, 1 x protease inhibitors were added. The medium was centrifuged, loaded onto a heparin column and eluted exactly as described above. Fractions containing domains VI-V were identified by dot blot analysis using rabbit polyclonal antibodies to netrin-1(VI-V) (1:1000). The fractions for each VI-V peak (the first spanned 450 mM NaCl to 530 mM NaCl, the second spanned from 650 mM NaCl to 686 mM NaCl) were combined, concentrated in centricon-30 microconcentrators (Amicon), and analyzed by silver staining under reducing conditions, and by immunoblot analysis under reducing and non-reducing conditions as described (Kennedy et al., 1994; Serafini et al., 1994). Samples analyzed under non-reducing conditions were solubilized in sample buffer containing no β-mercaptoethanol and were not boiled prior to loading onto the polyacrylamide SDS gel.

Crosslinking studies

Purified netrin-1 protein and VI-V protein were diluted to 50-100 ng/ml in PBS and incubated with 0, 3.7 μg/ml, or 37 μg/ml fresh Bis(sulfosuccinimidyl)suberate (BS3) (solubilized in DMSO) for 30 min at 37°C prior to quenching with 1 M TrisCl [pH 7.5] (added to 1/10 th the volume of the reaction). After addition of the SDS sample buffer, 1/5th of the sample was separated on a 6% polyacrylamide gel and subjected to immunoblot analysis using 9E10 supernatant directed to the myc epitope.

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Chapter V

Netrin-1 as a chemoattractant of commissural axons: is turning mechanistically linked to outgrowth?

Abstract

Netrin-1 is a multifunctional molecule capable of promoting the outgrowth and turning of spinal commissural, retinal, and cortical axons. Retinal axons turn towards a gradient of pure netrin-1 protein, and turning (like outgrowth) is mediated by the DCC receptor. However, it is not known whether commissural axons are also attracted to pure netrin-1 protein and whether commissural axon outgrowth and turning are mechanistically linked through the same DCC receptor pathway . In this study, we show that regenerating commissural axons from stage E13 grow directionally towards netrin-1 and that they can respond towards gradients of pure netrin-1 protein. In addition, we have shown that a mutant of netrin-1, partially defective for eliciting outgrowth, can elicit turning of commissural axons. Furthermore, under conditions where anti-DCC antibodies effectively block netrin-1 elicited outgrowth activity, turning is only partially blocked. These results suggest that netrin-1 activates two separate signaling pathways on commissural axons: a DCC-dependent pathway which mediates commissural axon outgrowth, and a DCC-independent pathway required for detecting gradients of netrin-1.

Introduction

Developing axons are guided to their intermediate and final targets by molecular cues present in their extracellular environment (Tessier-Lavigne and Goodman, 1996). As early as 1893, Ramon y Cajal proposed that some cues may be present in the form of diffusible gradients, secreted by target cells to attract axons from a distance. Evidence for diffusible attractants (i.e. chemoattractants) came from studies in which neurons were co-cultured with target cells at a distance within a collagen matrix. Collagen is thought to stabilize long range gradients, allowing diffusible cues to affect the growth of neurons at a distance (Ebendal and Jacobson, 1977). In this way, a diffusible floor plate derived cue was shown to elicit outgrowth of spinal commissural axons from a dorsal spinal cord explant (Tessier-Lavigne et al., 1988); maxillary epithelium was shown to elicit outgrowth of trigeminal sensory axons (Lumsden and Davies, 1983; Lumsden and Davies, 1986); and basilar pons was shown to cause turning of cortical axons from a distance (O'Leary et al., 1991). Chemotropic cues do not always attract developing axons. Septum secretes a factor which repels olfactory axons (Pini, 1993), ventral spinal cord explants repel sensory axons from dorsal root ganglia (Fitzgerald et al., 1993) and floor plate cells repel spinal motor axons (Colamarino and Tessier-Lavigne, 1995a; Guthrie and Pini, 1995). Nor are chemotropic cues always directed to the growth cone. For instance, basilar pons also causes directional branching of collaterals from corticopontine axon shafts (O'Leary et al., 1991).

The recent identification of a number of chemotropic cues and their receptors has provided a starting point for analyzing how axons detect and respond to molecular gradients. Netrin-1, the first diffusible guidance cue isolated, was purified based on its ability to elicit commissural axon outgrowth from dorsal spinal cords when presented at uniform concentrations (Serafini et al., 1994). Despite its tight cell binding properties, netrin-1 can diffuse from transfected COS cells and elicit commissural axon outgrowth from dorsal spinal cord explants cultured at a distance within a collagen matrix.

Furthermore, netrin-1 transfected cell aggregates can reorient E11 commissural axons within dorsal spinal cord explants from a distance up to ~250 μm (Kennedy et al., 1994), indicating that netrin-1 is a chemoattractant for pioneer commissural axons *in vitro*. Finally, defects in the guidance of commissural axon in netrin-1 -/- mice suggest that netrin-1 in part functions as a chemoattractant *in vivo* (Serafini et al., 1996).

Unfortunately, little is known about the mechanism by which netrin-1 attracts commissural axons. To begin with, we do not know whether later born commissural axons maintain a response to the netrin-1 protein. *In vivo*, follower axons follow the path laid by pioneers, but may do so by fasciculating with early pioneers. In this study, we have asked if commissural axons from E13 dorsal spinal cords are capable to growing towards increasing gradients of netrin-1 *in vitro*. Furthermore, we do not know whether pure netrin-1 protein, in the absence of possible accessory factors produced by COS cells, can reorient commissural axons. Finally, we do not know how the outgrowth promoting and reorienting activities of netrin-1 are related. Does netrin-1 activate different signaling pathways in commissural neurons and in this way elicit two different responses? Or are outgrowth and turning activity mechanistically related? For example, gradients of netrin-1 may cause outgrowth receptors to localize to one side of the growth cone, forcing directional growth to occur. In such a scenario, the receptors which mediate outgrowth would be necessary for axon turning. In this respect, it is interesting that HGF, a chemoattractant for developing motor axons, also elicits axon outgrowth at uniform concentrations (Ebens et al., 1996) and that semaphorin III, a chemorepellent for sensory axons in the CNS and PNS, inhibits outgrowth at uniform concentrations by causing growth cone collapse (Luo et al., 1993; Messersmith et al., 1995).

From the netrin-1 deletions constructed in chapter IV, we have identified a mutant, netrin-1(VI-V), which elicits greater axon outgrowth of commissural axons when it is presented from a point source. However, the total amount of outgrowth elicited by netrin-1(VI-V) is still less than that elicited by netrin-1, allowing us to test whether outgrowth

activity is important for turning activity. Our results suggest that the outgrowth and turning activities may be mediated by different signaling pathways.

Results

Netrin-1 is a chemoattractant for commissural axons at stage E13

Netrin-1 can change the direction of E11 commissural axons within the dorsal spinal epithelium (Kennedy et al., 1994). These axons normally grow dorsal-ventrally in an orderly array, but when co-cultured with netrin-1 cell aggregates, they reorient towards netrin-1. Unfortunately, the growth of older or regenerating commissural axons within E13 dorsal spinal cord explants is disordered and changes in their trajectory would be difficult to detect. However, commissural axons from the E13 explant into collagen from all sides evenly, allowing us to ask whether outgrowth can be made directional by gradients of netrin-1. E13 dorsal spinal cord explants were co-cultured with 293-EBNA $^{\text{netrin-1}}$ cell aggregates cut to ~1.2 mm^2. The percentage of axon bundles exiting the explant from the side facing towards and away from the aggregate was measured. Axonal outgrowth is directional with 60% of the bundles per explant growing towards the netrin-1 cells (figure 1a). Directionality in outgrowth is not due to differences in the absolute value of netrin-1 concentration at the proximal and far sides of the explant because explants distant from netrin-1 source also grow directionally.

The shape of the netrin-1 gradient depends on the size of the netrin-1 source. To confirm that E13 commissural axons respond to gradients of netrin-1, we asked whether directional growth would increase when netrin-1 was presented from a smaller aggregate. Directional outgrowth was measured towards three sizes of netrin-1 aggregates, 400 μm^2 800 μm^2 and 1.2 mm^2 [3]. The two larger aggregates were comprised of netrin-1 cells diluted with untransfected 293 cells so that the amount of netrin-1 produced would be kept constant. As a result, the total amount of axon outgrowth was roughly equivalent (not shown). However, directionality in outgrowth varied. Only 59% of axon bundles grow towards the 1.2 mm^2 aggregate, 79% grow towards the 800 μm^2 aggregate, and 87 % of

[3] The thickness of these cell aggregates was kept constant in all experiments by standardizing the prepartation of the hanging drops (see methods and materials).

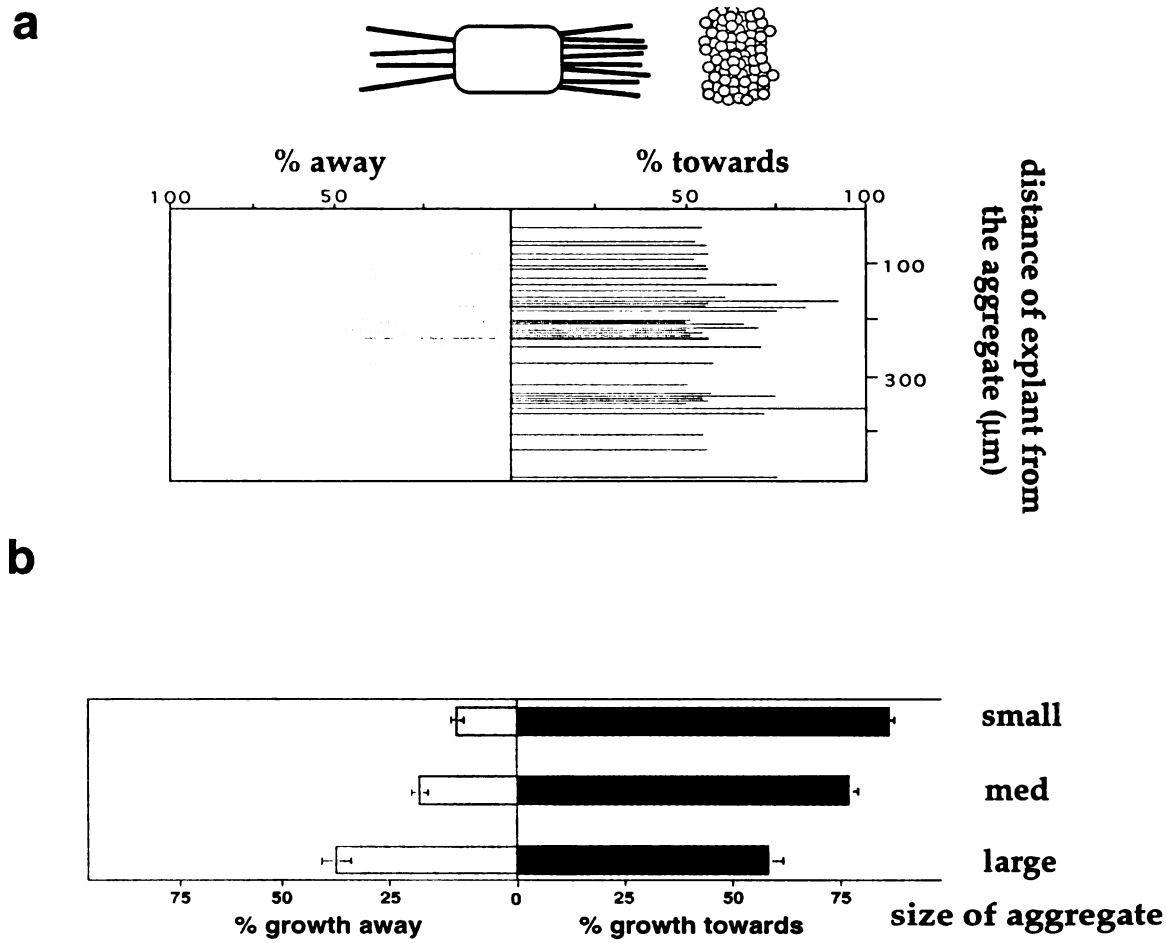


Figure 1. E13 commissural axons grow towards a source of netrin-1. (a) E13 dorsal spinal cord explants were co-cultured with a large aggregate (~1.2mm2) of COS-1 cells transfected with netrin-1, and the percentage of axon bundles growing towards (blue) and away (yellow) from the aggregate was measured from each explant (x-axis). In addition, the distance of each explant from the netrin-1 source is shown on the y-axis. (b) E13 dorsal spinal cord explants were co-cultured with small (400µm2), medium (800µm2) and large (1.2mm2) aggregates of 293 cells stably transfected with netrin-1. The larger aggregates were diluted with untrasfected 293 cells so as to keep the total amount of netrin-1 produced constant. For each condition, the percentage of axon bundles growing towards (blue) and away (yellow) from the netrin-1 source was measured from 8 explants. The means and standard errors are shown.

the axons grow towards the 400 μm^2 aggregate (figure 1b), suggesting that the shape of the netrin-1 gradient alters how E13 commissural axon are attracted to netrin-1.

Commissural axons are attracted to gradients of pure netrin-1 protein

Pure netrin-1 protein secreted from a pulsatile pipette is a chemoattractant for dissociated retinal ganglion axons and causes turning of their growth cones within 15 minutes (de la Torre et al., 1997). However, it is not known whether pure netrin-1 protein can attract commissural axons. Netrin-1 has always been presented to spinal commissural axons from an aggregate of transfected cells, which probably secrete many other proteins. Here, we have tested whether directional outgrowth of E13 commissural axons occurs towards pure netrin-1 protein. To present netrin-1 from a point source heparin acrylic beads were preincubated with netrin-1 or with PBS, then co-cultured with dorsal spinal cord explants (figure 2). In contrast to beads incubated in PBS, beads bound with netrin-1, elicited robust and directional outgrowth of commissural axon bundles (2b). Directional outgrowth also occurred when netrin-1 was bound to avidin acrylic beads[4] (2c) verifying that the presence of heparin *per se* is not important for attraction.

Netrin-1(VI-V) elicits greater axon outgrowth when it is presented from a point source

Netrin-1(VI-V), a deletion mutant of netrin-1 lacking the C terminal basic domain, elicits little commissural axon outgrowth from E13 dorsal spinal cord explants when presented uniformly in the culture medium but as we will next describe, seems to elicit greater outgrowth when it is presented from a point source. In figure 3a (right) pure netrin-1(VI-V) protein has been prebound to a heparin acrylic bead and cocultured with E13 dorsal spinal cord explants. Commissural axons grow directionally into the collagen towards the bead. Uniform netrin-1(VI-V) protein inhibits this outgrowth (figure 3b,

[4] The use of avidin beads was piloted by Orion Weiner, a rotation student in our laboratory.

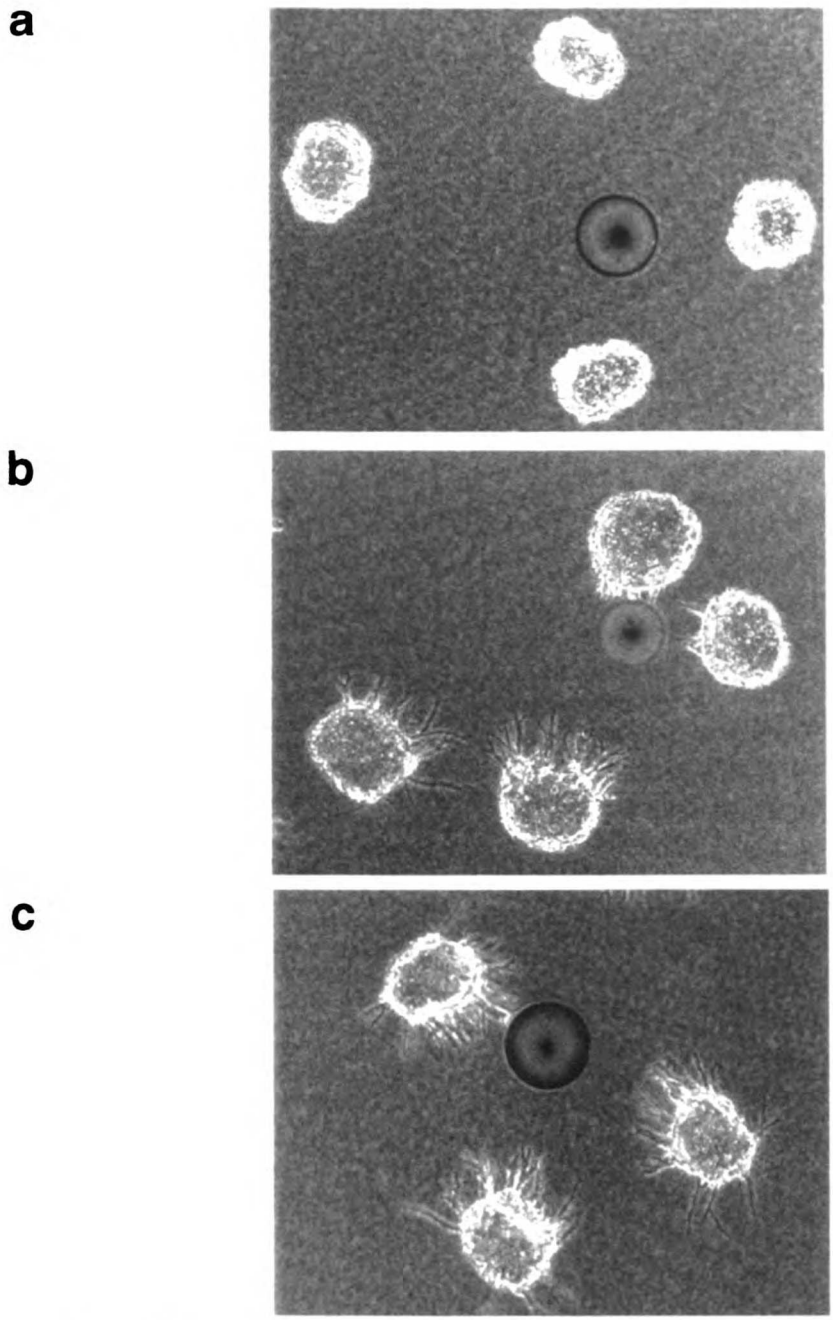


Figure 2. Pure netrin-1 protein elicits directional growth of commissural axons from E13 dorsal spinal cord explants into collagen. Commissural axon outgrowth from E13 dorsal spinal cord explants elicited by a heparin acrylic bead alone (a), a heparin acrylic bead preincubated with 100ng netrin-1 protein, or an avidin acrylic bead preincubated with 100ng netrin-1 protein. Directional outgrowth occurs towards the beads preincubated with netrin-1.

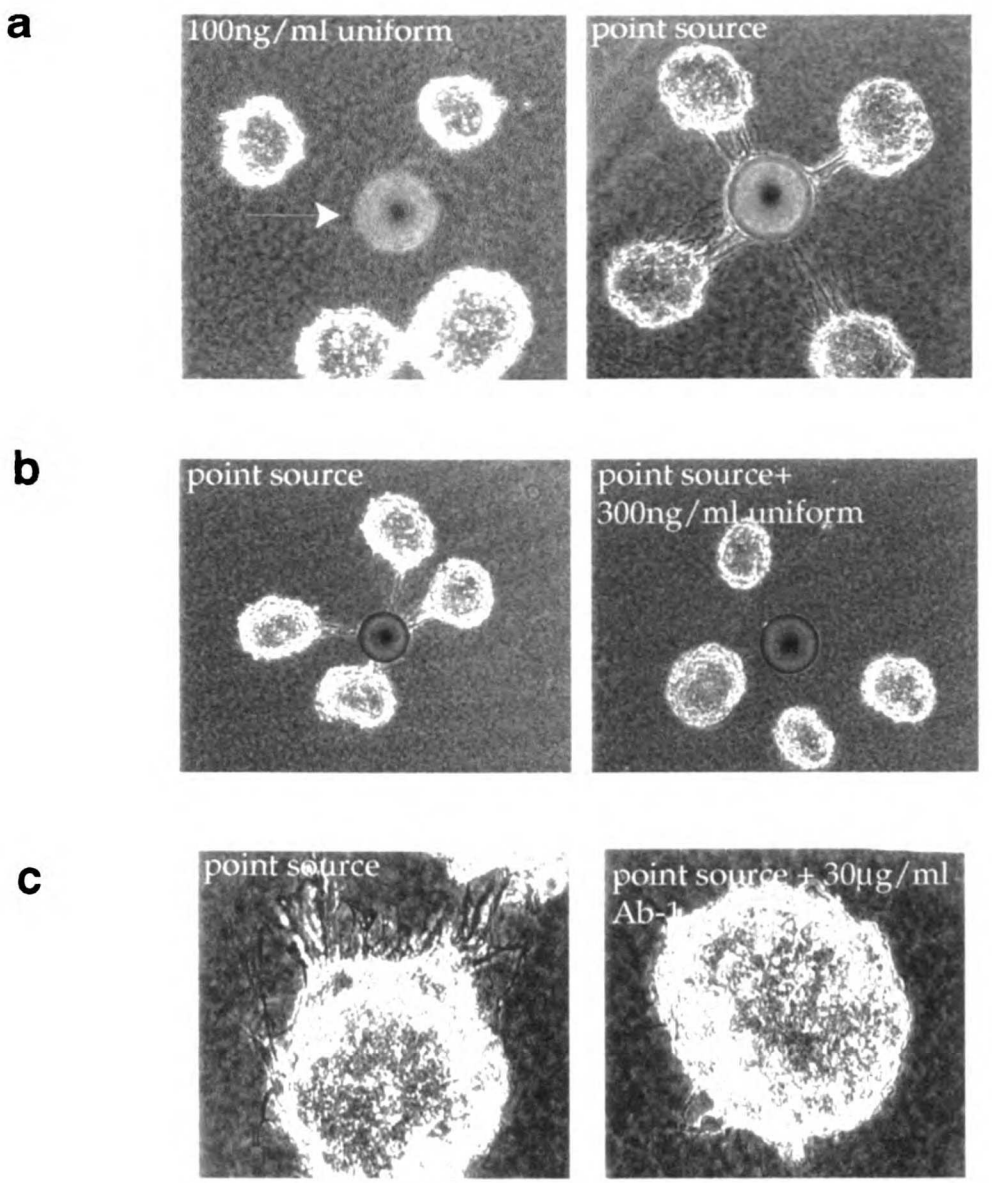
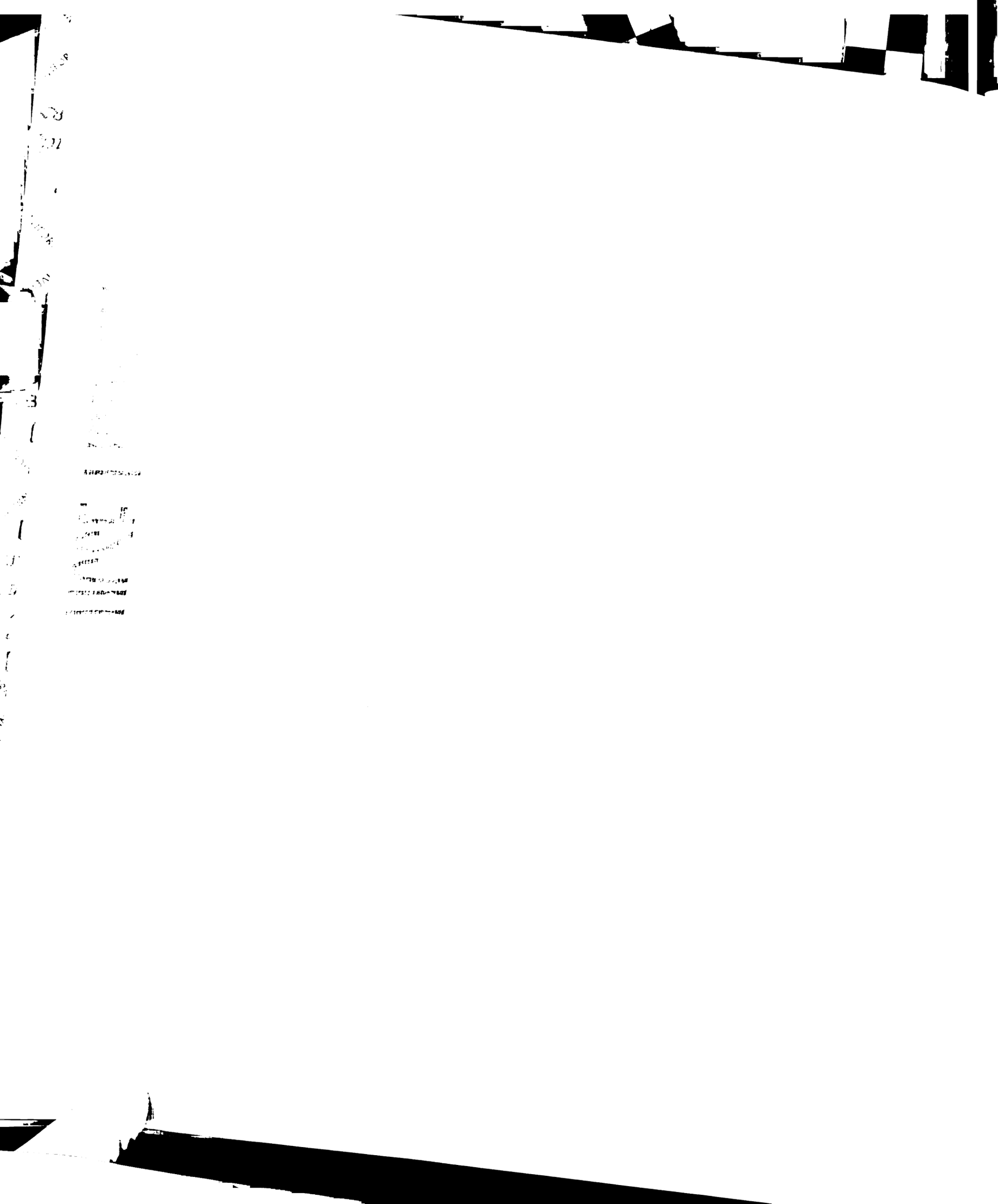


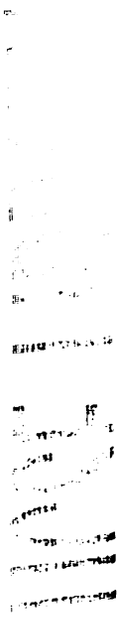
Figure 3. Netrin-1(VI-V) elicits more outgrowth when presented from a point source. Dorsal spinal cords explants were dissected from E13 rat embryos and co-cultured with a heparin acrylic bead (a, uniform) or heparin acrylic beads preincubated with 100ng of pure netrin-1 (VI-V) protein (a, right; b, right and left). We have indicated whenever netrin-1(VI-V) was also presented uniformly in the conditioned medium. Panel (a) shows that netrin-1(VI-V) elicits significantly more outgrowth when presented from a point source (right) than it does when presented uniformly (left). Outgrowth is low when netrin-1(VI-V) is presented uniformly irrespective of its concentration (see chapter IV). Panel (b) shows that the outgrowth elicited by a point source of netrin-1(VI-V) (left) can be inhibited by 300ng/ml uniform netrin-1(VI-V) protein(right). In pannel (c) , outgrowth of commissural axons has been elicited by aggregated 293 cells stably transfected with netrin-1(VI-V) (top). As shown onthe right, this outgrowth can be blocked with mabAb-1, a monoclonal antibody to DCC.



compare the right and left panels), confirming that the presentation of netrin-1(VI-V), and not simply its concentration, is important for outgrowth activity. Any heparin which may be released from the bead is unlikely to increase the activity of netrin-1(VI-V), because uniform concentrations of netrin-1(VI-V) preincubated with heparin elicits little outgrowth (not shown). Also, a heparin acrylic bead alone does not elicit axon outgrowth when netrin-1(VI-V) is presented uniformly (figure 3a, left), indicating that gradients of heparin do not attract commissural axons, even in the presence of netrin-1(VI-V) protein. Thus, netrin-1(VI-V) elicits substantial outgrowth when presented from a point source, even though it functions poorly when presented uniformly.

As shown previously, netrin-1 elicited axon outgrowth of commissural axons into collagen is inhibited by 10 μg/ml of Ab-1, a monoclonal antibody to the extracellular domain of the DCC receptor. Mab Ab-1 also seems to inhibit outgrowth elicited by netrin-1(VI-V), here presented from a cell aggregate, suggesting that the DCC receptor is involved in the commissural axon response towards netrin-1(VI-V) (figure 3c).

Within its active concentration range, Netrin-1(VI-V) elicited outgrowth differs from netrin-1 elicited outgrowth in three ways. First, commissural axons are poorly fasciculated as they grow into the collagen. This means is that larger numbers of thinner bundles invade the collagen. Second, the total outgrowth elicited by netrin-1(VI-V) is less than that elicited by netrin-1. Third, axonal growth is more directional towards netrin-1(VI-V). Axons emerge from the E13 explant only from the region directly facing the bead (compare figure 2 with figure 3). Differences in directionality of outgrowth between netrin-1 and netrin-1(VI-V) are most evident when E13 explants are co-cultured with large aggregates of transfected cells. As shown previously, only 60% of commissural axons grow towards 1.2 mm^2 aggregates of netrin-1 transfected cells (figure 4). This contrasts with 89% of commissural axons bundles which grow towards netrin-1(VI-V) cells also presented from a 1.2 mm^2 aggregate. Thus, deletion of domain C decreases outgrowth but



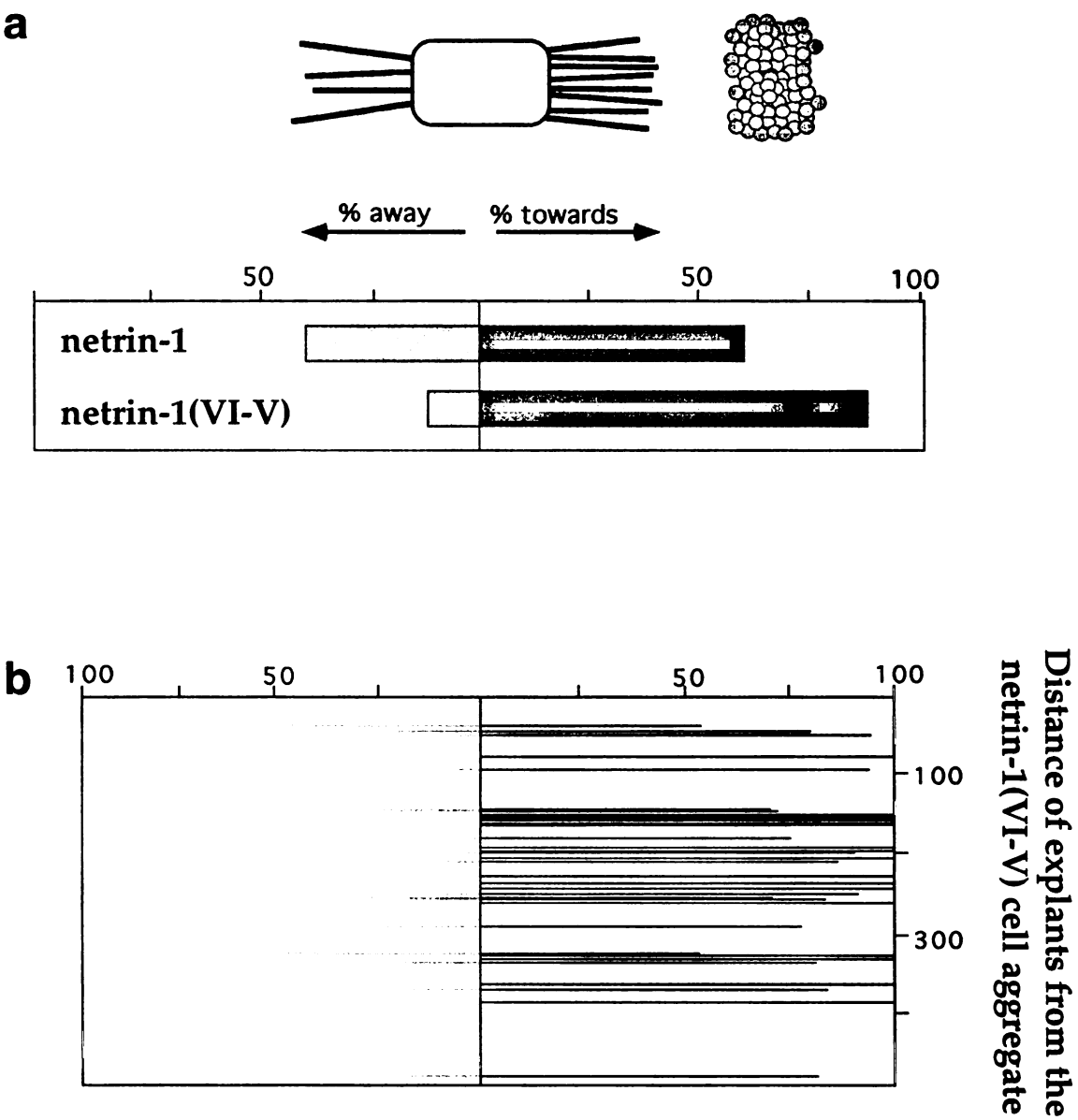
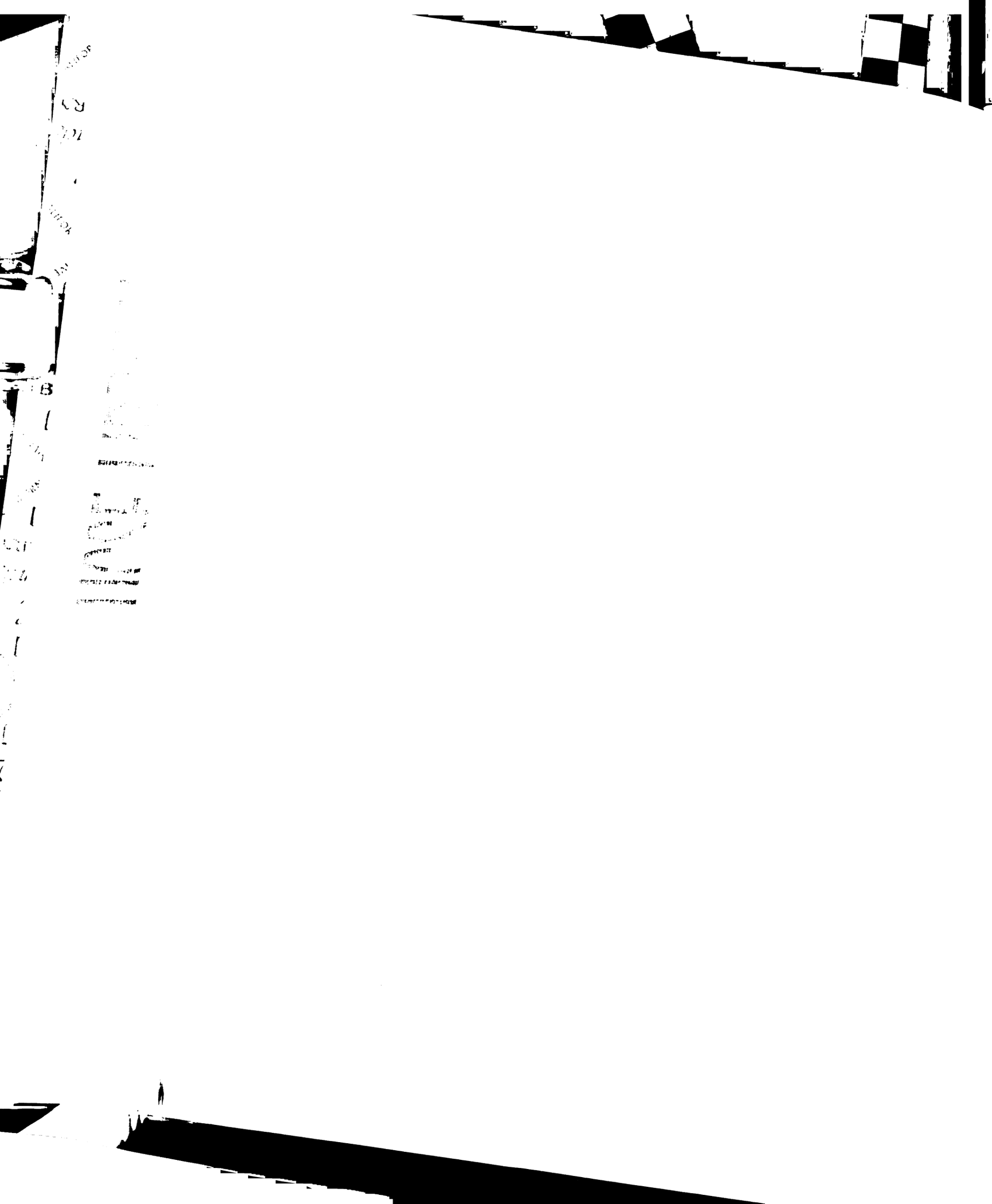


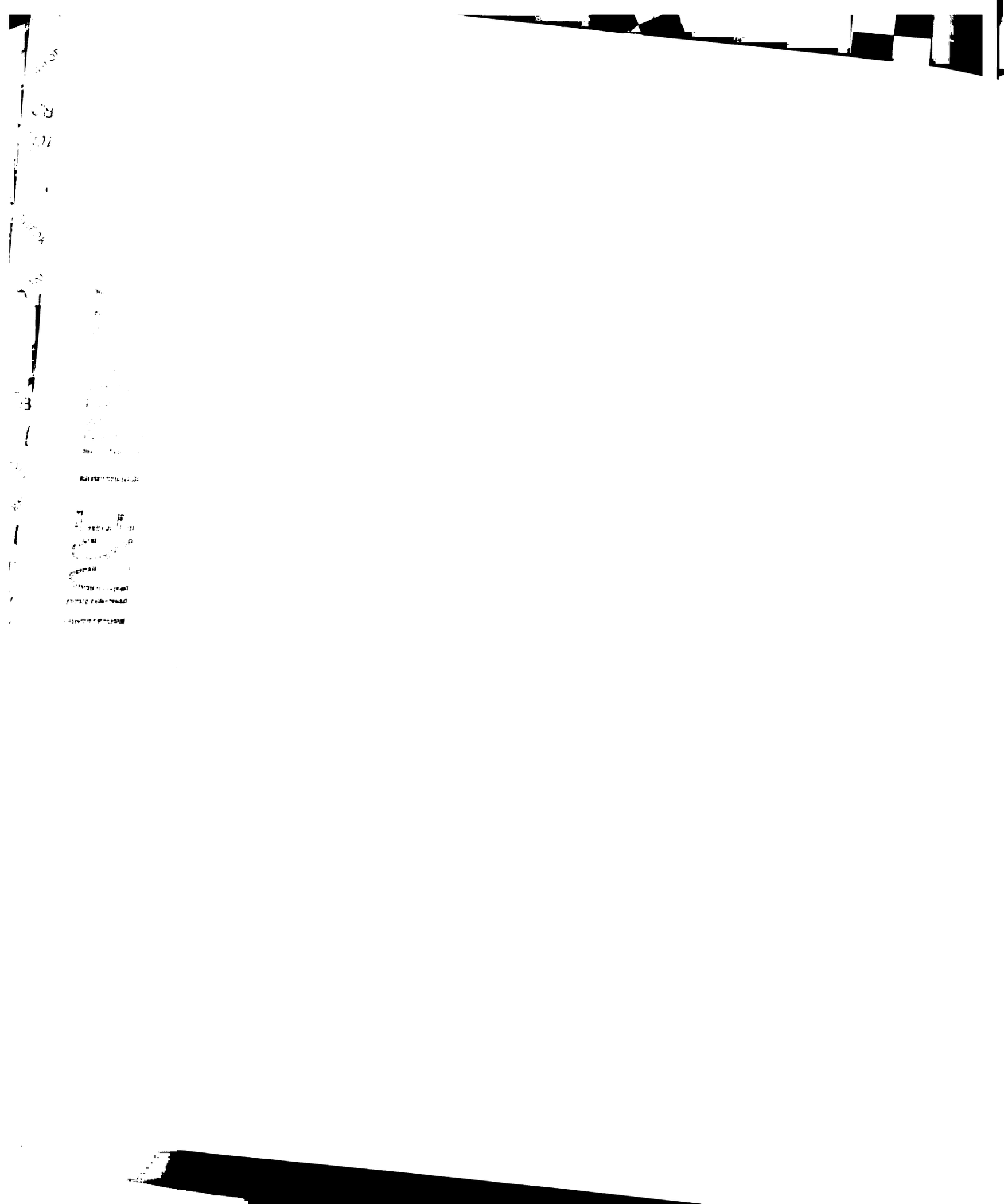
Figure 4. Netrin-1(VI-V) cell aggregates elicit more directional growth of spinal commissural axons than do netrin-1 cell aggregates. (a) Dorsal spinal cord explants were dissected from E13 rat embryos and co-cultured within collagen gels with large COS-1 cell aggregates (1.2mm2) transiently transfected with either netrin-1 or netrin-1(VI-V). From each explant, the percentage of axon bundles growing towards (blue) or away (yellow) from the cell aggregates was measured. As shown previously, axon bundles grow with only slight directionality towards netrin-1 if it is presented from a large cell aggregates. However, axon bundles grow with strong directionality towards netrin-1(VI-V) when it is presented from an equally large source. (b) Within 400μm, the percentage of outgrowth towards (blue) or away (yellow) from netrin-1(VI-V) (x-axis) is independent of the distance of the explant from the netrin-1(VI-V) source (y-axis).



does not diminish directionality in outgrowth, suggesting that these two responses are mechanistically separate.

Netrin-1(VI-V) and VI-V-Fc cause an equivalent degree of turning of E11 commissural axons

Even when netrin-1(VI-V) is presented from a point source, axon outgrowth is not as robust as the outgrowth elicited by netrin-1. Thus, using netrin-1(VI-V) as a tool, we asked whether strong outgrowth activity is important for netrin-1 elicited turning of commissural axons within the E11 dorsal spinal cord. When aggregates of COS cells transfected with netrin-1 are cultured at the cut edge of a dorsal spinal cord explant, TAG-1 expressing commissural axons reorient towards netrin-1 up to a distance of 250 μm. In contrast, aggregates of untransfected COS cells do not elicit turning (Kennedy et al., 1994). Like netrin-1, netrin-1(VI-V) transfected cells reorient commissural axons from their dorsal-ventral trajection (figure 5). While turning occurs at a shorter distance than that elicited by netrin-1 (205μm +/- 20μm SE), the angle of deflection is steep and turning occurs consistently in all explants tested (n=16), suggesting that netrin-1(VI-V) is an efficient chemoattractant for E11 commissural axons. Moreover, enhancing netrin-1(VI-V)'s axon outgrowth activity 2-3 fold, by expressing netrin-1(VI-V) as a fusion with human immunoglobulin Fc, does not seem to affect turning. Turning towards aggregates of VI-V-Fc cells (183μm +/- 12μm SE where n=16) is identical to turning towards netrin-1(VI-V) (figure 5). These results suggest strong axon outgrowth is not required and does not enhance the commissural axon turning response. While these results do not provide a definitive answer, they do suggest that the permissive and instructive activities of netrin-1 are mediated by different signaling pathways. Consistent with the presence of two signaling pathways, NSA (netrin-1 synergizing activity) (Serafini et al., 1994), an activity in extracts of embryonic chick brains, increases the axon outgrowth activity of netrin-1 on



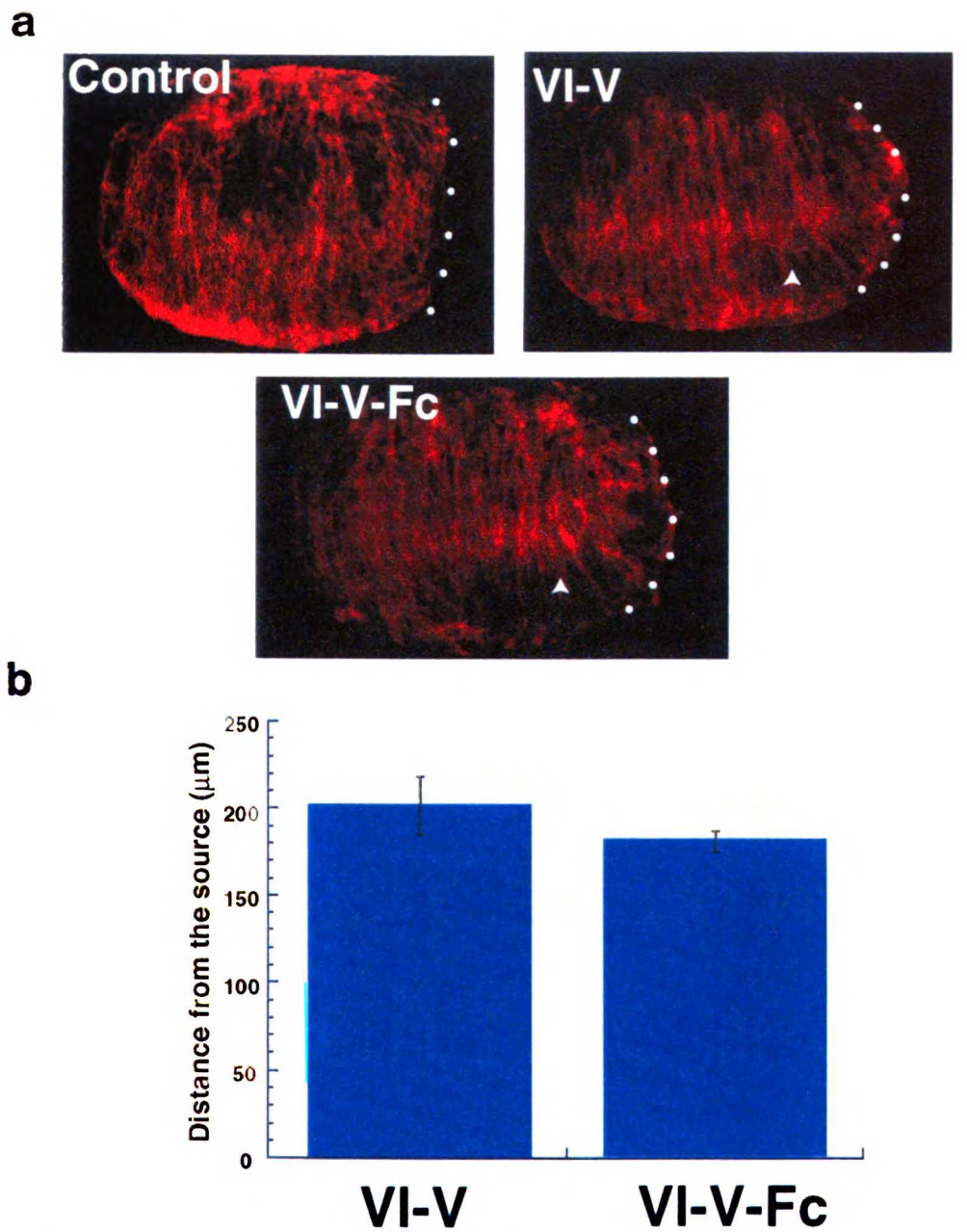
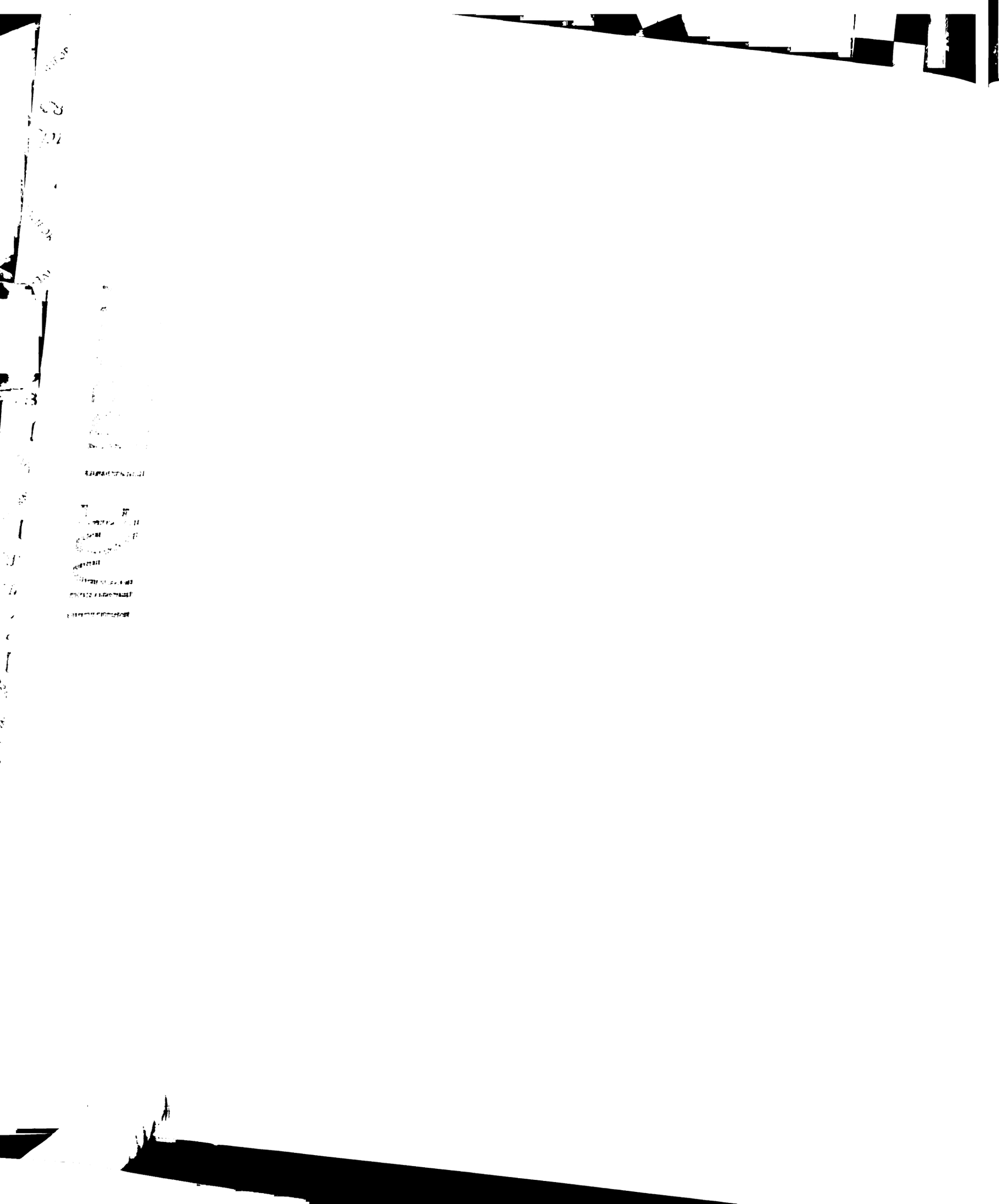


Figure 5. Commissural axons turn towards aggregates of COS cells transfected with netrin-1(VI-V) and VI-V-Fc. (a) COS-1 cells transiently transfected with netrin-1(VI-V) or VI-V-Fc were aggregated and co-cultured along the cut edge (white dots) of an E11 dorsal spinal cord explant. The trajectory of the commissural axons within the explant was visualized by indirect immuno-fluorescence using antibodies directed to TAG-1. Aggregates of control cells have no affect on the dorsal ventral growth of commissural axons, whereas aggregates of netrin-1(VI-V) and VI-V-Fc elicit axon turning (arrow). (b) The distance of commissural axon turning was measured from 18 explants each towards netrin-1(VI-V) and VI-V-Fc cell aggregates. The mean distance of turning and the standard errors are shown.



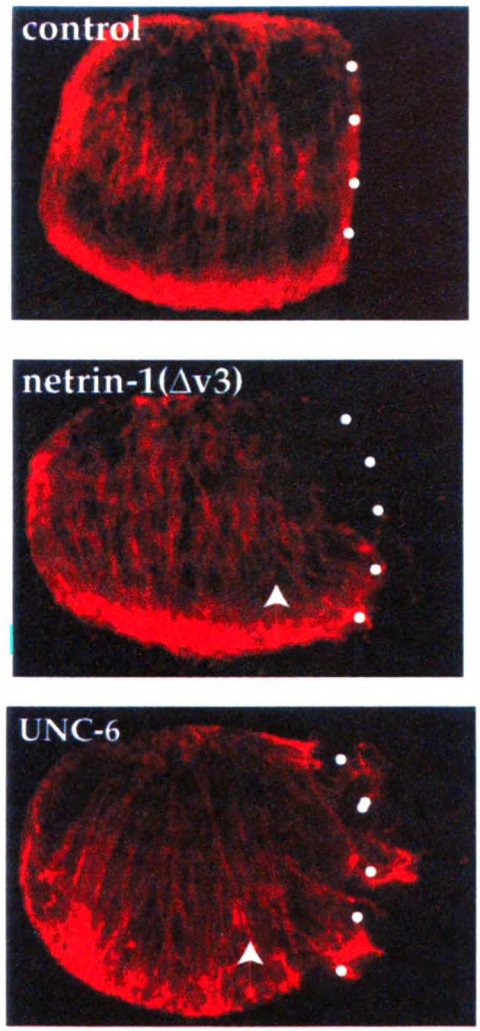
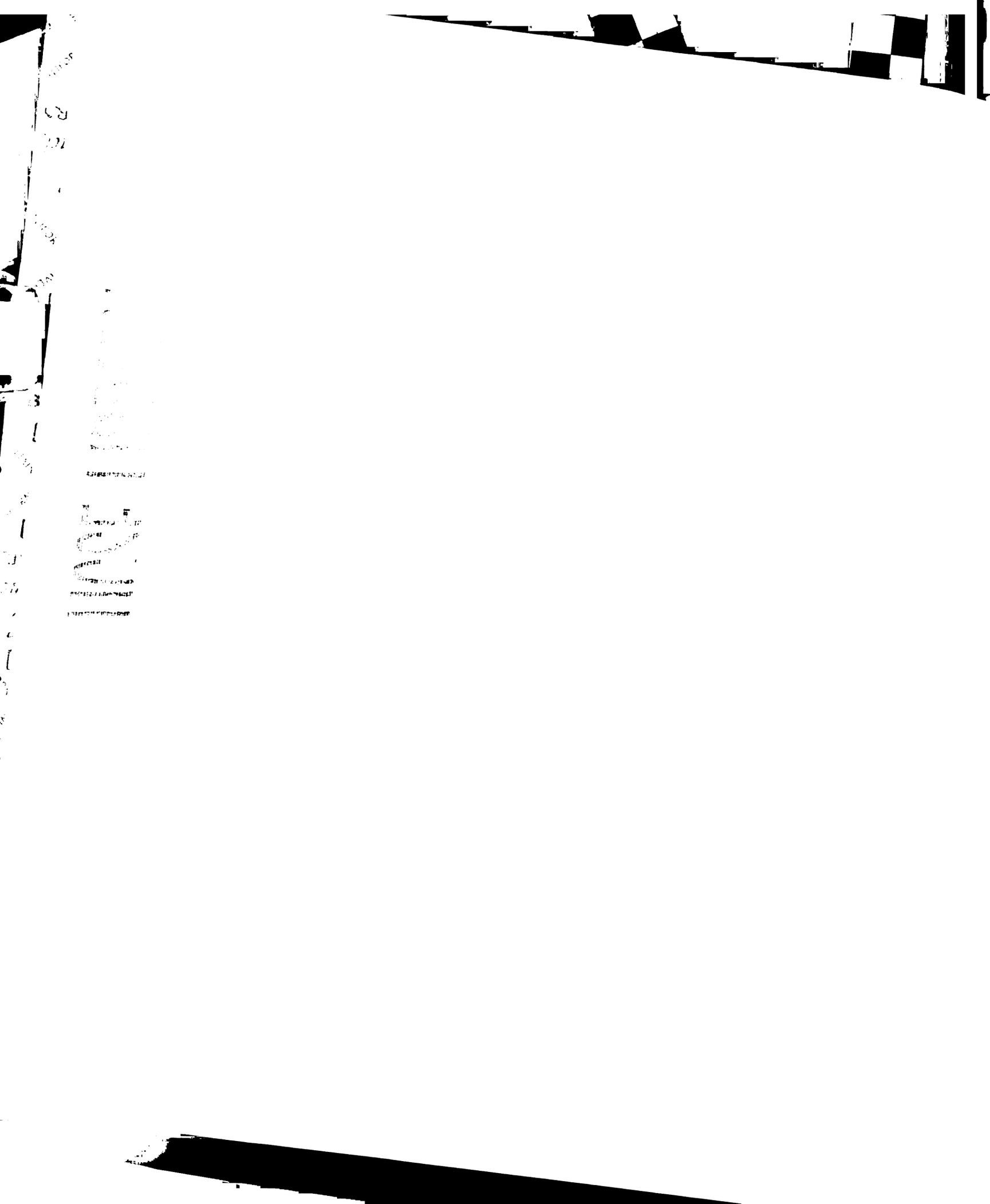


Figure 6. Commissural axons turn towards cells transfected with netrin-1(Δv-3) and the nematode netrin, UNC-6. (a) 293 cells stably transfected with netrin-1(Δv-3) or COS-1 cells transiently transfected with UNC-6 were aggregated and co-cultured along the cut edge (white dots) of an E11 rat dorsal spinal cord explant. The trajectory of commissural axons within the explant was visualized by indirect immunofluorescence using antibodies directed to TAG-1. Aggregates of control cells (293 cells shown) have no affect on the dorsal-ventral growth of commissural axons within the cord, whereas aggregates of netrin-1(Δv3) and UNC-6 producing cells elicit commissural axon turning (arrow).



E11 commissural axons but does not seem to affect commissural axon turning towards netrin-1 (M. Galko, unpublished results).

In addition to netrin-1(VI-V), we have identified two other netrin derived proteins which elicit commissural axon turning. Cell aggregates transfected with UNC-6 (the nematode homologue in *C.elegans*) and cell aggregates transfected with netrin-1(Δv3) elicit commissural axon turning (figure 6). In the case of netrin-1(Δv3), protein expression is relatively poor, preventing us from exhaustively testing turning activity. However, whenever protein levels were high enough to elicit axon outgrowth from E11 dorsal spinal cord explants, turning also occurred (n=3).

Antibodies to DCC which blocks the netrin-1 elicited outgrowth do not block netrin-1 elicited turning

Netrin-1 elicits outgrowth of commissural axons from dorsal spinal cord explants into collagen (Serafini et al., 1994). DCC is required for this activity since a monoclonal antibody to DCC, Ab-1, blocks outgrowth. In contrast, mab Ab-1 does not seem to inhibit the turning of commissural axons towards netrin-1 transfected cell aggregates (Keino-Masu et al., 1996). However, in these experiments, a failure to block turning may have been a reflection of the failure of mab Ab-1 to penetrate the dorsal spinal cord explant, where turning occurs. In light of recent studies showing that Ab-1 interferes with turning of retinal ganglion axons towards netrin-1 (de la Torre et al., 1997), we repeated these experiments more thoroughly and with the appropriate controls.

In both netrin-1 and DCC null mice, commissural axons show reduced outgrowth into the ventral spinal cord. We tested if mab Ab-1 can penetrate spinal cord explants cultured in collagen gels, by asking if it could inhibit commissural axon growth into the ventral spinal cord. Whole E11 spinal cords were cultured in collagen gels for 40 hrs, either alone or in the presence of 10-30 μg/ml mab Ab-1. The trajectory of commissural axons was then visualized using antibodies to TAG-1. Commissural axons in control

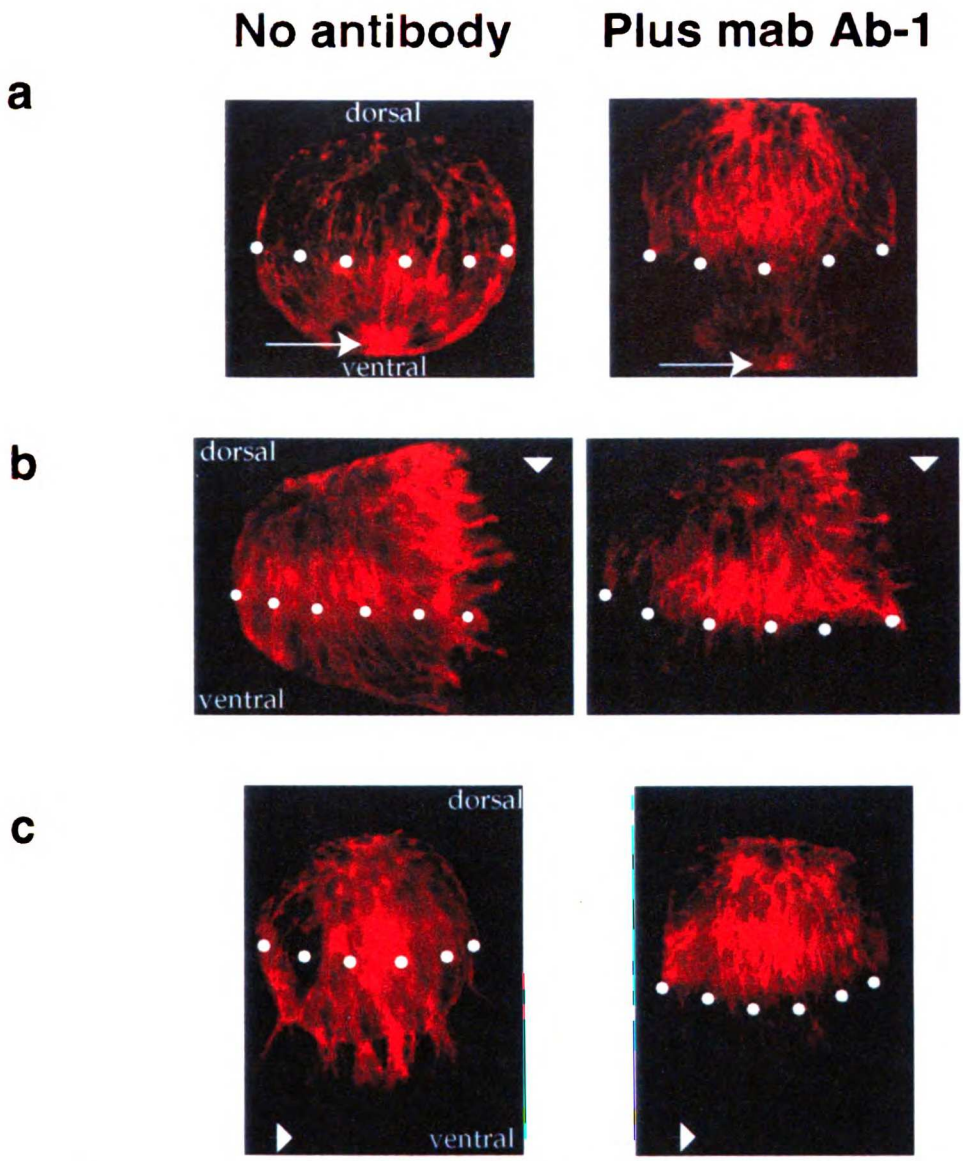
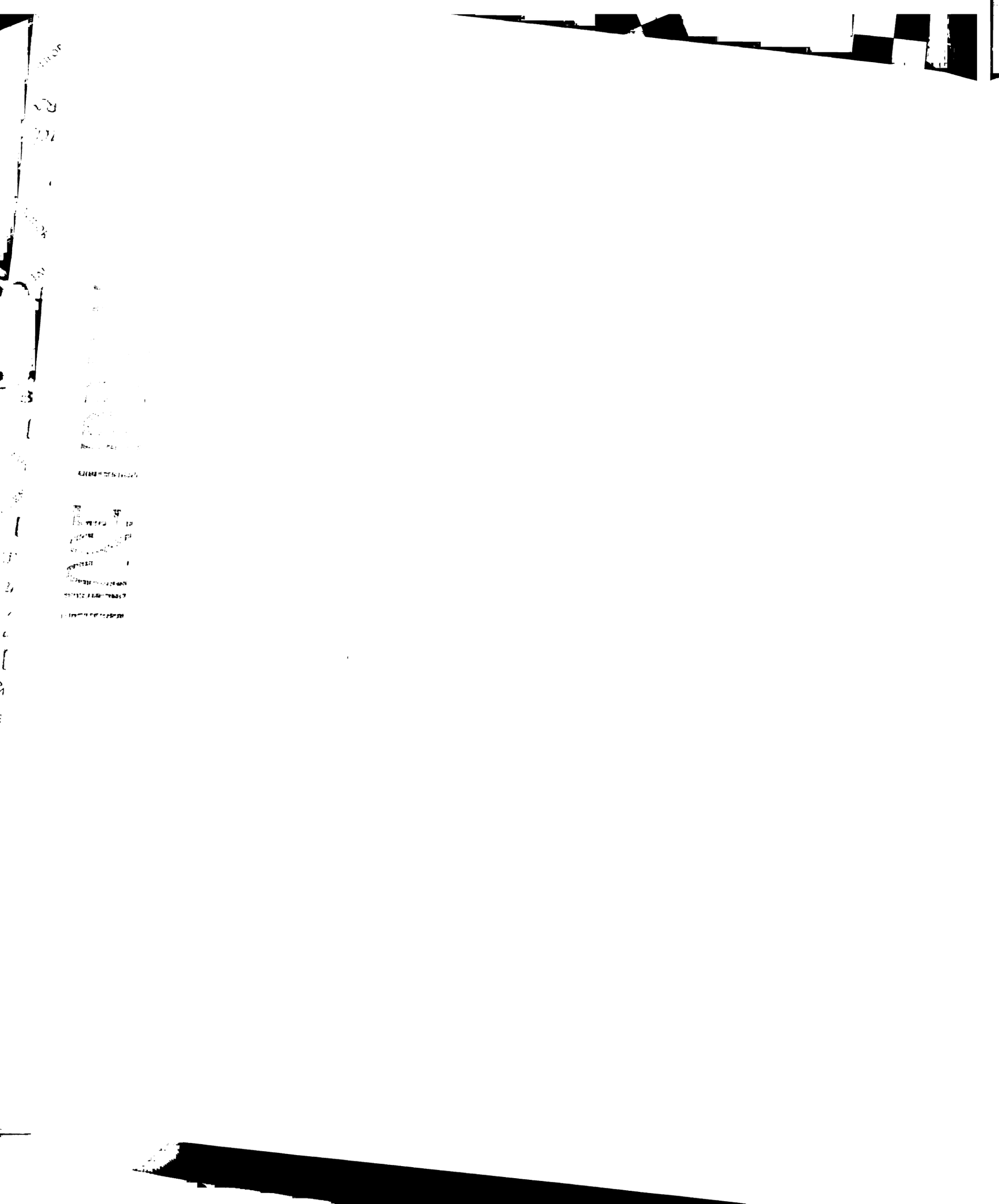


Figure 7. Mab Ab-1 partially blocks turning of commissural axons towards aggregated cells producing netrin-1. (a) Whole spinal cord explants from E11 embryos were cultured in collagen gels in the absence (left) and presence (right) of 30µg/ml mab Ab-1. Commissural axons within the explant were visualized by indirect immunofluorescence using antibodies to TAG-1. Most of the commissural axons of explants grown in the presence of mab Ab-1 do not enter the ventral half of the spinal cord (marked by the white dots) and cannot coalesce at the floor plate (arrow) as do axons of control explants. (b) 293 cells stably transfected with netrin-1 and diluted 1:2 with untransfected 293 cells were aggregated and co-cultured with dorsal spinal cord explants at their cut edge (short arrows), along their dorsal-ventral axes. As previously reported, commissural axons turn toward the netrin-1 cell aggregate. Commissural axons also turn towards netrin-1 in the presence of mabAb-1, even though the axons do not extend into the ventral region of the dorsal spinal cord explant (marked by the white dots). However, the axons do seem less organized and and the distance of turning and frequency of turning is less (see text). (c) Axon outgrowth from E11 dorsal spinal cord explants was also measured towards these cell aggregates. Outgrowth into collagen is robust in the absence of the antibody (left), but does not occur in the presence of mab Ab-1 (right). Most axons remain in the dorsal spinal cord (above the white dots) and do not reach the collagen interphase.



spinal cords (figure 7a, left) grow ventrally from the dorsal spinal cord inan ordered array and coalesce at the center of the floor plate (arrow). In contrast, commissural axons cultured in the presence of anti-DCC antibodies (figure 7a, right) grow poorly into the ventral spinal cord (below the white dots) and coalesce weakly at the floor plate (arrow). These experiments indicate that DCC is required for commissural axons to enter the ventral spinal cord, and confirm that mab Ab-1 can penetrate spinal cord explants cultured in collagen gels. Penetration of the antibody should be even greater in the turning assay, where the spinal cord is cut into halves.

Having shown this, we could now ask whether Ab-1 can interfere with turning towards netrin-1 cell aggregates. Aggregates of 293 cells stably transfected with netrin-1 (see chapter III) were co-cultured along the cut edge of a dorsal spinal cord explant in the presence and absence of mab Ab-1, and the trajectory of commissural axons within the cord was visualized with antibodies directed to TAG-1. Turning within the dorsal half of the explant (above the white dots) occurs in 11/11 explants at a distance of 244 μm +/- S.D=18 μm. In the presence of 10-30 μg/ml mab Ab-1, turning also occurs but less consistently (we saw no turning in 4/22 assays). In explants where turning occurred, distance of turning was slightly shorter (176μm +/- SD of 20μm, n=18), and the axons failed to extend into the ventral portion of the explant (below the white dots). As a control, outgrowth towards aggregates of netrin-1 producing cells was efficiently blocked in the presence of mab Ab-1.

Thus, under conditions where mab Ab-1 blocks commissural axon outgrowth into collagen and commissural axon growth into the ventral spinal cord, it only partially blocks commissural axon turning towards netrin-1. Although still preliminary, these results suggests that DCC, or the domain of DCC recognized by mab Ab-1, plays a less significant role in commissural axon turning than axon outgrowth. Alternatively, DCC may be required for both axon turning and outgrowth, but higher concentrations of mab Ab-1 may be required to block the turning activity.

Discussion

Netrin-1 is chemotropic for E13 commissural axons

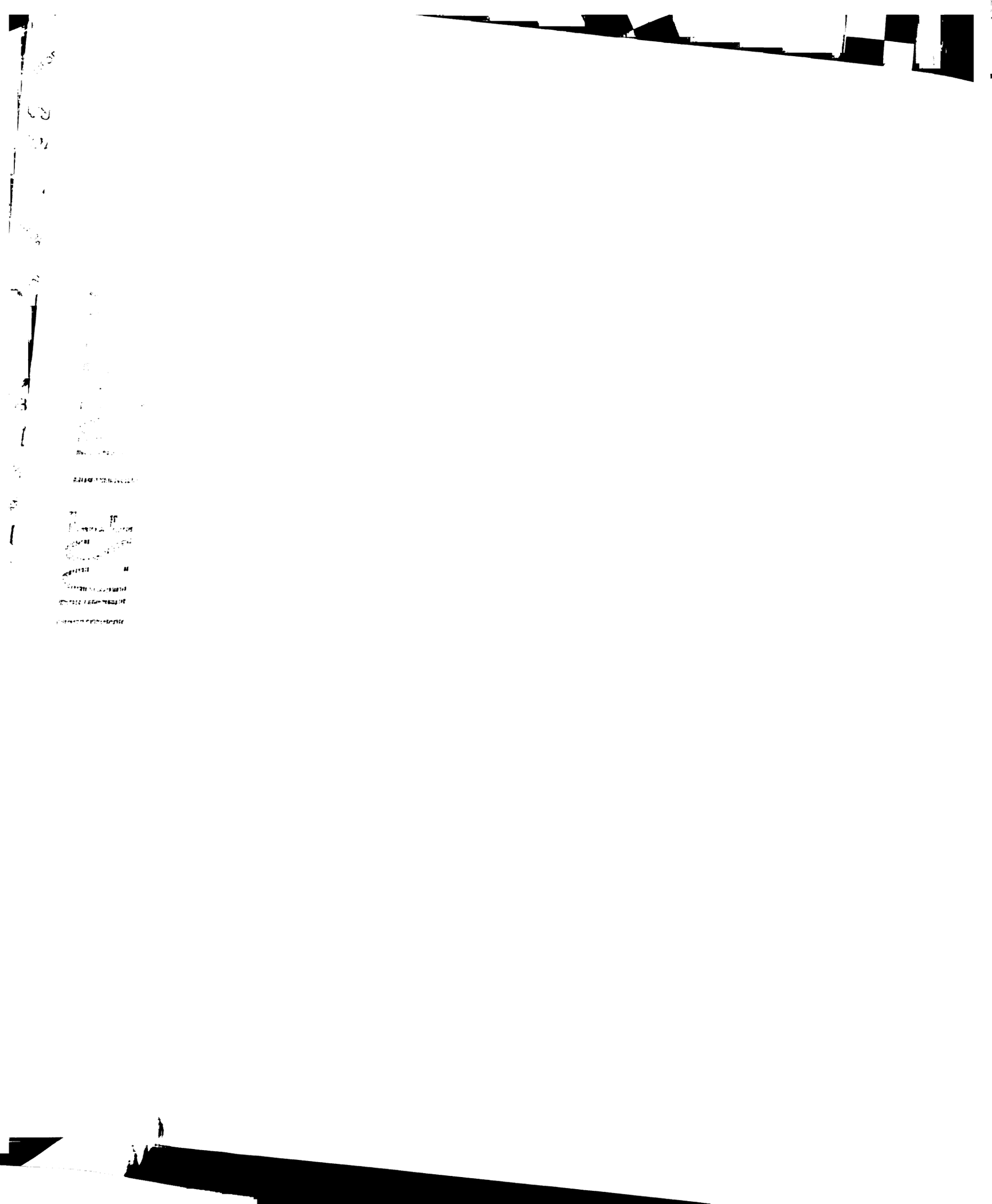
Commissural neurons, born in the dorsal spinal cord over a three day period, sequentially extend axons to their intermediate target, floor plate cells at the ventral midline. To reach the midline, commissural axons extend circumferentially to the motor column then turn ventromedially, a deflection directed in part by the floor plate chemoattractant, netrin-1. Follower axons form fascicles with these pioneers, but it is not known if fasciculation is required for guidance or if followers turn ventromedially in response to the same signals which led pioneers (Colamarino and Tessier-Lavigne, 1995b). In general, fasciculation with pioneers often facilitates the speed of pathfinding but is not necessary for formation of accurate connections. For example, ablation of pioneer axons of the intersegmental nerve (ISN) in *Drosophila* leads to frequent pathfinding errors of follower axons, but followers eventually compensate to form the ISN pathway (Lin et al., 1995). We have shown that netrin-1 acts as a potent chemoattractant for E13 commissural axons *in vitro*, suggesting that follower axons would be able to reach the floor plate even if fasciculation were prevented. However, netrin-1 cannot elicit turning at distances greater than 250 μm *in vitro* (Kennedy et al., 1994). It is certainly possible that as the spinal cord grows in size, fasciculation plays a more important role in initiating the ventromedial deflection towards the midline.

Pure netrin-1 is a chemoattractant for commissural axons

When presented from a point source, netrin-1 functions as a chemoattractant for commissural axons: it reorients commissural axons within E11 dorsal spinal cord explants, and causes E13 commissural axons to grow directionally into collagen. Directed growth from E13 explants is independent of distance from the netrin source and is diminished when netrin-1 is presented from a large source, confirming that commissural axons respond to gradients of netrin-1 protein. Until this study, netrin-1 has been presented to

commissural axons from aggregates of transfected COS/293 cells and the possibility that COS-1/293 derived cofactors are involved in reorienting commissural axons had not been ruled out. Netrin-1 elicited outgrowth from E11 dorsal spinal cords is greatly stimulated by an activity from embryonic chick brains, NSA (netrin synergizing activity), illustrating that it can be modulated by co-factors (Serafini et al., 1994). In this study, we have shown that E13 axons grow directionally towards pure netrin-1 protein presented from a heparin or avidin acrylic bead, indicating that netrin-1 works as a chemoattractant for commissural axons in the absence of cofactors. Despite this directional outgrowth, we rarely saw turning of commissural axons within the collagen matrix. This is probably due to thick fasciculation of the netrin-1 responding axons. In fact, commissural axons responding to netrin-1(VI-V), which are less fasciculated than axons responding to netrin-1, often do turn within the collagen.

Commissural axons are not the only axons that are attracted to gradients of pure netrin-1 protein. Dissociated Xenopus retinal ganglion axons turn towards a point source of netrin-1 protein, secreted from a calibrated pulsatile micropipette (de la Torre et al., 1997). Here, netrin-1 turning is initiated by a highly directed growth cone, indicating that differences in concentration of the netrin-1 protein are detected "spatially" (rather than temporally) over the diameter of the growth cone. The E13 chemotropic assay and the retinal ganglion axon turning assay, provide us with a starting point to study the parameters of the netrin-1 gradient required for growth cone response. Polymorphonuclear leukocytes, which are attracted to gradients of N-formylmethionyl peptides, detect relative slope over their 10 μm diameter (known as steepness), and do not chemotax if steepness is less than 1% (Zigmond, 1977). Retinal ganglion axons (from temporal retina) are inhibited by increasing gradients of a phosphatidylinositol anchored repulsive factor *in vitro*. In this case, growth is inhibited when the steepness of the gradient is more than 5% over 25 μm, the average diameter of a growth cone (Baier and Bonhoeffer, 1992). It will be interesting to see whether developing axons also respond to the relative slope of diffusible



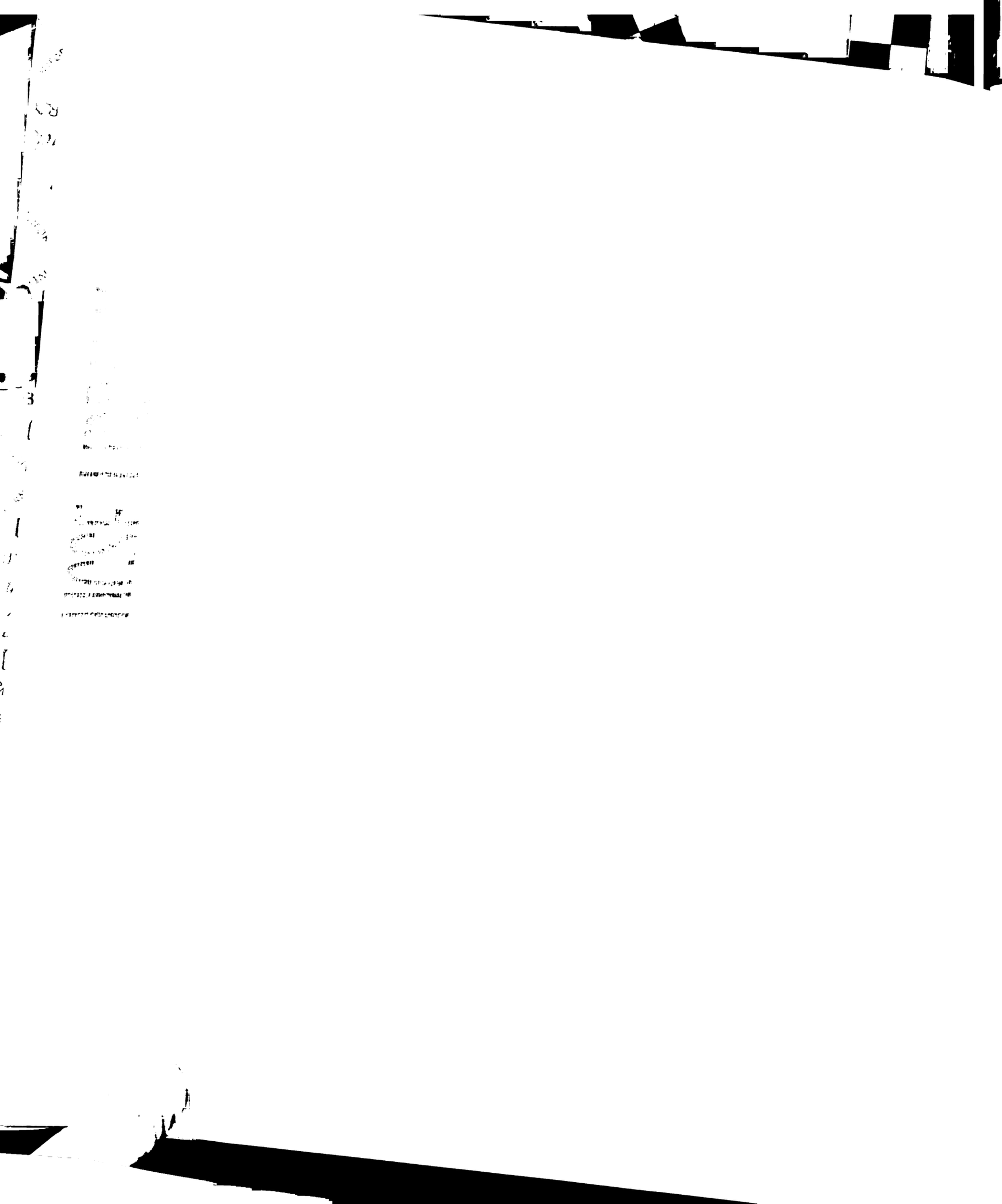
chemoattractants such as netrin-1 and whether they can sense netrin-1 with greater or less sensitivity.

Robust outgrowth activity of netrin-1 is not required for chemotropism

HGF, expressed in the developing limb, functions both as a chemoattractant for developing motor axons and as an outgrowth promoting factor for motor axons from ventral spinal cord explants (Ebens et al., 1996). Motor axons from mice lacking HGF's receptor tyrosine kinase receptor, cMet, are deficient for both activities suggesting that the outgrowth and chemotropic activities are mediated through one receptor pathway. Similarly semaIII, which acts as a chemorepellent for sensory axons when presented in the form of a gradient, inhibits growth of dissociated DRG neurons at uniform concentrations by causing growth cone collapse. These two responses are also mediated by the same receptor, neuropilin, since antibodies to neuropilin block both the collapse and chemorepellent activities (He and Tessier-Lavigne, 1997). These results suggest that growth and chemotropism may be functionally linked activities. For example, in response to gradients of HGF or semaIII, axons may localize their receptors to one side of the growth cone and elicit directional growth.

Alternatively, chemotropism may occur through completely different signaling pathways. There are at least two examples of chemotropic cues which have no affect on axonal growth. The repulsive cue of the tectum do not inhibit neuronal growth at uniform concentrations, but are potent axonal repellents when presented as a gradient (Baier and Bonhoeffer, 1992). Also, floor plate cells from netrin -/- mice secrete a factor which, like netrin-1, can reorient commissural axons within the dorsal spinal cord, but has no axon outgrowth activity (Serafini et al., 1996).

Two lines of evidence suggest that the netrin-1 elicited outgrowth and turning responses are mediated by different pathways. First, netrin-1(VI-V), a mutant which has diminished axon outgrowth activity (even when presented from a point source), can



function as a strong chemoattractant in both the E11 and E13 chemotropic assays. In fact, outgrowth of commissural axons from E13 explant is unusually directed with axons only exiting the side of the explant immediately facing the netrin-1(VI-V) source. Furthermore, growth is highly directional even when netrin-1(VI-V) is presented from a large aggregate. Second, when the outgrowth response of netrin-1(VI-V) was enhanced by generating chimeras with human immunoglobulin Fc, turning was not enhanced. Likewise, NSA (netrin synergizing activity), an activity in extracts of embryonic chick brains which increases netrin-1 elicited axon outgrowth on E11 commissural axons has no measurable effect on turning towards netrin-1 (Serafini et al., 1994; M.Galko and T.Serafini, unpublished results). These differences in response suggest that the outgrowth and chemotropic activities of netrin-1 may be mediated by separate pathways.

Whether DCC is or is not involved in turning of commissural axons is still unclear. Although we are now certain that the mab Ab-1 can penetrate the dorsal spinal cord explant, we cannot be sure that sufficient concentrations of mab Ab-1 were added to block commissural axon turning. Nonetheless, the fact that 10-30 μg/ml mab Ab-1 completely blocks outgrowth but only partially blocks turning suggests that DCC plays a less significant role in turning.

A model for netrin-1 function

Commissural axons grow poorly into the ventral spinal cord of netrin-1 and DCC null embryos. In addition, commissural axons grow poorly into the ventral spinal cord of cultured dorsal spinal cord explants when DCC function is blocked with antibodies or when inhibitory concentrations of exogenous netrin-1 protein are present (see chapter IV), suggesting that commissural axons do not grow into the ventral spinal cord unless netrin-1 (acting through the DCC receptor) makes the ventral cord permissive. How does netrin-1 activate the DCC receptor? Our studies from chapter IV indicate that netrin-1 binding to

DCC is insufficient for receptor activation and that homodimerization of DCC or heterodimerization with another netrin-1 receptor may be required (see chapter IV).

We propose the following model of netrin-1 function. DCC is an outgrowth receptor and gradients of netrin-1 are detected by a second receptor which monitors netrin-1 levels and serves as a compass for growth (red squares in figure 8). Through this receptor, commissural axons detect gradients of netrin-1 or netrin-1(VI-V) protein and tell the growth cone where to turn. Whether DCC is required for turning depends on whether commissural axons are growing within a favorable or unfavorable environment.

When a gradient of netrin-1 is present, the gradient receptor causes DCC to aggregate on the side of the growth cone facing netrin-1 (figure 8e). Netrin-1 protein also activates DCC directly by causing receptor dimerization. Commissural axons can thus grow directionally into unfavorable environments. In contrast, a gradient of netrin-1(VI-V) cannot directly dimerize and activate DCC. However, it does activate the gradient receptor which causes DCC receptors to aggregate on one side of the growth cone. The high local concentration of DCC occasionally allows spontaneous DCC dimers to form, allowing moderate levels of outgrowth to occur. Outgrowth towards netrin-1(VI-V) is weaker than that elicited by netrin-1, but is far more directional. It is also less fasciculated, presumably because of netrin-1(VI-V)'s inability to bridge DCC molecules on neighboring axons.

One prediction of our model is that domains VI-V of netrin-1 should be able to replace netrin-1 when presented as a gradient. Genetic studies in nematodes confirm this prediction. When expressed as a gradient off the UNC-6 promoter, domains VI-V of UNC-6 can rescue *unc-6* null animals (personal communication, W. Wadsworth). However, the responding axons are less fasciculated and, at least in one case, fail to remain bundled within an axon tract. These results confirm that the molecular mechanism of response to netrin is evolutionarily conserved, and suggest that fasciculation may be an important function of netrins in vivo.

The fact that netrin-1(VI-V) does not elicit outgrowth at uniform concentrations but is active when presented in the form of a gradient reminds us that the presentation of netrin-1 may be an important determinant of its activity *in vivo*. For instance, axonal growth into unusually inhibitory tissues may require optimal DCC aggregation which form only with gradients of netrin-1. As mentioned earlier, the commissural axons of whole spinal cord explants from netrin-1 null embryos grow within the dorsal spinal cord but the majority fails to grow into the ventral spinal cord. It will be interesting to test whether uniform concentrations of netrin-1 protein can rescue this defect *in vitro*, or whether netrin-1 must be presented in the form of a gradient from the floor plate. Presentation in the form of a gradient is known to be important for the phosphatidylinositol anchored inhibitory activity of posterior tectal membranes. Retinal ganglion neurons from the nasal retina extend axons on uniform carpets of posterior membranes, but growth is inhibited on gradients of these membranes.

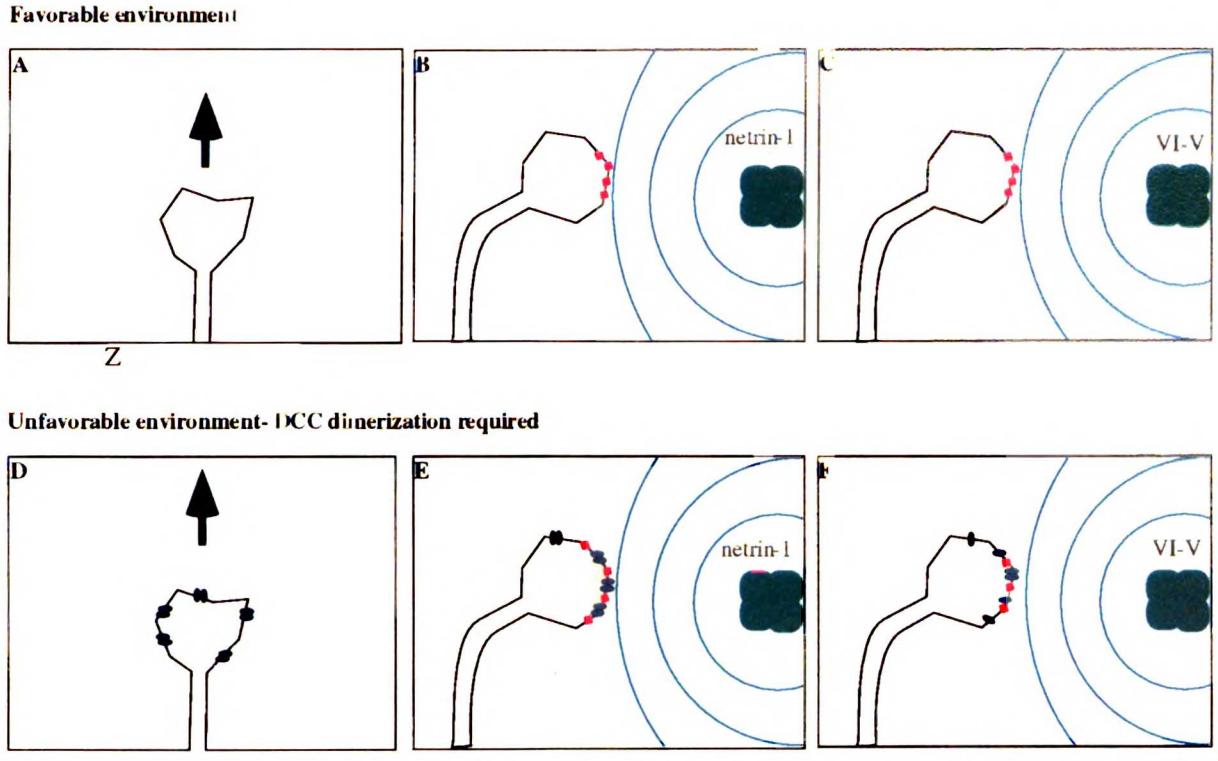


Figure 8. A model for commissural axon response to netrin-1. DCC (represented in blue) is an "outgrowth" receptor required for commissural axon growth in unfavorable environments such as collagen or the ventral spinal cord (D-F) but not required in favorable environments such as the dorsal spinal cord (A-C). Dimerization of DCC is required for receptor activation, and dimerization is elicited by netrin-1 but not netrin-1(VI-V). Gradients of netrin-1 (B and E) and gradients of netrin-1(VI-V) (Cand F) are detected by a second receptor (represented in red) which acts as a compass to orient the growth cone. As a result, DCC receptors aggregate towards the netrin-1 and netrin-1(VI-V) gradients (shown in E and F). As mentioned earlier, netrin-1(VI-V) cannotdimerize the DCC receptor directly and is normally unable to elicit outgrowth into an unfavorable environment. But, when gradients of netrin-1(VI-V) cause DCC to aggregate onto one side of the growth cone, some receptors spontaneously dimerize and outgrowth occurs.

Methods and Materials

Spinal cord explant culture

Outgrowth assays using E13 rat dorsal spinal cord (E0 is the day of vaginal plug) were done as described in (Serafini et al., 1994). Four explants were co-cultured in each well with netrin-1 or netrin-1(VI-V). Dissection and culture of E11 rat spinal cord explants were performed as described (Tessier-Lavigne et al., 1988). All explants were cultured in 64% OptiMEM-1 (GIBCO-BRL), 25% F12, 40 mM glucose, 2 mM Glutamax-1, 100 µg/ml streptomycin sulfate and 100 µg/ml penicillin G. Whole spinal cord explants were dissected as described (Tessier-Lavigne et al., 1988) except dorsal and ventral spinal cord were not separated from one-another, and incubated alone, in the presence of 10-30 µg/ml Ab-1 antibody directed against the extracellular domain of DCC.

Protein Presentation

COS-1 cells were transiently transfected with netrin-1, netrin-1(VI-V) or VI-V-Fc DNA using lipofectAMINE as described (Kennedy et al., 1994). Stably transfected 293 cells were made as described (see chapter III). To produce aggregates of transiently transfected COS cells or stably transfected 293-EBNA cells, cells grown to confluence in a 35 mm diameter dish were trypsinized, washed twice with 10 mls DME + 10%HIFBS, and resuspended in 200 µl of DME +10%HIFBS. Drops of the cell suspension (20 µl) were placed onto the lids of 10 cm dishes, which were inverted and incubated for 3-5 hrs. These aggregates were harvested into L15 medium with a plastic transfer pipette, with the tip cut off, and trimmed with tungsten needles prior to coculturing with dorsal spinal cord explants. When indicated, 293-EBNA$^{netrin-1}$ cells were diluted with untransfected cells prior to aggregating the cells.

In order to present pure netrin-1 and netrin-1(VI-V) proteins from a point source, heparin acrylic beads (Sigma) were first washed in large volumes of PBS. 100ng-1µg pure netrin-1 or netrin-1(VI-V) protein was diluted in 20 µl of PBS, and incubated with 3

medium sized beads (50 μm diameter) in siliconized polypropylene tubes for 30 minutes at RT, but could be stored on ice up to 4 hours. Prior to use, the beads were sequentially washed 5 times in 0.5 ml of PBS, and imbedded with E13 explants in collagen gels.

Whole mount immunohistochemistry

TAG-1 positive E13 and E11 axons were detected by indirect immunofluorescence. Explants were fixed for 1 hr at RT with fresh 4% paraformaldehyde in PBS, blocked in PHTX (PBS, 1% heat-inactivated goat serum, 1% Triton-X-100) for 4 hrs 4°C, incubated with 4D7 culture supernatant for 12 hrs 4°C, washed four or five times for 2 hrs each in PHTX, incubated in Cy3 conjugated goat anti-mouse IgM antibody (Jackson ImmunoReserach, 1:700 in PHTX), prior to mounting in Fluoromount G.

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Chapter VI
Summary and Conclusions

In the developing nervous system, axons grow in response to a combination of short range and long range guidance cues (Tessier-Lavigne and Goodman, 1996). Long range guidance cues exist in the developing spinal cord. Floor plate cells at the ventral midline secrete a diffusible cue, netrin-1, which attracts ventrally directed commissural axons born in the dorsal spinal cord (Tessier-Lavigne et al., 1988; Kennedy et al., 1994; Serafini et al., 1994).

Commissural axons of homozygous mice lacking a full length copy of netrin-1 show two types of guidance defects. First, while axons grow normally within the dorsal spinal cord, most are unable to grow into the ventral spinal cord. Second, those which do grow into the ventral spinal cord are misdirected and have difficulty finding the floor plate. These defects suggest that netrin-1 functions as a permissive cue, allowing commissural axons entry into the ventral spinal cord, and as an instructive cue attracting axons to the floor plate (Serafini et al., 1996). Using *in vitro* assays which mimic these functions (the outgrowth and turning assays) we have studied the molecular mechanism by which commissural axons respond to netrin-1.

We began by addressing an interesting paradox. In chicken embryos, netrin-1 expression is restricted to the floor plate but netrin-1 protein extends hundreds of microns into the spinal cord indicating that netrin-1 is highly diffusible (Kennedy et al., 1996). Yet, in a cellular environment, recombinant netrin-1 is not a very soluble protein: the majority associates with the cell surface and netrin-1 in the soluble fraction is capable of rebinding to cells (Kennedy et al., 1994; chapter III). Cell binding is not unique to recombinant netrin-1 protein. Netrin-1 endogenously made in E9.5 chick brains is cell membrane associated and the majority of netrin-1 produced by the floor plate associates tightly with floor plate and ventral spinal cord cells (Serafini et al., 1994; chapter III). How then can netrin-1 function as a chemoattractant and cause turning of axons at a distance?

We have generated a mutant of netrin-1, netrin-1(VI-V), which is missing its cell binding domain. Interestingly, netrin-1(VI-V) elicits commissural axon turning but at a

shorter distance than that elicited by netrin-1. Thus, cell binding which has commonly been thought to limit the diffusion range of proteins may actually aid in the formation of a stable, far ranging gradient of netrin-1 . To test this model, it will be important to identify the ubiquitous low affinity netrin-1 receptors on cells. Our preliminary experiments suggest that netrin-1 binds to cell associated proteoglycans. If this is true, netrin-1 gradients should extend less within animals deficient for proteoglycan synthesis and commissural axons should fail to reach the floor plate. In Drosophila, two genes known as sugarless and sulfateless seem to be involved in proteoglycan synthesis and sulfation (N. Perrimon, unpublished). Embryos deficient for these genes die prior to the time of commissural axon guidance, preventing us from determining their role within the spinal cord. However, by generating tissue specific knock outs of sulfateless and sugarless, it should be possible to determine the significance of proteoglycans in facilitating the diffusion of netrin-1 and in mediating commissural axon guidance.

As described in chapter IV, we have generated a bank of netrin-1 deletion constructs and have used these to study how netrin-1 mediates commissural axon outgrowth. Our results show that domain C is netrin-1's cell binding domain and its strongest heparin binding domain and that domain v3 is required neither for DCC binding or for axon outgrowth. Moreover, netrin-1(VI-V) binds tightly to DCC but cannot elicit robust axon outgrowth when presented uniformly in the medium, indicating that DCC binding is not sufficient for outgrowth.

Interestingly, dimers of netrin-1(VI-V) do elicit outgrowth, suggesting that outgrowth may occur either when DCC receptors are homodimerized or when DCC is heterodimerized with a second high affinity netrin-1 receptor. To test if dimers of netrin-1(VI-V) can homodimerize DCC receptors, Elke Stein a post-doc in our laboratory will be generating chimeras of the DCC extracellular domains with the intracellular domains of the Trk tyrosine kinase receptors. By assessing the levels of tyrosine phosphorylation, she will be able to readily monitor the dimerization state of the DCC receptor.

Our finding that dimers of netrin-1(VI-V) elicit outgrowth when monomers cannot also suggests that domain C may itself be a dimerization domain. Netrin-1 dimerization does not seem to occur directly but may occur indirectly, for instance by first binding to a heparin sulfate proteoglycan. This would provide an explanation of why the heparin binding domain of an unrelated molecule, human semaphorin III, can substitute the functions of netrin-1(C).

How are the outgrowth and turning activities related to one-another? While netrin-1(VI-V) presented from a point source elicits some axon outgrowth, outgrowth is less than that elicited by netrin-1, allowing us to test whether outgrowth is important for turning activity. Our results indicate that netrin-1(VI-V) elicits directed axon turning, though at a smaller distance (see chapter III) than that elicited by netrin-1. Increasing outgrowth activity, by generating dimers of netrin-1(VI-V), does not increase the distance or angle of turning. Furthermore, anti-DCC antibodies have a greater blocking effect on axon outgrowth than on axon turning. While these results are not definitive, they do suggest that outgrowth and turning are mediated by different signaling pathways.

To extend these studies, it will be important to identify any additional netrin-1 receptors present on commissural axons. DCC clearly plays a role in mediating the growth of commissural axons towards netrin-1, but for the following reasons, it does not seem to function alone as an attractive receptor. First, DCC is present on neurons which are unresponsive to netrin-1 (Keino-Masu et al., 1996). Second, the DCC homologue in nematodes, UNC-40, is sometimes required for UNC-6 mediated repulsion (Hamelin et al., 1993). Furthermore, some of the defects present in the *unc-40* mutants are not present in the *unc-6* mutants (Hedgecock et al., 1990). Our work suggests that there may be two additional netrin-1 receptors on commissural axons. A low affinity HSPG important for the outgrowth response and involved in dimerizing netrin-1 molecules, and perhaps a chemotropic receptor specialized in detecting differences in netrin-1 concentration over the length of the growth cone.

Finally, in chapter V, we describe the E13 chemotropic assay. When dorsal spinal cord explants are co-cultured with aggregates of netrin-1 producing cells, commissural axons grow into the collagen directionally. This assay indicates that older E13 commissural axons , known to fasciculate with pioneer axons *in vivo*, are also attracted to the netrin-1 signal. Commissural axons from E13 dorsal spinal cord explants can also respond to netrin-1 protein immobilized on a two dimensional substrate (C. Mirzayan, not shown; E. Stoeckli, personal communication), where the steepness and concentration of netrin-1 can be carefully controlled. Thus, this assay also provides us with a starting point to examine the parameters of the netrin-1 gradient required for attraction of commissural axons. Do commissural axons read the slope of the netrin-1 gradient? Does the concentration of netrin-1 affect the axons' ability to read this slope? Finally, does the concentration of netrin-1 at the cell body affect the decision of the growth cone to turn or not to turn? Since the days of Ramon y Cajal, scientists have wondered about these very questions. Finally, we may be nearing their answers.

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Appendix I

The elongating T4 holoenzyme: the polymerase and its accessory proteins exist in equilibrium between joint and disjoint states

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Abstract

The T4 holoenzyme, comprised of the polymerase (43p) in association with its three accessory proteins (44p, 62p and 45p) is capable of highly processive and accurate replication of single stranded and double stranded DNA templates. The 44 and 62 proteins co-purify as a tight complex and form an ATPase which is stimulated by DNA and by the 45 protein. On single stranded templates, ATP hydrolysis is required only to assemble the holoenzyme. We have shown that this is also true for replication on double stranded templates. Furthermore, using rapid dilution assays, we investigated whether the T4 polymerase and its accessory proteins are associated tightly once the holoenzyme has been assembled. Our results suggests that the elongating holoenzyme is a dynamic structure which exists in equilibrium between a "joint" state where the accessory proteins and polymerase are functionally bound, and a "disjoint" state where the components of the holoenzyme are transiently uncoupled, but can reassociate without additional ATP hydrolysis. Reassociation must occur quickly because both the polymerase and the accessory proteins have a tendency to fall off the double stranded template when separated. These results indicate that the components of the T4 holoenzyme reciprocally stabilize one-another onto the DNA template, and that termination of replication is a two step process.

Introduction

Our understanding of the detailed mechanism of DNA replication of the T4 bacteriophage has emerged from *in vitro* reconstitution studies of purified components (for reviews, see Alberts, 1987 #58; Nossal, 1992 #59) This work has led to the enzymatic and structural characterization of the T4 holoenzyme, defined as the polymerase (43 protein) in association with its three accessory proteins (44p, 62p and 45p). 44p and 62p co-purify as a tight complex, referred to as 44/62p (Jarvis et al., 1989); together they form a DNA-dependent ATPase which is stimulated by the 45 protein (Piperno, 1978; Mace and Alberts, 1984b). On single stranded templates, DNA replication that is catalyzed by a complete T4 holoenzyme is several fold more efficient than that catalyzed by the polymerase alone (Mace and Alberts, 1984a). This stimulation by the accessory proteins is ATP dependent, requires that the polymerase couple physically with the accessory proteins (Goodrich et al., 1997), and has manifested itself in two sets of assays. First, the rate and fidelity of 3' to 5' exonuclease activity of the polymerase is increased in the presence of the accessory proteins (Bedinger and Alberts, 1983). Second, the processivity of the polymerase is increased from 800 to 3000 nucleotides per initiation event (Huang et al., 1981; Venkatesan and Nossal, 1982; Bedinger and Alberts, 1983; Mace and Alberts, 1984c). Replication of double stranded templates *in vitro* requires the assembly of the holoenzyme and the rate of replication depends on the concentration of 32p, the single stranded binding protein (Venkatesan and Nossal, 1982; Topal and Sinha, 1983). The 41p helicase is not required, though in its presence, replication of the leading strand proceeds at the maximum rate of (>400 nucleotides/sec), even in the absence of 32p (Cha and Alberts, 1989).

The functions of the holoenzyme are remarkably conserved in evolution, with similarities ranging from mammals to T4 bacteriophage. One of the best characterized of these is the E.coli *in vitro* replication system. Here, the γ accessory protein hydrolyzes ATP to load a second accessory complex, β,onto the DNA template (for review (O'Donnell

and P.T. Stukenberg, 1992). β forms a tight protein clamp and can freely slide on the DNA but fall off the ends of linearized DNA molecules (Stukenberg et al., 1991). Once β is loaded, it tethers the polymerase to the primer template junction and replication proceeds efficiently in the absence of γ and ATP.

Several studies suggest that the T4 accessory proteins 44/62p hydrolyze ATP to load 45p as a sliding clamp onto DNA. First, by cryo-electron microscopy, the structure of 45p resembles a circular clamp (Gogol et al., 1992). Second, ATP hydrolysis by 44/62p is not required in the presence of a molecular crowding agent and very high concentrations of 45p, suggesting that 44/62p is necessary only to load 45p onto the template (Reddy et al., 1993). Finally, like β,the T4 holoenzyme falls off of ds DNA templates which have been linearized with a restriction enzyme (Hacker and Alberts, 1994a). The rate with which the holoenzyme fall off is greater if the template is linearized at a site close to the holoenzyme, suggesting that it too slides along the DNA molecule. Despite these similarities, attempts to isolate 45p primed onto DNA have been unsuccessful, suggesting that 45p binds to circular DNA templates with less affinity than the β clamp.

In this study, we attempted to understand in greater depth the mechanism by which the T4 holoenzyme elongates double stranded DNA templates. We began by verifying that ATP hydrolysis is only required for assembly of the holoenzyme. Once the holoenzyme was assembled, we could selectively deplete its components from our ongoing reactions by rapid dilution and assay the effects on the total level of DNA synthesis and on product size. Our results suggest that the elongating holoenzyme is a dynamic structure found in equilibrium between "joint" and "disjoint" states. In the joint state, the polymerase and the accessory proteins are functionally bound. In the disjoint state, the accessory proteins and the polymerase are physically uncoupled but can reassociate without additional need for ATP hydrolysis.

Results

A sigmoidal response to ATP during replication on nicked double stranded templates

On primed single stranded templates, the accessory proteins stimulate the rate and processivity of the T4 polymerase (Huang et al., 1981; Mace and Alberts, 1984c). For synthesis on nicked double stranded templates, they are absolutely required. Once these 5 proteins are present, replication proceeds in a rolling-circle mode starting from a nick on a circular double stranded DNA template (Sinha et al., 1980; Meyer et al., 1981).

The requirement for ATP hydrolysis by the T4 accessory proteins has been analyzed on single stranded templates but not on double stranded templates (Jarvis, 1991). We began by investigating the requirement for ATP during strand displacement DNA synthesis. To determine the concentration of ATP required to give maximal stimulation of DNA replication of double stranded templates, replication reactions were carried out in the presence of ATP ranging from 5μM-200μM at two concentrations of accessory proteins. dATP can act as an alternate energy source for 44/62p. In its place, we used dAMPPNP as one of the four deoxynucleoside triphosphates, an imidiophosphate analog which contains a nonhydrolizable bond in the β-γ position (Piperno, 1978). The amount of labeled dTTP incorporated into DNA, after 2, 4, and 6 minutes of synthesis was plotted as a function of ATP concentration (figure 1). At low concentrations of both accessory proteins (2.5μg/ml 44/62; 1.1μg/ml 45), the Km for ATP is 36.5μM ATP (figure 1a). The slope of the hill plot is greater than 1 (2.4 +/- 0.4*) implying that ATP binding to 44/62p is cooperative and that multiple ATP molecules are hydrolyzed per initiation event. At 10 fold higher concentration of each accessory protein, (25μg/ml 44/62; 11μg/ml 45), 3.3-fold less ATP is required for maximum DNA synthesis and at 20μM ATP, replication occurs at a 10-fold higher rate than at the lower accessory protein concentration (figure 1b). Adding more

* curve fitted by the Ultrafit Non-Linear Curve-Fitting Package; Biosoft

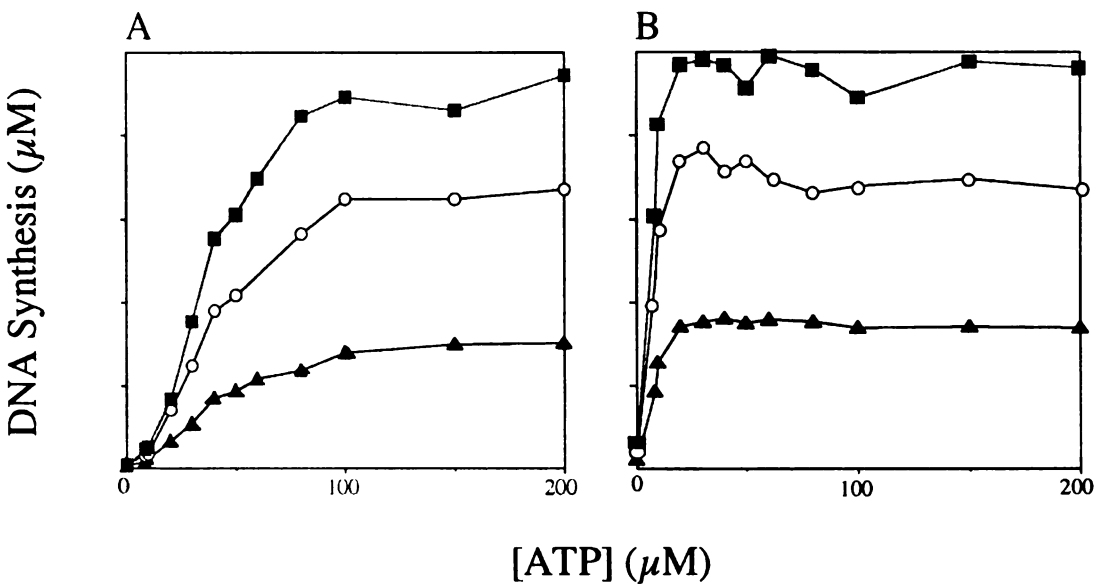


Figure 1. ATP concentration required for maximal stimulation of replication at two levels of accessory proteins. DNA synthesis was measured using the standard incorporation assay (see Materials and Methods) with a range of ATP concentrations present from the start of replication: 8, 10, 20, 30, 40, 50, 60, 80, 100, 200 μM ATP. From each reaction, aliquots were removed at 2 minutes (black diamond), 4 minutes (blue circles) and 6 minutes (red squares) after the start of replication. In (A), low concentrations of accessory proteins were present (1.1μg/ml 45p, 2.5μg/ml 44/62p). In (B), 10-fold higher concentrations of accessory proteins were present (11μg/ml 45p, 25μg/ml 44/62p).

accessory proteins causes only a small increase in DNA synthesis at saturating concentrations of ATP, suggesting that the reaction is saturated for accessory proteins.

Rapid Dilution reveals that during the elongation phase of DNA replication, there is an occasional need to re-load the accessory proteins onto the DNA

At standard concentrations of proteins and nucleotides (figure 1), replication proceeds with about 50% efficiently at 50μM ATP and minimally at 5μM ATP. To determine if ATP hydrolysis is required only to assemble the replication proteins at the start of replication, reactions were initiated and allowed to proceed for 1 minute at 50μM ATP, and then diluted to 5μM ATP. Radioactive deoxyribonucleoside triphosphates were added during the dilution and DNA synthesis was thereafter measured (figure 2). As shown in figure 2a (red), this dilution causes a gradual decline in replication with a 2-3 fold decrease in the replication rate within 5 minutes, indicating that ATP is continually required.

We suggest two possible explanations for this ATP requirement. First, 44/62p may act as a translocase during elongation and require regular ATP hydrolysis events for movement. Second, the polymerase holoenzyme may dissociate from the replication fork every few minutes and need to reassemble *de novo* with the DNA, a step known to require ATP hydrolysis. To distinguish between these possibilities, we initiated replication reactions under standard conditions then measured DNA synthesis upon 10-fold dilution of either ATP, accessory proteins, or ATP and accessory proteins. Ten fold dilution of either of these components is sufficient to prevent holoenzyme assembly on the template prior to the onset of replication. We shall see later that even greater dilutions have no additional effects on our replication reactions (see figure 4).

DNA synthesis from these dilution experiments is plotted in figure 2a, and an electrophoretic analysis of the size of the DNA made in a duplicate experiment is presented in figure 2b. The dilution of ATP or the accessory proteins causes a 2-fold decrease in the

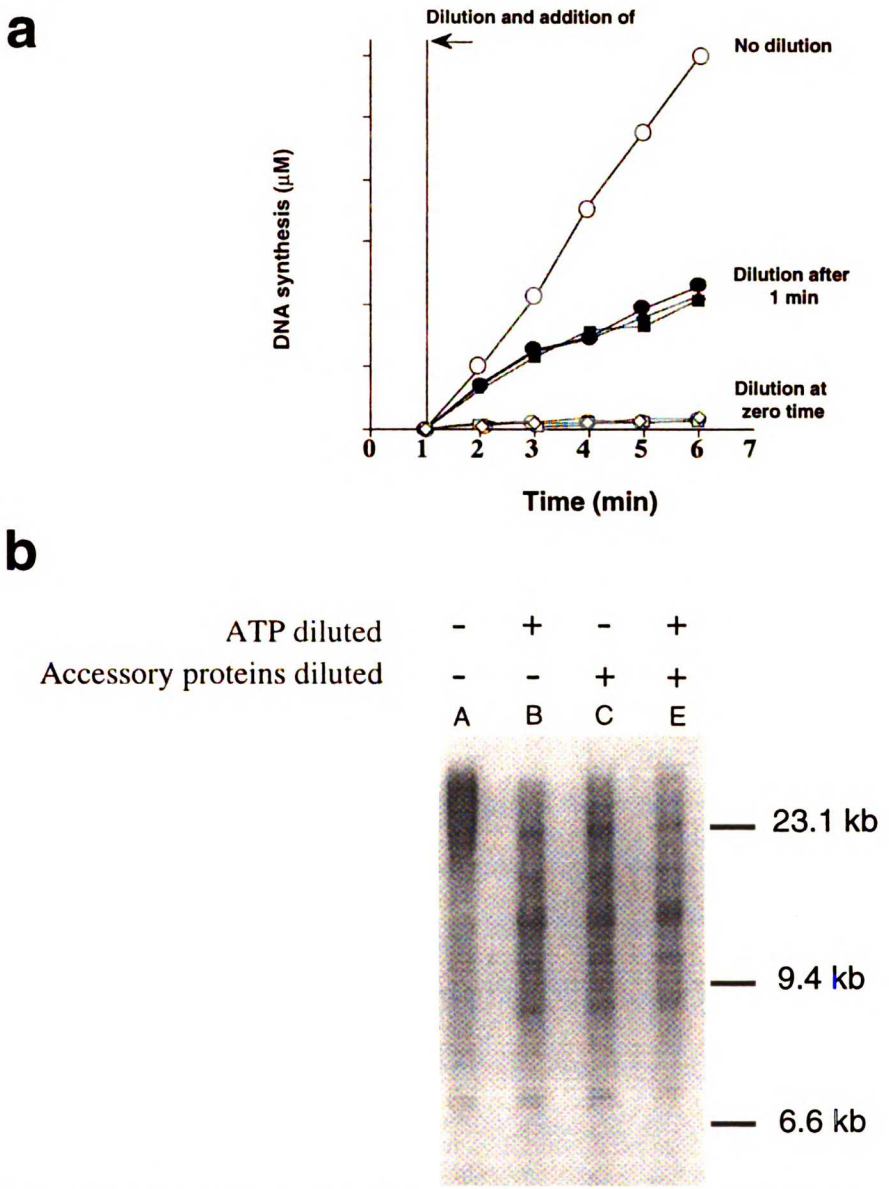


Figure 2. After the onset of replication, ATP hydrolysis is occassionally required for reassembly of the accessory proteins. (a) The continuous incorporation assay was performed as described in the Materials and Methods section, except the reaction mix was preincubated for only one minute prior to the start of replication and the label was added one minute afterward. Thereafter, samples were collected every minute for 5 minutes allowing us to measure the rate of DNA synthesis. At the same time the label was added, either the accessory protiens (solid blue), ATP (solid red), or both the accessory proteins and ATP (solid green) were diluted 10-fold. The black circles show total replication from a reaction where no dilution was conducted. Control reactions (dilutions at time zero) show that replication cannot begin at the diluted concentrations of accessory proteins (blue), ATP (red), or both accessory proteins and ATP (green). (b) [α-32P] dTTP is present from the start of the replication reactions. One minute after the onset of replication, the label was chased with excess unlabled dTTP. At that time, either the accessory proteins (lane C), ATP (lane B) or both the accessory proteins and ATP (lane D) were diluted 20-fold. The reactions were stopped after an additional 3 minutes and separated on an alkaline agarose gel. Lane A shows control reactions where no dilutions occured.

total DNA synthesized (at 4 min). Simultaneous dilution of accessory proteins and ATP has no additional affect beyond that of either dilution alone. These results suggest that on double-stranded templates, ATP hydrolysis is necessary during elongation to reload free accessory proteins from solution onto the DNA since the concentration of accessory proteins should not affect the requirement for ATP hydrolysis by an already assembled polymerase holoenzyme. This means that these accessory proteins occasionally leave the holoenzyme complex, requiring reassembly to keep the DNA polymerase moving.

Accessory protein dissociation sites correlate to site specific pause sites on the M13Mp19 template

In figure 2b, examination of the DNA products in lanes C,D, and E reveals that each of the dilutions gives rise to a number of discrete bands of DNA products, indicating that the holoenzyme preferentially dissociates at specific sites. The approximate location of these dissociations on the M13mp19 template can be determined from the sizes of the aborted products. Each time the polymerase replicates the 7.25 kb circle, the accessory proteins dissociate at three dominant sites, 0.85, 3.3, 5.5, kb from the origin of replication (marked by chew-back fill in at the gp2 nick site; see astrix). Two of these correspond to site specific pause sites previously characterized in this laboratory (Bedinger, 1989), suggesting that pausing may weaken accessory protein-DNA interactions leading them to dissociate from the template. If so, the holoenzyme should persist unperturbed for a much longer time under conditions where pausing is greatly reduced or eliminated.

The 41 helicase does not increase the stability of the moving DNA polymerase

Site specific pausing does not occur in the presence of 41 protein (Bedinger, 1989), a DNA dependent GTPase and the highly processive DNA helicase of the T4 primosome (Liu and Alberts, 1981). As shown in figure 3, the 41 helicase also eliminates site specific

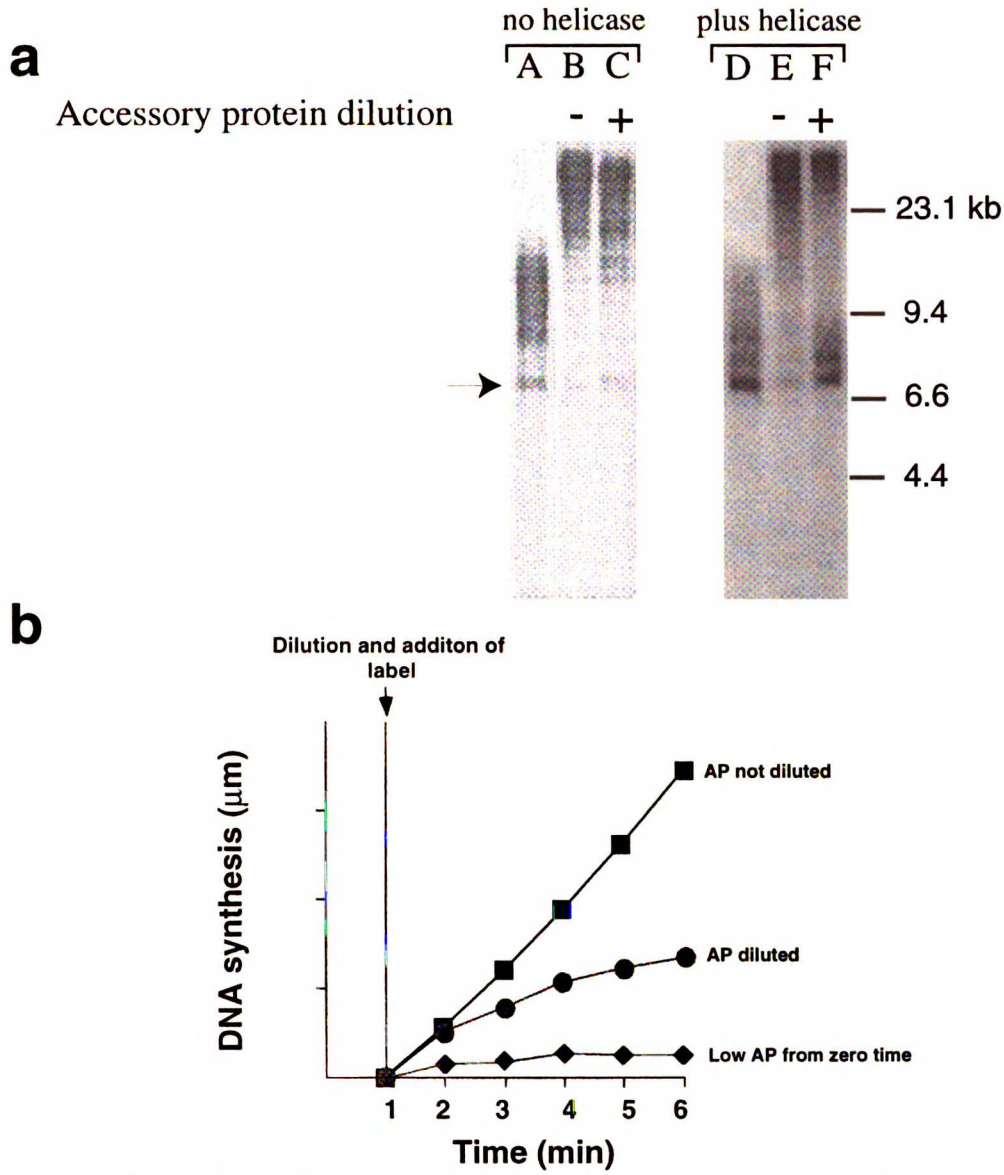


Figure 3. The T4 DNA helicase does not affect the dissociation rate of accessory proteins. (a) Pulse-chase replication reactions were conducted in the presence and absence of the T4 helicase (41p) and its accessory protein, 59p. 2mM GTP was present as an energy source for the helicase. [α-32P]dTTP was present from the onset of replication but excess unlabeled dTTP was added at the time of accessory protein dilution. DNA is synthesized roughly three times faster in the presence of the T4 helicase. Thus, in order to compare DNA products of equivalent sizes, dilutions for helicase plus reactions were conducted at earlier times. Helicase plus reactions were diluted 15sec after the start of replication, and a sample was removed after an additional 45sec. Reactions without helicase were diluted at 45sec after the start of replication and a sample was removed after 135sec. Lanes B and E are control reactions where the accessory proteins were not diluted. Lanes A and D show the sizes of the products at the time of dilution. The arrow marks the unit length of the plasmid which becomes labeled by chew-back/fill in synthesis. (b) Total DNA synthesis was measured in reactions containing 41p and 59p using the continuous incorporation assay. 2mM GTP was present as an energy source for the helicase. One minute after the start of the reaction [α-32P]dTTP added to the reactions. At this time, the accessory proteins were either diluted 20-fold (green circles), or maintained at their normal concentrations (black diamonds). Control reactions (red diamonds) show that replication cannot begin at the diluted concentrations of accessory proteins, even though the helicase is present.

dissociation of accessory protein upon dilution of the accessory proteins (figure 3a: compare lanes C and F). To test if the accessory proteins become destabilized on the DNA due to pausing, we measured the processivity of the accessory proteins by repeating the figure 2 type experiment in the presence and absence gene 41 protein (figure 3b). 59 protein was present to facilitate rapid loading of the DNA helicase onto 32p covered DNA (Barry and Alberts, 1994). The level of nucleotide incorporation is approximately three fold higher for helicase plus reactions irrespective of whether accessory proteins are diluted. By fitting the data presented in figures 2a and 3b to a first order dissociation curve, we calculated the rates of accessory protein dissociation in the presence and absence of DNA helicase. In both cases, dissociation occurs at an average of 2.8 times/minute. We conclude that the stability of the holoenzyme is unchanged in the presence and absence of pausing.

Replication halts upon dilution of both accessory proteins and polymerase

Our present view of the elongating holoenzyme is that the accessory proteins increase the "sticking distance" of the polymerase by tethering it to the DNA. Thus, we have been assuming that the accessory proteins and the polymerase dissociate from the DNA as a single unit. To test this idea, replication was initiated under our standard conditions and the DNA and either the polymerase or the accessory proteins (44/62p and 45p) were diluted later (20-fold). If the polymerase and the accessory proteins fall off the DNA simultaneously, then identical results should be obtained with either dilution: neither free polymerase nor free accessory proteins from the diluted solution can bind to the primer template junction and the lack of either one should suffice to prevent a restart of DNA synthesis. As controls, no DNA synthesis occurs if the reactions are initiated at diluted concentrations of DNA and either polymerase (50ng/ml) or accessory proteins (55ng/ml 45p, 125ng/ml 44/62p).

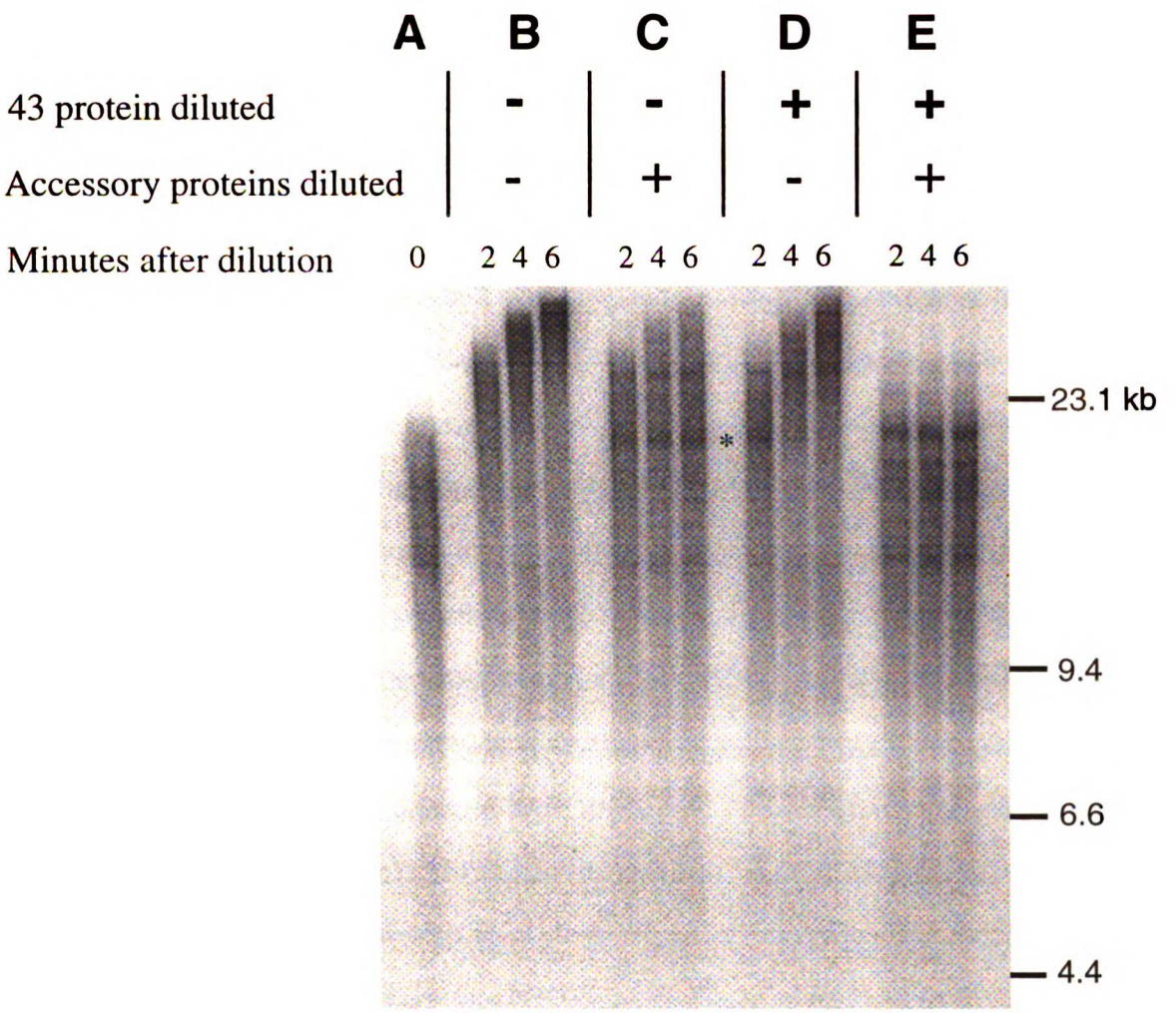


Figure 4. Dilution of both the accessory proteins and the polymerase has a greater affect on replication than dilution of either alone. [α-32P]dTTP is present from the start of replication. One minutes after the onset of replication, the label was chased with excess unlabeled dTTP and either the polymerase (lane D), the accessory proteins (lane C) or both the polymerase and the accessory proteins (lane E) were diluted 20-fold. Neither component was diluted from reaction B. From each reactions, aliquots were removed 2, 4, and 6 minutes after dilution. As a control, DNA products at the time of the dilution were stopped and are shown in lane A. The astrix marks a DNA product which stops growing in reactions where the accessory proteins have been diluted, but continues growing in reactions where the polymerase was diluted.

The results in figure 4 show that the products of reactions in which only polymerase is diluted differ qualitatively from those in which only the accessory proteins are diluted (compare lanes D with lane C). As evident from the time course performed for each reaction, replication forks gradually stop upon accessory protein dilution whereas they pause and resume growth upon polymerase dilution. Only the largest products of the accessory protein dilution (lane C) at 2 minutes grow upon further incubation. In contrast, all products of the polymerase dilution, irrespective of their size (lane D), grow with time. These differences argue that the components of the holoenzyme dissociate off the DNA as separate units. In support of this conclusion, when the polymerase and the accessory proteins are diluted simultaneously, the replication reactions come to an rapid halt (lane E). If the polymerase and accessory proteins had dissociated together, dilution of both sets of proteins should have had no greater effect than dilution of either alone. In order to estimate the number of base pairs replicated upon dilution of the polymerase and the accessory proteins, the sizes of the DNA products from lane E were compared to those of lane A (stopped at the time of dilution). These data show that the average processivity of 44/62p, 45p, and 43p combined is 4.3kb. If we assume a replication fork rate of 170 bp/sec, this suggests that one of the 4 components of the holoenzyme dissociate from the DNA every 25 seconds, and that accessory proteins and polymerase must constantly be recycled from solution to allow replication to proceed.

Based on the these results, we can propose a tentative model to account for the interactions of the polymerase with the accessory proteins and the role of ATP hydrolysis during the elongation phase of replication. Since the remaining experiments of this study are tests of this model, we describe it next.

A model: the elongating T4 holoenzyme is found in equilibrium between joint and disjoint states

Assembly of the holoenzyme begins with the recognition of the primer template junction by an ATP bound 44/62p complexed with 45p. The ATPase activity of 44/62p stimulated by the primer-template junction, causes a conformational change which allows 45p to associate with the DNA polymerase and for DNA synthesis to begin (Capson et al., 1991; Munn, 1991a; Munn, 1991b). We propose that during elongation, the interaction between the 45p and the polymerase is not stable and that the holoenzyme is repeatedly disrupted and reformed. This implies that the elongating holoenzyme is a dynamic structure found in equilibrium between "joint" and "disjoint" states and that the concentration of free accessory proteins and polymerase affects this equilibrium. At high concentrations of ATP, accessory proteins, or polymerase, the equilibrium is shifted towards the "joint" state and strand synthesis ensues. At low concentrations of accessory proteins or polymerase, the equilibrium is shifted towards the "disjoint" state. Reassociation must occur rapidly because in the disjoint state, neither the accessory proteins or the polymerase bind tightly to the DNA , and are inclined to dissociate from the template.

Replication halts upon dilution of polymerase and ATP

According to the disjoint holoenzyme model, dilution of only accessory proteins should not halt replication because 43p from solution can bind and stabilizes the 45p clamp. Likewise, dilution of only 43p should not stop strand synthesis since 44/62p from solution can hydrolyze ATP, continually regenerating 45p clamps until a holoenzyme forms. Thus, one prediction of the model is that strand synthesis would halt if ATP hydrolysis was prevented upon dilution of the polymerase.

As a test, we performed simultaneous dilution experiments of the polymerase with ATP (figure 5). ATP is the only available energy source for 44/62p in these reactions because dAMPPNP (200μM) is used as the replication substrate in place of dATP. As demonstrated previously, dilutions of only polymerase, only accessory proteins, or only

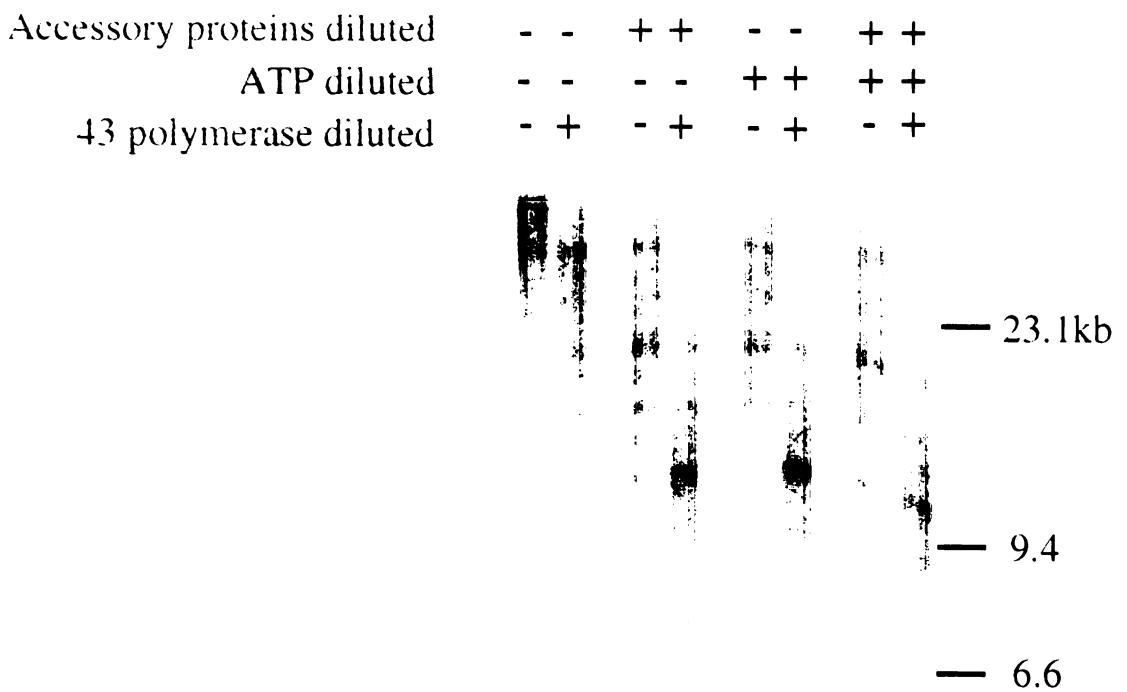


Figure 5. Dilution of ATP and the polymerase generates products identical to those generated upon dilution of the accessory proteins and the polymerase. [α-32P] dTTP is present from the start of replication and was chased one minutes after with excess unlabeled dTTP. At that time, either the polymerase, the accessory proteins, ATP, or combinations of these were diluted 20-fold. Two minutes after dilution, aliquots were collected from each reaction and separated on an alkaline agarose gel. Lane A shows replication products when neither ATP, accessory proteins or polymerase was diluted.

ATP does not halt replication. Furthermore, as previously shown, simultaneous dilution of 43p and accessory proteins halts replication rapidly. The new result is that replication halts rapidly when polymerase and ATP are diluted simultaneously.

45 protein requires the presence of both 43p and ATP for stable interactions with ssDNA cellulose

With respect to the binding coefficient of the accessory proteins to the DNA template, the model makes two clear predictions. First, the accessory proteins bind relatively tightly to the primer template junction in their ATP bound state, but following ATP hydrolysis, one or both accessory proteins become unstable. Second, the polymerase can stabilize the 45p clamp onto the DNA template.

Using a rapid filtration DNA-cellulose binding assay devised in this laboratory (Mace and Alberts, 1984a), we were able to measure binding of 44/62p and 45p onto ssDNA under different conditions. At subsaturating concentrations of 32p, hairpins form along the ssDNA cellulose which act as binding sites for the accessory proteins. From studies of Mace and Alberts, we knew that in the presence of ATP, both 44/62p and 45p bind 32p covered ssDNA. In the absence of 44/62p or ATP, 45p does not associate with the DNA (Mace; data not shown). We conducted rapid filtration of accessory proteins onto ssDNA cellulose in the presence of ATP, ATPγS, or ADP. Bound protein was eluted from the ssDNA cellulose with SDS lysis buffer, separated on a 13.5 % acrylamide gel, blotted then probed with a mixture of antibodies recognizing the 62 and 45 proteins. As reflected by the presence of 62p, 44/62p has a strong affinity for ssDNA irrespective of the nucleotide present (figure 6). In contrast, 45p binds tightly to DNA in the presence of ATPγS but we detect no binding in the presence of ATP[5], ADP or buffer To test if the presence of the DNA polymerase can increase the binding of 45p, we repeated these

[5] In contrast to these results, Mace and Alberts found that 45p binds to ssDNA cellulose in the presence of ATP. It is likely that this discrepency is due to differences in the temperature at which the two experiments were conducted. Our experiments were conducted at room temperature where ATP hydorlysis is rapid, and those of Mace and Alberts were conducted at 4°C where ATP hydrolysis is slow.

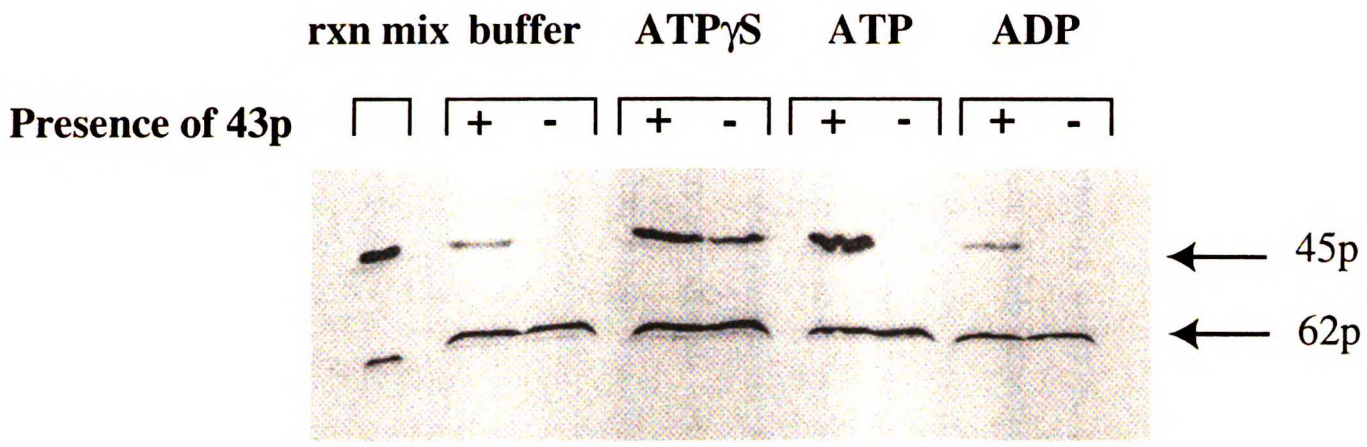


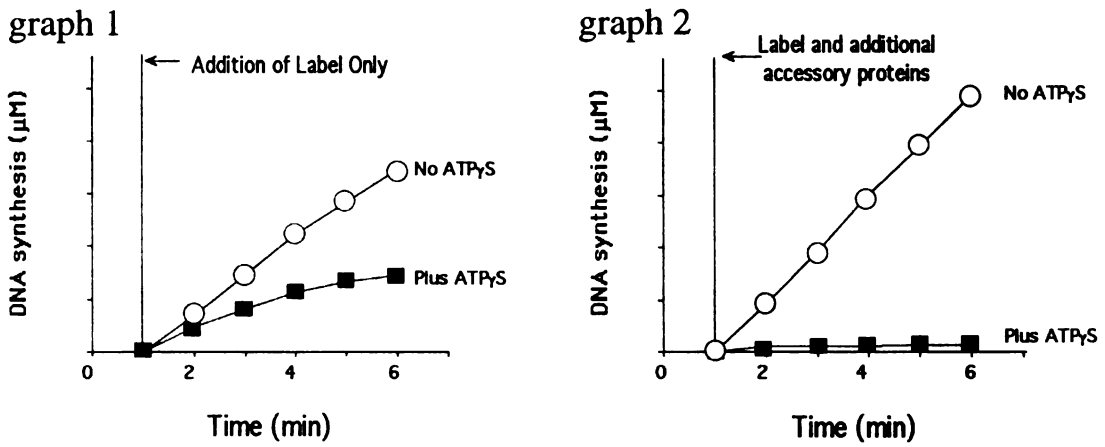
Figure 6. Rapid filtration assays indicate that 45p binds to 32p covered ssDNA cellulose in the presence of ATP and the T4 polymerase. The T4 accessory proteins, 44/62p and 45p were incubated with 32p covered ssDNA cellulose in the presence and absence of the T4 polymerase and in buffer containing 500μM ATPγS, ATP, ADP or nothing (buffer). This mix was then subjected to rapid filtration and washed. Proteins which bound to the ssDNA cellulose were resolubilized in SDS buffer, Zseparated on a 13.5% polyacrylamide gel, and immunoblotted using antibodies directed to 45p and 62p (a component of the 44/62p ATPase). The protein mixture prior to filtration is shown in the lane marked "rxn mix".

binding assays in the presence and absence of the polymerase. The results in figure 6 indicate that binding of 45p to the ssDNA cellulose is increased when the DNA polymerase is present in the pre-incubation mix. This stimulation is minimal in the presence of ATPγS, ADP and buffer but strong in the presence of ATP. These results agree with the experiments of Richardson et al. (Richardson, 1990) who showed that a functional complex of 44/62p and DNA can be isolated by gel filtration but that 45p can be isolated only in the presence of ATP, 44/62p and 43p.

In the presence of accessory proteins, addition of ATPγS causes a dominant negative effect which interferes with replication

Upon formation of the disjoint holoenzyme, the accessory proteins and the polymerase are predicted to move apart from one other and from the primer template junction, leaving the 3'-OH temporarily unoccupied. In its ATP bound state, 44/62p binds tightly to 45p at the primer template junction (Munn, 1991b). Thus, we reasoned that addition of excess accessory proteins and the nonhydrolyzable analog of ATP, ATPγS, could act as an enzymatic trap which would block unoccupied 3' OH's. At the normal concentration of accessory proteins, 250μM ATPγS is sufficient to inhibit replication if present from the onset of the reaction (not shown) but if added one minute after the start of the reaction, an exponential decay with a half life of 3.5 minutes occurs, producing a 2.2 fold decrease in the total rate of replication after 5 minutes (figure 7a). In contrast, if the concentration of accessory proteins is increased 10-fold when ATPγS is added, replication halts instantaneously. High concentrations of accessory proteins in the presence of ATPγS act as dominant negative agents, interfering with replication. This effect involves the synergistic action of both 44/62p and 45p since a 10-fold increase in the concentration of either protein alone fails to halt replication (figure 7b).

a



b

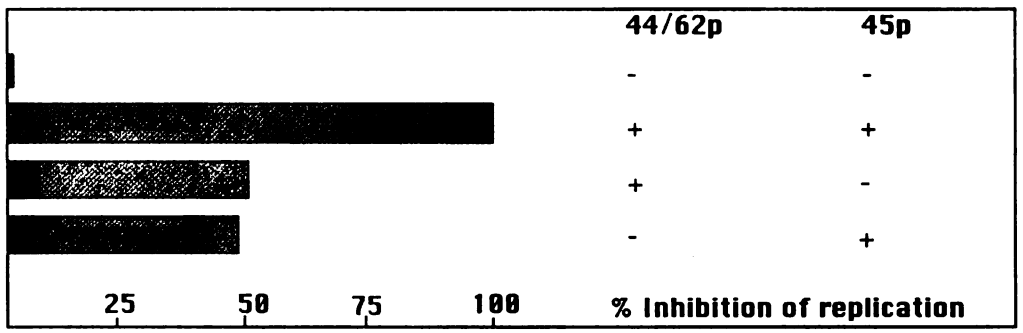


Figure 7. In the presence of ATPγS, high concentrations of accessory proteins interfere with ongoing replication reactions. (a) Replication reactions were initiated under our standard conditions. After one minute of synthesis, [α-32P]dTTP was added to the reactions along with 250μM ATPγS (Graph 1). In a parallel reaction (Graph 2), the concentration of accessory proteins was raised (to 11μg/ml 45p; 25μg/ml 44/62p) simultaneous to the addition of the label and ATPγS. Five aliquots were removed from each reaction every 30 seconds, and the total DNA synthesized in each sample was measured (blue squares). Control reactions where no ATPγS was added are shown for comparison (white circles). (b) The experiment in figure a was repeated in the presence of ATPγS, except here we raised the concentrations of 45p and 44/62p separately. Samples were collected 1, 3, and 5 minutes afterwards. Inhibition of replication is shown as a percentage. Zero percent inhibition is defined by reactions where ATPγS was added but the concentrations of accessory proteins were kept low.

Discussion

ATP hydrolysis is necessary only to load accessory proteins onto double stranded DNA templates

The replication of DNA is directed through the coordinated efforts of the DNA polymerase and its accessory proteins. These accessory proteins are required for replication of double stranded templates, and increase both the accuracy and processivity of the polymerase on single stranded templates (Huang et al., 1981; Venkatesan and Nossal, 1982; Bedinger and Alberts, 1983; Topal and Sinha, 1983; Mace and Alberts, 1984c). In *E.coli*, the γ accessory protein, an ATPase, hydrolyzes ATP to load a second accessory complex, β,onto DNA templates (for review O'Donnell and P.T. Stukenberg, 1992). Once loaded, β which is thought to encircle the DNA like a ring and can be purified on circular templates by gel filtration. When the polymerase is added to this "preinitiation complex," DNA is synthesized without additional ATP hydrolysis, indicating that ATP is hydrolyzed only at the onset of replication to load the β clamp onto the DNA template (Stukenberg et al., 1991).

The T4 accessory proteins, 44/62p and 45p, are thought to function analogous to the γ and β complexes; 44/62p, an ATPase, hydrolyzes ATP to load 45p onto DNA templates. Unfortunately, it has not been possible to purify the T4 "preinitiation complex" away from the 44/62 protein, leaving open the possibility that 44/62p also acts as a translocase during elongation, hydrolyzing ATP to move along the DNA. This possibility has been ruled out for replication on single stranded templates (Jarvis, 1991), but not double stranded templates.

Our results indicate that ATP stimulates replication on double stranded templates sigmoidally, suggesting that 44/62p hydrolyzes ATP molecules cooperatively. This is consistent with our knowledge of the structure of 44/62p comprised of one 62 molecule and four 44 molecules, each containing an ATP binding site (Jarvis et al., 1989). To test whether this ATP hydrolysis occurs only when holoenzymes are loaded onto the DNA or

whether 44/62p acts as a translocase on double stranded templates, we selectively removed accessory proteins or ATP from ongoing replication reactions by rapid dilution. Dilution of accessory proteins causes a gradual decline in replication with a 2-3 fold decrease within 5 minutes, indicating that the accessory proteins occasionally fall off the DNA template. However, when ATP is diluted along with the accessory proteins, no additive affect occurs indicating that ATP is required only to load accessory proteins from solution onto the template. Thus, similar to the γ complex of *E.coli*, 44/62p functions during holoenzyme assembly but does not act as a DNA translocase.

The polymerase stabilizes 45 protein onto the DNA template

To initiate replication, 44/62p (in its ATP bound form) binds tightly to 45p at the primer-template junction. Once ATP is hydrolyzed, the conformation of 45p changes, enabling it to associate with the T4 polymerase to form a functional holoenzyme (Capson et al., 1991; Munn, 1991b). Truncations of the DNA polymerase which retain full polymerase and exonuclease activity but cannot bind to 45p are unable to form a holoenzyme, suggesting that the components of the holoenzyme must interact physically (Goodrich et al., 1997).

In its "loaded" conformation, 45p is thought to function analogous to the β complex of *E.coli*. It resembles a sliding clamp by cryo-electron microscopy (Gogol et al., 1992). These clamps often appear in clusters, indicating that multiple clamps can be injected into a single nick on double stranded templates and can slide freely on the DNA template. Furthermore, if the holoenzyme is stalled at a missing nucleotide on a circular DNA template, it dissociates at a faster rate when the template is linearized (Hacker and Alberts, 1994b). The rate of holoenzyme dissociation depends on whether the template is linearized close to or far from the stall site, suggesting that 45p, like β,slides off the ends of the DNA. Despite of these similarity to the *Ecoli* β complex, the 45p clamp is not as stable as the β clamp (Munn, 1991a) and cannot be purified as a complex with the DNA template by

gel filtration (Richardson, 1990) or by rapid filtration on ssDNA cellulose (figure 6). By cryo-electron microscopy, accessory proteins which appear as dash-marks on the DNA disappear in less than a minute in the absence of ATP, indicating that they have a relatively short lifetime (Gogol et al., 1992).

Using rapid filtration studies, we have shown that the T4 polymerase stabilizes the 45p clamp and allows it to remain bound to the DNA template. These results follow earlier studies by Richardson et al. (Richardson, 1990) who showed that the 45p clamp can be purified by gel filtration on circular templates if 43p and 32p are present. Also, Munn and Alberts (1990) showed that the 45p clamp footprints onto DNA templates if 43p is present.

Thus, despite similarities, the T4 holoenzyme and the bacterial holoenzyme differ in a fundamental way. In *E.coli*, the β accessory proteins bind tightly to the DNA template and tethers the polymerase to the DNA. In contrast, the T4 holoenzyme is comprised of components which reciprocally stabilize one another. Neither the 45p or the polymerase are stable, but the holoenzyme as a whole binds tightly to DNA and can synthesize DNA processively and accurately. Besides their role in DNA replication, the T4 accessory proteins (44/62p and 45p) function to enhance the transcription of the late T4 promoters, a process which requires two additional T4 proteins, 33p and 55p (Herendeen et al., 1989; Herendeen et al., 1990). Our results suggest that the RNA polymerase, 33p or 55p may bind to the 45 clamp and maintain it on the DNA because 45p will have a tendency to fall off the template otherwise.

The elongating T4 holoenzyme is a dynamic structure, found in equilibrium between joint and disjoint states

The T4 polymerase and its accessory proteins associate with one-another to form a stable holoenzyme complex. But, do the components of the holoenzyme remain tightly associated during the elongation phase of replication? To answer this question, we conducted rapid dilutions to selectively remove either the accessory proteins or the

polymerase from ongoing strand invasion reactions. Our experiments show that the processivity of the holoenzyme (the polymerase and its accessory proteins) is not equivalent to the processivity of either the accessory proteins or the polymerase, indicating that the elongating holoenzyme does not behave as one unit. Our results are consistent with the following model (figure 8).

The holoenzyme is a dynamic structure found in equilibrium between "joint" and "disjoint" states (figure 8: "elongation"). In the joint state, the accessory proteins and the polymerase are complexed in a functional holoenzyme. In the disjoint state, the polymerase and the accessory proteins temporarily move away from one-another and from the primer-template junction but can reassociate without additional ATP hydrolysis. When disjoint, both the accessory proteins and the polymerase are weakly bound to the DNA and will fall off the template unless they reassociate rapidly.

In our *in vitro* reactions if the polymerase is diluted but the concentrations of accessory proteins and ATP are kept high, 45p can continually load onto the DNA to stabilize the polymerase at the replication fork. Likewise, if the accessory proteins are diluted but the concentration of polymerase is kept high, polymerase from solution can continually bind to 45p, preventing it from dissociating from the template. However, when both the accessory proteins and polymerase are diluted, neither component of a disjoint holoenzyme can associate with free proteins from solution and are unlikely to reassociate with one another. Thus, they fall off the DNA rapidly. The existence of a "disjoint state" is supported by the experiments of Hacker and Alberts. When the holoenzyme is stalled at a missing nucleotide on a double stranded template which is then linearized, the accessory proteins slide off the end of a linearized template. To do this, the accessory protein clamp must disjoin from the T4 polymerase since the polymerase is restricted to single stranded regions of the template.

If the holoenzyme does exist in equilibrium between joint and disjoint states, DNA synthesis must occur in spurts. Synthesis would temporarily stop when the components of

the holoenzyme disjoined, and the primer-template junction would be temporarily unoccupied. The accessory proteins bind tightly to the primer template function in the presence of ATPγS (Capson et al., 1991; Munn, 1991b). We probed for the presence of unoccupied primer template junctions by asking if ATPγS added simultaneous with high concentrations of 44/62p and 45p can interfere with ongoing replication reactions. Consistent with our dilution studies which indicated that ATP hydrolysis is occasionally required to reload accessory proteins onto ongoing replication forks, we show that addition of ATPγS decreases DNA synthesis 2-3 fold within the first 5 minutes. However, raising the concentration of accessory proteins upon addition of ATPγS abruptly inhibits replication, suggesting that unoccupied primer-template junctions exist. Though it seems unlikely, it is also possible that unoccupied primer-template junctions do not exist and that ATP bound accessory proteins displace active holoenzyme from the growing replication fork.

Implications for the termination of replication

The stability of the holoenzyme on DNA templates is context dependent. When the holoenzyme encounters a DNA hairpin helix, it dissociates from the DNA within 1 second, a rate fast enough to allow the holoenzyme to recycle on the lagging strand. In contrast, the processivity of the elongating holoenzyme is roughly 25 seconds, based on our experiments where the accessory proteins and the polymerase were diluted simultaneously (10 sec as measured by (Jarvis, 1991)). Finally, holoenzymes which have been stalled at a missing nucleotide remains associated with the DNA for an astonishing 2.5 minutes (Hacker and Alberts, 1994b). How then is termination of replication controlled and why does the dissociation rate of the holoenzyme vary?

The holoenzyme equilibrium model suggests that termination of replication is a two step process. The holoenzyme first separates into its accessory proteins and polymerase components. Each component then falls off the DNA separately. This means that

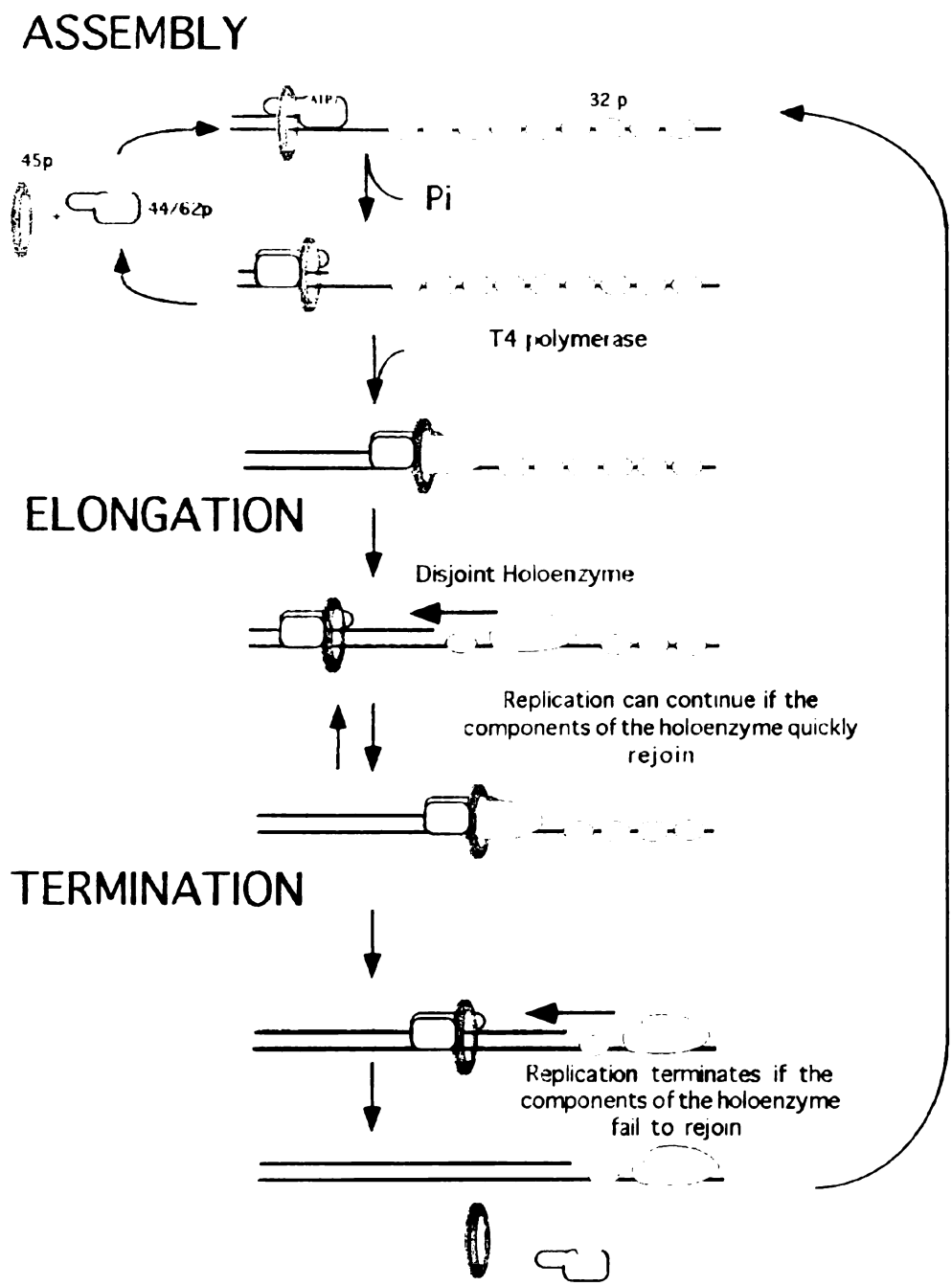


Figure 8. The holoenzyme equilibrium model. Assembly of the holoenzyme begins by the recognition of the primer template junction by 44/62p (in its ATP bound state) and 45p. Hydrolysis of ATP results in a conformational change which allows 45p to interact with the T4 polymerase. In this activated conformation, 45p is unstably bound to the DNA and has a tendency to fall off the template, requiring additional ATP hydrolysis for reassembly. However, once 45p binds to the T4 polymerase (forming a holoenzyme), it can remain bound to the DNA and facilitate DNA synthesis. During elongation, the protein-protein interactions between the polymerase and the accessory proteins are occasionally severed and the holoenzyme becomes "disjoint." In this disjoint state, the polymerase and the accessory proteins temporarily move away from one another and from the primer-template junction, but can reassociate without additional ATP hydrolysis. If they fail to reassociate rapidly, 45p may dissociate from the template, causing the termination of replication

termination of replication can be regulated at either of these two steps. Rapid holoenzyme dissembly at a DNA hairpin helix or at the end of an Okazaki fragment may occur either because the components of a disjoined holoenzyme cannot rejoin, or because they are inclined to fall off the DNA immediately upon disjoining. In contrast, a holoenzyme stalled at a missing nucleotide may have an increased tendency to remain in the joint state or to remain bound to the DNA template after disjoining. We know that as the holoenzyme synthesizes DNA, it frequently disjoins into its polymerase and accessory protein components because high concentrations of exogenous accessory proteins and ATPγS bind to unoccupied primer-template junctions and interfere with replication. It will be interesting to see whether the addition of ATPγS and high concentrations of accessory proteins also interferes with replication when the holoenzyme is stalled at a missing nucleotide.

The dissociation of the T4 holoenzyme off of the DNA template follows the simple kinetics of a first-order reaction, excluding the so-called "clock mechanism", which states that ATP hydrolysis by 44/62p places the holoenzyme into a high energy state which decays once the holoenzyme pauses, causing the holoenzyme to dissociate from the template (Munn, 1991a). We found that the accessory proteins of an elongating holoenzyme dissociate from the M13Mp19 template preferentially at sequence specific sites, corresponded to pause sites described previously be (Bedinger, 1989). However, when these pause sites were eliminated by including the T4 helicase in our reactions, the processivity of the accessory proteins did not improve. Thus, our results corroborate the conclusions of Hacker and Alberts (1994) and confirm that pausing of the elongating holoenzyme does not decrease the stability of the accessory proteins.

Materials and Methods

Enzymes

Bacteriophage fd gene 2 protein was purified in this laboratory by a modification of the protocol described in Meyer and Geider (Meyer, 1979). The T4 DNA replication proteins corresponding to genes 32, 44/62, 45, 43, and 41 were purified according to published procedures (Bittner, 1979; Morris, 1979). Gene 59 protein, whose function is to efficiently load the helicase (41p) onto the DNA was purified as described (Barry and Alberts, 1994). All of these preparations were free of detectable nuclease contamination and nearly homogeneous as judged by Coomassie blue staining of SDS polyacrylamide gel electrophoresis (Laemmli, 1970).

DNA Template

Double-stranded supercoiled M13mp19 DNA (7250kb) was purchased from Boehringer Mannheim. To nick this DNA at one specific site, fd gene 2 protein (200μg/ml) was incubated with 80μg/ml of the fd DNA in 20mm Tris-Cl (pH 8.5), 80mM NaCl, 2.5mM $MgCl_2$, 2mM β-mercaptoethanol, 50μg/ml human serum albumin, and 5% glycerol for 2.5 minutes at 30°C. The gene 2 protein was then inactivated by extraction of buffer saturated phenol. Analysis of the products of this reaction by agarose gel electrophoresis showed that more than 90% of the fd DNA was nicked in the reaction.

Standard Replication Reactions

Unless stated otherwise, *in vitro* DNA replication was carried out in the presence of 33 mM Tris Acetate (pH 7.8), 66 mM KOAc, 10mM MgOAc, 1.5μg/ml DNA, 100μg/ml HSA, 0.5 mM DTT, 50μM ATP, and 150μM each dATP, dCTP, dGTP, and dTTP. (a-^{32}P)TTP purchased from Amersham was used to radiolabel the reaction products. In standard reactions, the following T4 DNA replication proteins were present at the indicated concentrations: T4 DNA polymerase, 1μg/ml; T4 gene 44/62 protein, 2.5μg/ml; T4 gene

45 protein, 1.1μg/ml; T4 gene 32 protein, 200μg/ml. Where indicated, the reactions contained in addition 40μg/ml T4 gene 41 and 1μg/ml T4 gene 59.

Replication reactions were started synchronously at a specific nick in the fd DNA template. Reaction mixtures lacking only replication proteins and dCTP (150μM) were prepared at 0° C. All of the proteins were added and the mixture incubated at 37°C for 2 minutes unless specified otherwise; dCTP (150μM) was then added to allow replication forks to proceed. At various intervals, aliquots were removed from the reaction, pipetted onto glass microfibre filters (GF/A Whatman) which was immediately submerged in ice cold 5% trichloroacetic acid containing 0.1 volume of saturated sodium pyrophosphate. The filters were washed four times with ice cold 1N HCl, two times with 95% ethanol, and dried. Radioactivity was determined by liquid scintillation counting using Ecoscint.

For experiments in which products were analyzed on gels, aliquots were removed from the reaction into tubes containing gel loading buffer to produce a final concentration of 20mM Na_3EDTA, 10 % sucrose, and .04% Bromocresol green dye. The DNA was analyzed by electrophoresis through a 0.5% agarose gel under denaturing conditions as described in (Formosa and Alberts, 1986). The gels were then dried onto a DE81 ion exchange chromatography paper and autoradiographed either at room temperature or at -70° C using a Du Pont Lightning Plus intensifying screen. The sizes of the radioactively labeled DNA strands were determined by comparison with ^{32}P-labeled HindIII molecular weight standards of known size.

Dilution Assays

In order to carry out dilution experiments, reactions were prepared and replication forks were initiated as described above. Replication was allowed to proceed under the concentrations of substrates described above for one minute. At that time, the volume of the reaction was increased either 10 fold (for experiments represented in figures 2a, 2b, and 3) or 20 fold (for experiments represented in figures 4a and 4b) by addition of a dilution

mixture containing all components of the replication mixture (see above) except for DNA, and wherever indicated, ATP, 44/62p and 45p, and/or 43p. At various intervals, aliquots were removed, processed, and analyzed, exactly as described above. In experiments where ATP is diluted, 200μM dAMPPNP was used as replication substrate in place of 150μM dATP.

In dilution experiments where total incorporation of label was measured, (a-^{32}P)TTP was excluded from the starting mixture but included in the dilution mix. In pulse/chase experiments, (a-^{32}P)TTP was present in the starting reaction containing only 75μM dTTP; the dilution mixture for these experiments lacked additional label and contained 150μM dTTP. 20-fold dilutions were conducted.

Quantitation of Proteins Bound to ssDNA Cellulose

Rapid filtration was used to quantitate the binding of 44/62p and 45p to ss DNA cellulose. The procedure was performed exactly as described in Mace and Alberts (Mace and Alberts, 1984c) except that the reactions were performed at room temperature instead of 4°C and that the nucleotide in each reaction varied; ATP, ATPγS, ADP were used at 0.5mm. When present, the T4 genes 44/62, 45, 43, and 32 proteins were added at 42μg/ml, 44μg/ml, 5μg/ml and 360μg/ml respectively. Briefly, purified proteins were incubated with ssDNA bound to cellulose and transferred to a vacuum filtering apparatus containing a Millipore HA filter. Rapid filtration was then performed under vacuum, the trapped DNA-cellulose was scraped from the filter onto a spatula and suspended in 50μl of SDS-containing electrophoresis sample buffer. Samples were next boiled for 10 minutes, separated on a 13.5% polyacrylamide gel containing SDS (Laemmli, 1970), and blotted onto nitrocellulose (Burnette, 1981). The blots were blocked in 5% nonfat milk and 0.05% NP40 in PBS. Primary and secondary antibody incubations were done in the same buffer. Polyclonal rabbit antibodies against 62protein and 45protein were obtained from the laboratory of William Koningsberg and used at a dilution of 1:10,000.

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Appendix II

Two dimensional gel analysis of rolling circle replication in the presence and absence of bacteriophage T4 primase

I performed the *in vitro* rolling circle replication reactions (with and without lagging strand synthesis) which were then analyzed in figure 3.

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Two-dimensional gel analysis of rolling circle replication in the presence and absence of bacteriophage T4 primase

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ABSTRACT

The rolling circle DNA replication structures generated by the *in vitro* phage T4 replication system were analyzed using two-dimensional agarose gels. Replication structures were generated in the presence or absence of T4 primase (gp61), permitting the analysis of replication forks with either duplex or single-stranded tails. A characteristic arc shape was visualized when forks with single-stranded tails were cleaved by a restriction enzyme with the help of an oligonucleotide that anneals to restriction sites in the single-stranded tail. After calibrating the gel system with this well-studied rolling circle replication reaction, we then analyzed the *in vivo* replication directed by a T4 replication origin cloned within a plasmid. DNA samples were generated from infections with either wild-type or primase-deletion mutant phage. The only replicative arc that could be detected in the wild-type sample corresponded to duplex Y forms, consistent with very efficient lagging strand synthesis. Surprisingly, we obtained evidence for both duplex and single-stranded DNA tails in the samples from the primase-deficient infection. We conclude that a relatively inefficient mechanism primes lagging strand DNA synthesis *in vivo* when gp61 is absent.

INTRODUCTION

Bacteriophage T4 is extremely useful for studying the mechanism of DNA replication, in part because the phage encodes all of its own replication proteins in a relatively small genome. These proteins have been purified and used to establish replication forks on artificially primed templates (for a review see 1). The *in vitro* reaction depends on the phage-encoded DNA polymerase (gene product 43, gp43), polymerase accessory proteins (gp44/62 and gp45), single-stranded DNA (ssDNA) binding protein (gp32), helicase–primase complex (gp41/61) and helicase assembly factor (gp59). gp45 acts as a sliding clamp, allowing the polymerase to synthesize DNA in a highly processive manner (2–4). The ssDNA binding protein is important for T4 DNA replication, recombination and repair (for reviews see 1,5). In the *in vitro* replication system, gp32 coats ssDNA and is thought to play important roles in both template unwinding and coordination of primer synthesis on the lagging strand. The replicative helicase, gp41, unwinds the parental helix ahead of the leading strand polymerase by tracking along the lagging strand template (6). In association with gp41, the primase (gp61) synthesizes pentaribonucleotide primers for Okazaki fragment synthesis on the lagging strand (7–10).

Within the infected cell, T4 DNA replication initiates by two different strategies (for a review see 11). At early times of infection, replication is initiated from any of several origins scattered throughout the genome. As the infection progresses, these origins are shut off and a recombination-dependent mode of replication becomes dominant. The recombination-dependent mode is believed to involve invasion of a duplex phage chromosome by a single-stranded genomic 3′-end, followed by assembly of a replication complex in the resulting D loop and use of the invading 3′-end as a primer for leading strand synthesis.

Two T4 replication origins, *ori(34)* and *ori(uvsY)*, are able to direct autonomous replication of pBR322 derivatives into which they are cloned (12,13). Replication of such T4 origin-containing plasmids begins shortly after phage infection and requires T4 DNA polymerase and the polymerase accessory proteins (14). Somewhat surprisingly, a deletion of the T4 primase gene does not eliminate either T4 origin-containing plasmid DNA replication or phage DNA replication, although in both cases replication is severely delayed (14; see also 15–18).

The DNA delay phenotype of T4 primase mutants has two interesting features. First, replication is quite deficient at early times of infection, arguing that primase is normally a key component of the replication machinery. Lagging strand synthesis would be expected to require primase and the enzyme could also be necessary to prime leading strand synthesis from T4 origins at early times. Second, replication eventually reaches a vigorous rate despite the absence of the T4-encoded primase. Although it has not been proven, it seems likely that leading

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strand replication from the origins is primed by an origin transcript at late times during primase-deficient infections. Several models can be considered for the generation of duplex replication products in the absence of primase: (i) priming of lagging strand replication by an alternative DNA primase; (ii) cleavage of recombinational junctions by endonuclease VII (gp49) to provide primers for subsequent replication (19); (iii) priming of the lagging strand by RNA polymerase-generated transcripts; (iv) annealing of single-stranded products of oppositely oriented replication forks.

Both *in vivo* DNA replication and the *in vitro* replication system can use plasmid templates to produce concatemeric products through a rolling circle mechanism of replication (20–23). In the *in vitro* system, replication is initiated artificially by providing a strand-specific nick. The 3′-hydroxyl at the nick is extended by the DNA polymerase holoenzyme complex to replicate the DNA on the leading strand, while lagging strand synthesis using the displaced strand as template depends on periodic RNA priming by the helicase–primase complex. The generation of rolling circles from the origin-containing plasmid *in vivo* is not understood. Replication probably initiates in a bidirectional mode to generate a θ form, which is then converted into a rolling circle, perhaps by breakage of the leading strand template at one of the two forks.

In order to address the role of T4 primase *in vivo*, the two-dimensional agarose gel method of Brewer and Fangman (24) was utilized to examine replicative structures in primase-proficient and primase-deficient infections. Because the arcs generated in these two-dimensional gels by rolling circle intermediates have not been well characterized, we first calibrated the system using the products of *in vitro* reactions with and without primase.

MATERIALS AND METHODS

Materials

Restriction enzymes, random primed labeling kit, [α-^{32}P]dATP and oligonucleotides were purchased from commercial sources. Oligonucleotides were also synthesized by the Duke University Botany Department Oligonucleotide Synthesis Facility. The T4 origin-containing plasmid, pGJB1, has been described elsewhere (25,26). L Broth contains NaCl (10 g/l), BactoTryptone (10 g/l) and yeast extract (5 g/l) and was supplemented with ampicillin (25 mg/l) for growth of plasmid-containing cells.

Strains

The non-suppressing *Escherichia coli* host strain AB1 [*araD139 Δ(ara-leu)7697 ΔlacX74 galU galK hsdR rpsL*] was described by Kreuzer *et al.* (13). T4 strain K10, which is considered the wild-type control in these experiments, has the following mutations: *am*B262 (gene *38*), *am*S29 (gene *51*), *nd*28 (*denA*) and *rII*PT8 (*rII–denB* deletion) (27). K10-*61*Δ is isogenic except that it harbors an extensive in-frame deletion of gene *61* (14).

In vitro replication assay

Double-stranded M13mp19 DNA was incubated with phage fd gene 2 protein to produce a uniquely nicked substrate as previously described (28,29). The gene 2 protein nicking reaction was ~80% complete, explaining the presence of intact monomeric topoisomers in the two-dimensional gels. A small percentage (~1–5%) of the substrate contained randomly located nicks. The *in vitro* replication reactions contained 0.15 μg M13mp19 DNA that had been nicked as described above, 33 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 1 mM GTP, 150 μM of each of the other seven deoxyribo- and ribonucleotide triphosphates and the following T4 replication proteins: 20 μg/ml helicase (gp41), 100 μg/ml ssDNA binding protein (gp32), 1 μg/ml DNA polymerase (gp43), 25 and 11 μg/ml polymerase accessory proteins (gp44/62 and gp45, respectively), 0.5 μg/ml helicase–primase loading protein (gp59) and, where indicated, 1.5 μg/ml primase (gp61). After incubation at 37°C for 8.5 min, 35 mM Na_3EDTA and 0.1% SDS were added to stop DNA synthesis and the reaction products were stored at –70°C prior to gel analysis.

Alkaline agarose gel electrophoresis

Alkaline agarose gel electrophoresis was performed as described by Formosa and Alberts (30) with the following variations. Samples were denatured by bringing them to 300 mM NaOH and a 1/5 vol. stop solution (5% SDS, 15% Ficoll, 0.25% bromophenol blue and xylene cyanol FF) was then added. The denatured samples were loaded onto a 0.5% agarose gel (13 × 20 cm) which was subjected to electrophoresis in a solution of 30 mM NaOH and 2 mM Na_3EDTA with recirculation for 20 h at 30 V. Gels were neutralized in 1.5 M NaCl, 0.5 M Tris–HCl, pH 7.5, prior to Southern blotting as described by Sambrook *et al.* (31) and the blots were probed with labeled DNA (Boehringer Mannheim Random Primed Kit) prepared from an M13 ssDNA template (from US Biochemical).

Two-dimensional gel analysis of *in vitro* samples

Some DNA samples were loaded directly onto the first dimension agarose gel; others were treated with proteinase K at 37°C for 15 min, extracted once with phenol, passed through a CL6B spin column and digested with *Alw*NI for 1 h at 37°C in the presence or absence of 90 pmol oligonucleotide, 5′-GCGCAGTCTCT-GAAT-3′, which anneals to the *Alw*NI site in M13 plus strand DNA (the displaced strand that normally serves as lagging strand template). The conditions for two-dimensional gel electrophoresis were based on those described by Brewer and Fangman (24). Briefly, the first dimension gel contained 0.4% agarose and was run in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM Na_3EDTA) at 1 V/cm for 48 h. The second dimension gel contained 1.0% agarose and ethidium bromide (0.3 μg/ml) and was run in TBE buffer containing ethidium bromide (0.3 μg/ml) for 22 h at 6 V/cm at 4°C with buffer recirculation. The two-dimensional gels were analyzed by Southern hybridization (31) and the blots were probed with labeled DNA (Boehringer Mannheim Random Primed Kit) prepared from an M13 ssDNA template (from US Biochemical).

In vivo plasmid replication

Plasmid replication assays were performed essentially as described by Kreuzer and Benson (14). *Escherichia coli* AB1 cells containing plasmid pGJB1 or pBR322 (as indicated) were grown to a density of 4×10^8 cells/ml with vigorous shaking at 37°C in L broth. Where indicated, the cells were infected with either T4 K10 or K10-*61*Δ at a multiplicity of 3 plaque-forming units/cell.

After a 3 min attachment period without shaking, the infected cells were incubated at 37°C with vigorous shaking for 20 min. DNA was isolated by pelleting the cells in a microcentrifuge, resuspending the pellet in lysis buffer (100 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.2% SDS, 10 mM Na_3EDTA, 0.3 mg/ml proteinase K) and incubating at 37°C for 10 min. The lysates were then placed in a microtiter plate and trioxsalen (Sigma Chemical Co.) was added to each sample (final concentration 2 µg/ml). The DNA was cross-linked by illuminating with long wavelength UV light at 4 cm for 10 min (4.5 mW/cm^2). The samples were incubated at 65°C for 1 h, sequentially extracted with phenol, phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol and finally dialyzed against TE (10 mM Tris–HCl, pH 7.8, 0.5 mM Na_3EDTA) at 4°C overnight. The DNA samples were stored at –70°C prior to gel electrophoresis.

Two-dimensional gel analysis of *in vivo* samples

The DNA samples were digested with *Dra*I for 2 h at 37°C. When samples were digested in the presence of oligonucleotides to render ssDNA susceptible to restriction enzyme cleavage, 110 pmol of the oligonucleotide DraA (5′-TTGATTTAAAACTTCATTTTTAATTTAAAAGGA-3′) was added after the initial 2 h digest and the samples were heated to 65°C and then allowed to cool slowly to 37°C. The DraA oligonucleotide hybridizes to one strand of pBR322-derived plasmids in the interval 3228–3260 (pBR322 map coordinates), thereby rendering two potential *Dra*I sites double helical. After this annealing, the digest was supplemented with additional enzyme and incubated at 37°C for another 2 h. The annealing step was then repeated after the addition of 220 pmol of the second oligonucleotide, DraB (5′-TCCTTTTAAATTAAAAATGAAGTTTTAAATCAA-3′), which hybridizes to the same region of pBR322 but on the opposite strand. The heating, cooling and additional enzyme additions were also performed for those samples that were digested in the absence of oligonucleotide, so that the products would be directly comparable. The conditions for two-dimensional gel electrophoresis were identical to those described above, except that the first dimension gel was run for only 24 h (because the monomer DNA length is shorter). The two-dimensional gels were analyzed by Southern hybridization (31) and the blots were probed with randomly labeled DNA (Boehringer Manheim Random Primed Kit) prepared from a denatured *Ase*I-linearized pBR322 DNA template.

RESULTS

Analysis of *in vitro* replication structures

The two-dimensional agarose gel electrophoresis system of Brewer and Fangman (24) provides a powerful technique for analyzing the structure of DNA replication intermediates because it allows separation of molecules according to both mass (first and second dimensions) and shape (second dimension). We wished to use this system to analyze the structure of plasmid replication intermediates that form when phage T4 infects cells that carry a T4 origin-containing plasmid. However, these intermediates were believed to be rolling circles (20,21) and relatively little information was available on the patterns generated in two-dimensional gels by rolling circles. Therefore, we began by using the well-characterized T4 *in vitro* replication system to calibrate the system. When provided with a nicked circular substrate, the

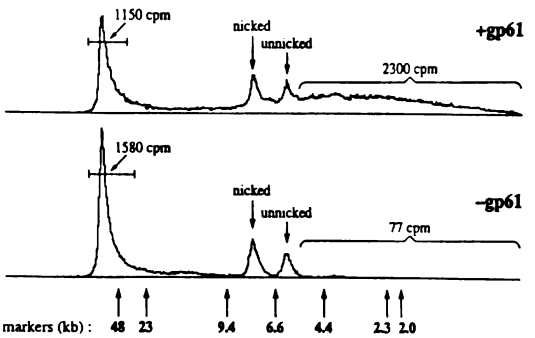

Figure 1. Analysis of *in vitro* replication products by alkaline agarose gel electrophoresis. Uncleaved *in vitro* replication reaction samples (+gp61, top and –gp61, bottom) were denatured and subjected to electrophoresis through an alkaline 0.5% agarose gel. The DNA was visualized by Southern blotting with an M13 minus strand probe. Direct radioisotope counting was performed using an AMBIS imager. The peak labeled nicked consists of unreplicated substrate plus strand (nicked by the gene 2 protein), while the peak labeled unnicked consists of covalently closed interlocked ssDNA circles that result from denaturing the substrate molecules that escaped gene 2 nicking. Size markers (denatured λ DNA and λ *Hin*dIII fragments, in kb) are noted along the bottom of the profile.

in vitro T4 replication system generates rolling circles by chain extension from the nick (22,23). For a template, we used double-stranded M13mp19 DNA with a site-specific nick introduced by the fd gene 2 protein. The reactions were performed either in the presence or absence of the T4-encoded primase (gp61) in order to generate rolling circles with either single-stranded or duplex concatemeric tails.

The expected reaction products were first verified by one-dimensional alkaline agarose gel electrophoresis. Quantitation of a Southern blot of the gel revealed the expected reaction products, along with remaining substrate DNA (Fig. 1). The profiles show products longer than 50 kb in both reactions, consistent with the expected leading strand of rolling circles (substrate monomer length 7.25 kb). In addition, short products consistent with Okazaki fragments were generated only in the primase-containing reaction. The two peaks labeled 'nicked' and 'unnicked' comprise substrate DNA (see Fig. 1 legend). With verification of the expected products of rolling circle replication, we turn to the two-dimensional gel analysis.

To help explain the two-dimensional gel results below, Figure 2 shows the replicative forms expected with either efficient, delayed or no lagging strand synthesis (Fig. 2A–C respectively). N represents the site of the nick introduced by fd gene 2 protein and filled triangles represent *Alw*NI cleavage sites that have not been cleaved. The left panel presents molecules prior to restriction enzyme cleavage, while the right panel shows the same molecules after cutting with *Alw*NI.

Figure 3 shows typical two-dimensional gel patterns generated from the *in vitro* reaction products, visualized by Southern hybridization with an M13 probe. The samples in Figure 3A (+ primase) and B (– primase) were not digested with restriction enzyme prior to electrophoresis. In both electropherograms, a ladder of intact supercoiled *topoisomers* and a spot of *nicked circles*, presumably consisting of unreplicated substrate, are

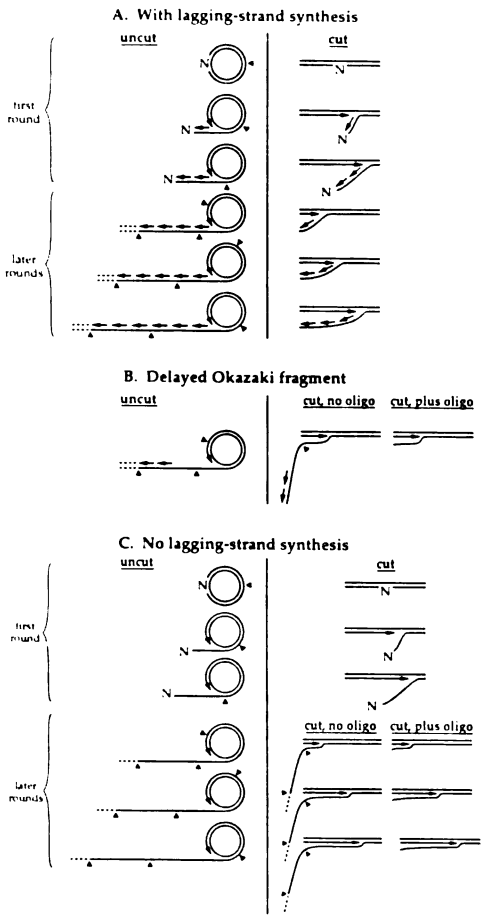

Figure 2. Diagram of rolling circle forms both before and after restriction enzyme cleavage. The first and later rounds of rolling circle replication are depicted, with and without lagging strand synthesis (**A** and **C**). A molecule with a partially completed lagging strand is also shown (**B**). The nicking site used to initiate replication is indicated by the letter N and uncleaved *Alw*NI sites are indicated by filled triangles. The dashed lines at the end of concatemer tails indicate that the tails may be much longer than represented in the diagram.

clearly visible (Fig. 3A and B). These and additional forms below were assigned based in part on their migration compared with molecular weight markers in both dimensions (data not shown).

In addition, a *ds linear arc* is particularly prominent in the products generated with primase (Fig. 3A). This *ds linear arc* very likely consists of molecules that were generated by 'onion skin' synthesis, as previously analyzed by Barry and Alberts (32). This type of replication, diagrammed in Figure 4A, occurs when an Okazaki fragment becomes displaced from the lagging strand, either by improper extension of the subsequent Okazaki fragment or by assembly of a new replication complex at the junction between two Okazaki fragments. In either case, the displaced Okazaki fragment is then converted into a duplex form by the excess primase and DNA polymerase holoenzyme activities in the reaction.

DNA from the primase-containing reaction (Fig. 3A) generated a characteristic curve, the *ds eyebrow*, emanating from the nicked circle spot. [The name *ds eyebrow* was used by Preiser *et al.* (33) for a similar curve from malarial mitochondrial DNA]. We are certain that this *ds eyebrow* curve consists of rolling circles with duplex tails (see Fig. 2A, left panel) for the following reasons: (i) the curve begins at the position of the nicked circle and increases in mass as expected for rolling circles with increasingly long tails; (ii) previous studies (22,23,34,35) have demonstrated rolling circle replication in this system; (iii) the curve is dependent on the presence of primase; (iv) restriction enzyme treatment apparently converts the curve into a simple Y arc (see below).

The *ds eyebrow* curve disappeared when the samples were prepared without primase (Fig. 3B). In this case, priming cannot occur on the lagging strand and the concatemer tail should thus remain single-stranded. In place of the *ds eyebrow*, a continuously upward-moving arc emanates from the nicked circle spot. The presence of a single-stranded tail apparently causes the rolling circles to run differently through the gel, producing a characteristic curve which we call the *ss eyebrow* (see Fig. 2C, left panel).

The gels shown in Figure 3C and D display the results obtained when the same two DNA samples were cleaved prior to electrophoresis with *Alw*NI, which cuts double-stranded M13 DNA once (~50% of the M13 DNA length away from the nicking site), but is unable to cleave M13 plus strand ssDNA. When primase had been present in the *in vitro* reaction, a standard *duplex Y arc* was generated (Fig. 3C). The *duplex Y arc* begins at the *ds monomer* spot and returns to the *ds linear arc* at 2× M13 size, behaving exactly as expected for cleaved rolling circles with a completely replicated concatemer tail (see Fig. 2A, later rounds). An additional Y arc is observed beginning at the *ds monomer* spot and returning to the *ds linear arc* at ~1.5× M13 size. This additional Y arc (*first round Y arc*) is expected to be generated from molecules that were in the first round of replication, since the nicking site used to initiate replication, rather than an *Alw*NI site, forms the end of the concatemer tail and thus one end of the Y molecule (see Fig. 2A, first round). An unlabeled arc is faintly visible in Figure 3C, beginning near the end of the *first round Y arc* and staying beneath the *duplex Y arc*. We infer that this faint arc consists of first round molecules in which replication has passed the *Alw*NI site, but the site has not yet become duplex (which is necessary for *Alw*NI cleavage; see below).

Several notable features are obvious with the *Alw*NI-cleaved reaction products that had been generated without primase (Fig. 3D). First, a prominent arc of long ssDNA linear molecules is evident (*ss linear arc*). Analysis by one-dimensional alkaline agarose gels indicated that purification of the DNA prior to restriction enzyme treatment caused a low level of random breakage of the single-stranded rolling circle tails (data not shown). Second, a long continuous arc emanates from the *ds monomer* (linear) spot and continues diagonally up the gel (Fig. 3D, denoted *uncut ss tails*). This long arc must have been generated from rolling circles with single-stranded tails, with *Alw*NI cleaving the duplex template circle but not the single-stranded concatemer tail (see Fig. 2C, first round, cut; later rounds, cut, no oligo). Third, a distinct arc begins at the same *ds monomer* site but hooks over at a relatively small size. This *first*

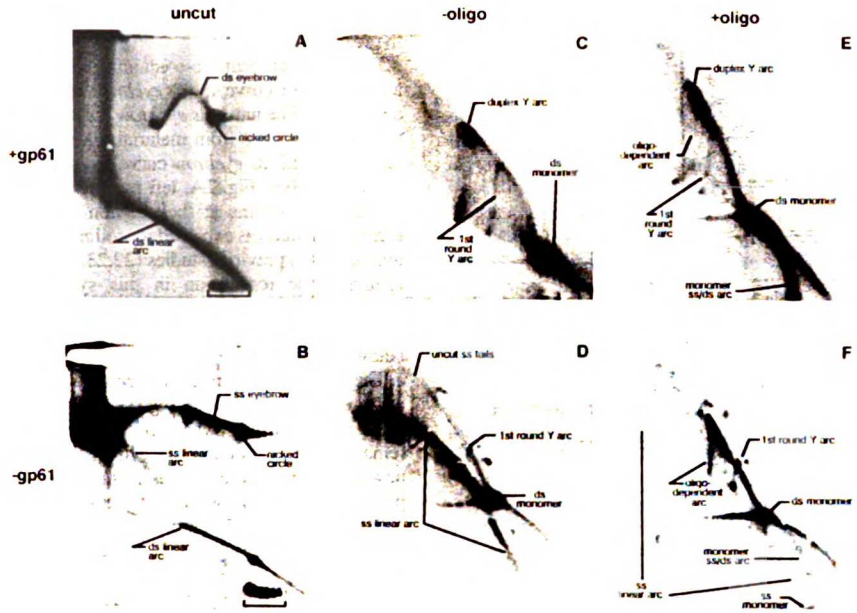

Figure 3. Two-dimensional gel analysis of *in vitro* samples. The conditions for the *in vitro* reactions and for the two-dimensional gel electrophoresis are described in Materials and Methods. The first dimension is represented horizontally (left to right) and the second dimension vertically (top to bottom). The DNA samples for (**A**), (**C**) and (**E**) were produced in the presence of T4 gp61, whereas those for (**B**), (**D**) and (**F**) were generated without gp61. The samples in (A) and (B) were not cleaved with restriction enzyme, while those in panels (C)–(F) were cleaved with *Alw*NI; an oligonucleotide complementary to the *Alw*NI site was added to the digests for (E) and (F). Each panel is a Southern blot of the two-dimensional gel, using randomly labeled M13 minus strand DNA as probe.

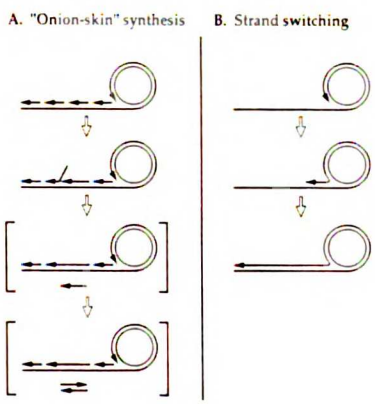

Figure 4. Diagram of replicative structures generated from 'onion skin' synthesis (**A**) and strand switching (**B**) mechanisms. During 'onion skin' synthesis, displacement of an Okazaki fragment and subsequent replication of the fragment into a duplex results in short, linear duplex molecules. The strand switching mechanism invokes template strand switching by the T4 DNA polymerase, leading to the production of duplex concatemer tails (and duplex Y forms) even in the absence of primase.

round Y arc is expected from first round replication structures that contain a discrete end on their concatemer tail (the nicking site; molecules represented in Fig. 2C, first round, cut).

We wished to further analyze the branched DNA forms that contain single-stranded regions by adding an oligonucleotide complementary to the restriction enzyme site during the *Alw*NI digest. Control experiments demonstrated that *Alw*NI is unable to cleave M13 plus strand ssDNA, but annealing of the appropriate oligonucleotide allows efficient cleavage (Fig. 5, compare lanes 1, 3 and 5). Furthermore, *Alw*NI cleavage of the ssDNA in the presence of oligonucleotides is highly site specific, as shown by the production of two appropriate discrete fragments in a double digest with *Alw*NI and *Hin*dIII (Fig. 5, lane 7; oligonucleotide for *Hin*dIII site was also present).

When the oligonucleotide was added to the digestion of the *in vitro* replication products produced with primase, two differences emerged (Fig. 3E; compare with Fig. 3C). First, an intense new arc (*monomer ss/ds arc*) was generated, beginning at the *ds monomer* (linear) spot and ending at the *ss monomer* (linear) spot (which ran off this particular gel). The molecules within this arc must be partially duplex, monomer length fragments with one *Alw*NI end in a single-stranded form. We presume that this *monomer ss/ds arc* originates from partially primed concatemer tails, for example regions where the 5′-end of the most recent Okazaki fragment is relatively distant from the fork (in Fig. 2B, right panel, the portion of the concatemer tail that would be released when oligonucleotide is added to allow *Alw*NI cleavage of ssDNA; see also Discussion for a description of other ss/ds monomer fragments). The second notable difference generated by the addition of oligonucleotide is the generation of a (faint) novel Y arc, referred to as the *oligo-dependent arc* (Fig. 3E). This arc appears to start at the *ds monomer* spot and presumably

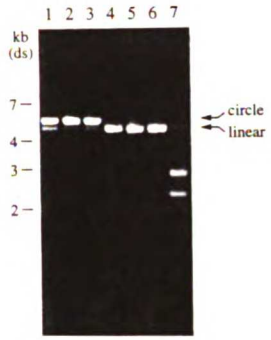

Figure 5. Specific cleavage of single-stranded M13 DNA by *Alw*NI and *Hin*dIII aided by oligonucleotides. All reactions contained 0.4 μg M13 ssDNA. In addition, the reactions contained: *Hin*dIII (lane 2); *Alw*NI (lane 3); *Hin*dIII plus an oligonucleotide for the *Hin*dIII site (lane 4); *Alw*NI plus an oligonucleotide for the *Alw*NI site (lane 5); *Hin*dIII, *Alw*NI plus an oligonucleotide to the *Hin*dIII site (lane 6); *Hin*dIII, *Alw*NI plus oligonucleotides to both the *Hin*dIII and *Alw*NI sites (lane 7). Samples were loaded onto a 1.0% agarose gel in TBE and electrophoresed for 18 h at 25 V. Circular and linear M13 ssDNA forms are noted with arrows. A molecular weight scale generated from the migration of double-stranded size markers (in kb) is indicated to the left of the gel.

contains molecules with concatemer tails that are single stranded, at least up to the first *Alw*NI site (see Fig. 2B, cut, plus oligo; see also below). The darkened broad area between this *oligo-dependent arc* and the *duplex Y arc* presumably contains Y forms with partial duplex tails.

When oligonucleotide was added to the sample prepared without primase (Fig. 3F), the long arc denoted *uncut ss tails* (Fig. 3D) disappeared due to *Alw*NI cleavage of the ssDNA tails. In its place, an intense *oligo-dependent arc* was generated. This arc, which begins at the *ds monomer* spot, must contain Y-shaped molecules with one ssDNA arm, as expected from rolling circles without lagging strand synthesis (see Fig. 2C, later rounds, cut, plus oligo). In addition, the DNA that formed the *ss linear arc* in Figure 1D was digested in the presence of oligonucleotide and most of the resulting molecules presumably migrated at the position of the *ss monomer* spot (much of this spot ran off the gel shown in Figure 3F, but repeats with shorter electrophoresis times showed an intense *ss monomer* spot). A very faint *duplex Y arc* was detected in both cleaved samples from the primase-deficient reactions, indicating that a replication fork occasionally had a double-stranded concatemer tail even in the absence of primase (Fig. 3D and F; the arc contains discrete spots and is not labeled). We surmise that these molecules result from strand switching by DNA polymerase, followed by replication of the single-stranded tail (see Fig. 4B and Discussion).

From these *in vitro* studies we were able to characterize a variety of unique forms associated with rolling circle replication. In particular, the addition of appropriate oligonucleotides during restriction enzyme digestion allows the clear visualization of single-stranded replicative structures. Thus informed, we turned our attention to the characterization of the replication structures found *in vivo*.

Analysis of *in vivo* replicated plasmid DNA

Either of two T4 origins, *ori(34)* or *ori(uvsY)*, can direct autonomous replication of a pBR322 plasmid upon bacteriophage T4 infection. The origin plasmid likely replicates, at least in part, by a rolling circle mechanism, because long concatemeric DNA products can be visualized by electron microscopy or by pulsed field gel electrophoresis (20,21). We began the *in vivo* experiments by comparing the replicative forms of pGJB1, an *ori(uvsY)*-containing plasmid, to those generated by a pBR322 control plasmid. DNA samples were prepared 20 min after infection of the plasmid-bearing *E.coli* cells by a T4 mutant deficient in host DNA breakdown. A DNA sample from the pGJB1-containing cells was also analyzed without T4 infection to determine whether the branched DNA forms are dependent upon phage infection. The DNA samples were cross-linked with trioxsalen immediately after cell lysis to reduce the possibility of DNA branch migration and then the samples were treated with the restriction enzyme *Dra*I and subjected to two-dimensional gel electrophoresis.

In the absence of T4 infection, the pGJB1 plasmid generated a faint arc, presumably due to replication triggered from the ColEI origin (Fig. 6A). This *theta* arc is exactly as predicted from the analysis of pBR322 replicative forms by Martin-Parras *et al.* (36). Furthermore, the results of Martin-Parras *et al.* (36) predict that the *theta* arc should give way to a single-Y arc and then a late stage double-Y form termination intermediate upon *Dra*I digestion; these are very likely the spots within the region labeled *Y forms* in Figure 6A. Note that a simple *duplex Y arc* was not detected in this control sample without T4 infection.

Turning to the analysis of samples from T4-infected cells (Fig. 6B and C), we need to first explain the cytosine modifications introduced during T4 replication and their effect on restriction enzyme digestion. T4 replicated DNA contains glucosylated hydroxymethylcytosine residues in place of normal cytosine, making the DNA refractory to most restriction enzymes. *Dra*I, which cleaves a hexameric sequence with only AT base pairs, is one of the few restriction enzymes capable of cleaving modified T4 DNA. Nonetheless, *Dra*I cleaves T4-modified (replicated) and unmodified (unreplicated) plasmid DNA differently. This differential cleavage is the cause of the two discrete *ds monomer* spots that are visible along the *ds linear arc* in Figure 6B (and in the gels below). Plasmid pGJB1 actually has three *Dra*I sites, but two of them are only 19 bp apart and therefore behave as a single site in this study. The third site is resistant to *Dra*I cleavage only when the substrate is modified, presumably because modified cytosines adjacent to this site block the restriction enzyme. Therefore, modified (replicated) pGJB1 is cleaved only once with *Dra*I, generating the larger (4.48 kb) band, while unmodified (unreplicated) plasmid is cut at both sites, generating the smaller (3.79 kb) band (and a 0.69 kb band that runs off these gels).

Figure 6B displays a typical result from a T4 infection of cells harboring the *ori(uvsY)* plasmid. We detected a strong *duplex Y arc* similar to that generated in the *in vitro* reactions, consistent with rolling circle replication. In addition, we detected a clear *X arc*, presumably containing plasmid recombination intermediates. Note that the *duplex Y arc* originates only from the modified DNA spot, implying that virtually all Y form DNA consists of replicated plasmid DNA (see also below). Importantly, no *duplex Y arc* was detected when T4 infected cells with the control plasmid that lacks *ori(uvsY)* (Fig. 6C). The *Y forms* generated with the T4 origin plasmid are therefore dependent on the presence of the T4 origin of replication as well as on T4 infection.

Essentially identical *duplex Y arcs* were detected after cleaving replicated pGJB1 DNA with restriction enzymes that cut (modified DNA) at other locations in the plasmid (data not

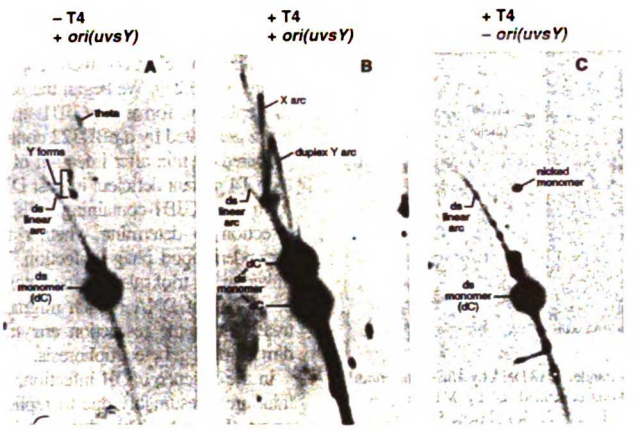

Figure 6. Two-dimensional gel analysis of plasmid replication with and without T4 infection. DNA was prepared from uninfected cells containing the T4 origin plasmid pGJB1 (**A**), from a T4 (gene *61**) infection of cells containing pGJB1 (**B**), or from a T4 (gene *61**) infection of cells containing pBR322 (**C**). All samples were treated with restriction enzyme *DraI*. Each panel is a Southern blot of the two-dimensional gel, using randomly labeled pBR322 DNA as a probe. Cytosine-containing (unmodified) monomer DNA is denoted *dC*, while the monomer DNA that contains glucosylated hydroxymethylcytosine (modified) residues is indicated by *dC**. The unlabeled monomer spot in (A) that is slightly larger than *ds monomer (dC)* represents unmodified DNA that has been partially cleaved by *DraI*. There are three unlabeled monomer spots in (C), representing (from high to low molecular weight) modified monomer DNA, hemi-modified monomer DNA and partially cleaved unmodified DNA. The first two of these spots result from a very inefficient replication of pBR322 in T4-infected cells and they migrate more slowly than partially cleaved unmodified DNA because of the glucosyl modifications.

shown). This result demonstrates that the Y forms originate from σ-shaped (rolling circle) molecules rather than uni- or bidirectional θ forms. Indeed, we have been unable to detect arcs consistent with θ form plasmid replication from T4-infected cells (data not shown). The *ds eyebrow* form, previously characterized with uncut rolling circle DNA *in vitro*, was also detected with uncut *in vivo* samples of origin-containing plasmid DNA (data not shown; the *ds eyebrow* was not nearly as pronounced as in the *in vitro* samples, presumably due to a low abundance of rolling circles with short tails). We conclude that the major branched plasmid DNA form from wild-type infections is the *duplex Y arc*, very likely generated by rolling circle replication with efficient lagging strand synthesis (see Discussion).

As described in the Introduction, we are interested in the nature of replication that occurs in a primase-deficient (gene *61* deletion mutant) infection. We therefore modified the oligonucleotide system for cleaving ssDNA so that it could be used with *in vivo* plasmid DNA samples. Unlike *in vitro* replication, we could not predict which strand (if either) of the origin-containing plasmid might remain single stranded in the absence of primase. Therefore, two oligonucleotides were used sequentially so that either DNA strand would be cleaved.

Figure 7 displays typical Southern blots of two-dimensional gels of pGJB1 plasmid DNA after infection by wild-type (Fig. 7A and B) or primase-deficient phage (Fig. 7C–E), without (A and C) or with added oligonucleotides (B, D and E). With wild-type phage, we again detected a *duplex Y arc* (whether or not the restriction digest contained oligonucleotide to allow ssDNA cleavage; Fig. 7A and B). Furthermore, the addition of oligonucleotide to allow cleavage of single-stranded forms caused no detectable changes in the two-dimensional pattern (compare Fig. 7A and B).

When we examined plasmid DNA from the infection with primase-deficient phage, the overall pattern was surprisingly similar to that of the wild-type (compare Fig. 7A and C). A *duplex Y arc* was readily detectable even without oligonucleotides that allow ssDNA cleavage (Fig. 7C). This result suggests that lagging strand synthesis occurred despite the absence of gp61 (see Discussion). In the digest without added oligonucleotides, DNA from the *61* mutant infection produced a line (labeled *ss tails*) that appears to emanate from the *ds monomer* spot and move up into the area beneath the *duplex Y arc*. Based on the analysis of the *in vitro* samples above, this line behaves like Y structures with one single-stranded arm, consistent with rolling circles that do not contain a fully functional lagging strand complex.

As mentioned above, the addition of oligonucleotide did not change the pattern of the wild-type sample (compare Fig. 7A and B). Therefore, in a wild-type infection, single-stranded structures are rare or absent. However, in the sample from the primase-deficient phage infection, the addition of oligonucleotides noticeably altered the electropherogram (compare Fig. 7C with D and E; Fig. 7E displays a lighter exposure of the blot in Fig. 7D). First, the line labeled *ss tails* became more prominent, consistent with the assignment of the molecules in this line as replication forks with non-functional lagging strand complexes. Second, we detected a prominent arc beneath the *ds monomer* (Fig. 7D and E). As with the *in vitro* samples (Fig. 3E), a *monomer ss/ds arc* appears to connect monomer linear dsDNA and monomer linear ssDNA, strongly arguing that the arc contains partial-duplex linear fragments. This assignment is supported by the fact that this arc disappears when the DNA sample is denatured prior to electrophoresis (data not shown).

These results indicate that the presence of abundant ssDNA is unique to the primase-deficient mutant, but that the detectable

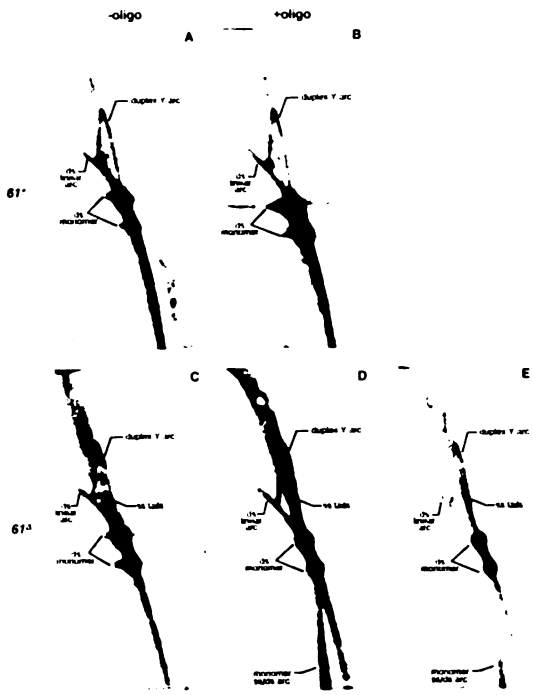

Figure 7. Two-dimensional gel analysis of plasmid replication by the T4 wild-type and a primase-deficient mutant. DNA was prepared from wild-type T4 infections of cells containing the T4 origin plasmid pGJB1 (**A** and **B**), or from primase-deficient mutant phage infections of the same plasmid-bearing host strain (**C**–**E**). All samples were treated with the restriction enzyme *DraI*. The restriction enzyme digests for (A) and (C) did not contain added oligonucleotide, whereas those for (B), (D) and (E) contained oligonucleotide to permit *DraI* cleavage of ssDNA. (E) A lighter exposure of (D). Each panel is a Southern blot of the two-dimensional gel, using randomly labeled pBR322 DNA as a probe.

single-stranded regions are often contiguous with duplex regions. As already discussed, an apparently normal *duplex Y arc* was readily visualized in DNA from the primase-deficient infection, arguing that some of the rolling circles have a functional lagging strand replication complex.

DISCUSSION

In this study, we analyzed the two-dimensional gel profiles associated with rolling circle replication and probed the mechanism of T4 DNA replication in the presence and absence of primase (gp61). Characteristic shapes, the *ss eyebrow* and the *ds eyebrow*, were observed when rolling circles had not been cleaved by restriction enzyme and the expected *duplex Y arcs* were observed after restriction enzyme treatment. In addition, the presence of an oligonucleotide during restriction enzyme treatment allowed unambiguous identification of structures with single-stranded regions. Our extensive characterization of various rolling circle structures and the method for cleaving single-stranded regions should be helpful to other workers analyzing unknown structures from a variety of systems.

We observed two features of the *in vitro* replication products that were not expected. First, the primase-containing reactions generated a very prominent arc of monomeric DNA that was partially single stranded when ssDNA cleavage was directed by the presence of the oligonucleotide. Given the fact that this arc is roughly as intense as the *ds monomer* spot, it seems unlikely that all of the DNA within this arc can be explained as regions of the lagging strand very close to the replication fork that were in the process of Okazaki fragment synthesis when the reaction was terminated. Most of the DNA in this *monomer ss/ds arc* probably originated from rolling circle tails in which the replication apparatus failed to complete one or more Okazaki fragments, thereby leaving ssDNA stretches in between adjacent Okazaki fragments. Such incomplete replication structures are commonly seen during electron microscopic analysis of T4 *in vitro* replication products (M.L.Wong and B.M.Alberts, unpublished data). A second unexpected feature was the presence of a faint *duplex Y arc* in the products of reactions lacking primase. This arc cannot result from gp61 contamination in one of the replication protein preparations, because all of the replication proteins were purified from overproducing clones. In addition, the arc consists primarily of a series of spots, rather than the more continuous line seen in the products of primase-containing reactions. The simplest explanation is that the T4 DNA polymerase undergoes strand switching (see Fig. 4B) at specific sites in the template circle, perhaps at inverted repeats where the newly synthesized 3′-end can hybridize with the ssDNA template tail (37–39). Once strand switching occurs, the site of the strand switch becomes a permanent Y junction and the tail of the rolling circle should be quickly replicated to completion.

Numerous investigators have used two-dimensional Brewer and Fangman (24) gels to characterize *in vivo* replication intermediates and the characterization of replication structures presented here should help in interpreting unusual arcs in both past and future studies. For example, Han and Stachow (40) observed an 'E-arc' from mitochondrial DNA of *Schizosaccharomyces pombe* which migrated much like our *ss tails*. Indeed, those authors provided evidence that the 'E-arc' contains DNA molecules with extensive single-stranded regions and argued that the arc consists of rolling circles with an unprimed tail. Our characterization of *bona fide* rolling circles with unprimed lagging strand tails supports the assignment made by Han and Stachow (40). In addition, Preiser *et al.* (33) recently detected *ds eyebrow* forms from uncut mitochondrial DNA of the malarial parasite *Plasmodium falciparum* and also concluded that these represent rolling circle intermediates.

The characterization of replicative structures from the *in vitro* system was designed to help decipher the plasmid DNA forms that are generated when phage T4 infects cells bearing a T4 origin-containing plasmid. The major branched DNA form detected from the primase-proficient infection was the *duplex Y arc*, as expected for rolling circle replication with normal lagging strand synthesis. Previous studies also suggested that T4 origin-containing plasmids use the rolling circle replication mode during phage infection, in that the major product of replication was found to be long concatemers of plasmid DNA (20,21).

Despite the strong evidence for rolling circle replication, we believe that replication from the T4 origin initiates with bidirectional θ forms which are converted to rolling circles at some frequency. The most direct evidence for this view comes from experiments in which a T4 origin was inserted into the *lac*

operon of the *E.coli* chromosome. The presence of the inserted origin increased replication of adjoining DNA during a T4 infection and all of the replicated DNA had undergone replication in both directions from the origin (unpublished data). Perhaps origin-containing plasmids initiate replication with a θ form which is then converted into a rolling circle at some frequency (analogous to replication of λ DNA; for a review see 41). Rolling circle intermediates should be much easier to detect because a rolling circle continues replication indefinitely. In addition, the products of rolling circle replication could predominate even if only a small fraction of θ forms are converted into rolling circles, because each rolling circle can generate numerous plasmid copies, whereas θ forms only duplicate the starting circle.

Several results argue that the *duplex Y arc* from the *in vivo* samples results from plasmid replication and not from recombination intermediates (e.g. by invasion of a linear end into an intact circle). First, the *duplex Y arc* was readily visualized from infections using a T4 mutant deficient in the recombination protein UvsY (synaptase accessory protein, required for UvsX-promoted recombination *in vivo*) (data not shown). Further, the modification state of the plasmid DNA argues strongly that the arcs are generated by replication. T4-directed modifications require *de novo* DNA synthesis, because the hydroxymethylcytosine is incorporated at the nucleotide level and glucosylation occurs subsequently on the hydroxymethyl residue. As shown in Figures 6B and 7, the arc emanates from the *Dra*I monomer spot containing T4-modified DNA and not from the spot containing unmodified plasmid DNA. Furthermore, the addition of *Hae*III, which cleaves unmodified (unreplicated) plasmid DNA into numerous small fragments, did not affect the shape or intensity of the *Dra*I-generated arc (data not shown). Finally, no arc was detected when we used restriction enzymes that linearize unmodified plasmid but are unable to cleave T4-modified DNA (data not shown). We cannot rule out the possibility that the *duplex Y arc* originates via recombination between two plasmid DNA molecules that have already replicated. However, in this model, the recombination event would need to strongly prefer T4-modified DNA and would need to be independent of UvsY protein. It seems much more likely that the *duplex Y arcs* contain true replicative intermediates (or aborted replication products).

We are very interested in the mechanism of origin plasmid DNA replication after infection by the *61* deletion mutant. A previous study showed that plasmid replication is deficient at early times but is quite robust by late times in the infection (14). In this study, we analyzed samples from a time (20 min) when plasmid replication is just beginning in the *61* mutant infection; similar arcs were detected from a much later time point (data not shown). Two features of the two-dimensional gel patterns of DNA from the primase-deficient mutant are notable. First, the addition of oligonucleotide to the restriction enzyme reaction revealed the presence of ssDNA. Second, the *duplex Y arc*, indicative of double-stranded concatemer tails, was visualized even though T4 primase was absent.

The ssDNA detected in the primase-deficient infection consists of Y forms with ssDNA tails (presumably rolling circles with little or no lagging strand synthesis) and monomer length partial duplexes (presumably tails of rolling circle with partially replicated lagging strands). One interesting possibility which we are currently testing is that the rolling circles replicate in only one direction when primase is not present. Both *ori(uvsY)* and *ori(34)* contain a middle mode promoter which is necessary for function of the origin (25,26). In several systems (ColE1, T7 and vertebrate mitochondria), a transcript made by RNA polymerase can act as a primer for leading strand DNA synthesis from the origin (42–46). It seems very likely that the RNA transcript from the T4 promoter within *ori(uvsY)* and *ori(34)* is used to initiate leading strand synthesis in the absence of primase. We do not know whether the origin transcript or a gp61-generated oligoribonucleotide primer is used to initiate leading strand synthesis in a primase-proficient infection (14).

The presence of a discrete *monomer ss/ds arc* in the primase-deficient infection suggests that some lagging strand synthesis occurs on the rolling circle tail, but that the mechanism is inefficient and leaves substantial single-stranded gaps. It is also interesting to note that these ssDNA forms were not detected from the primase-proficient infection, arguing for very efficient lagging strand synthesis when gp61 is available.

The presence of the *duplex Y arc* in the samples from the primase-deficient infection indicates that some of the concatemer tails from the rolling circles are completely duplex. Some lagging strand synthesis apparently occurs even in the absence of gp61, presumably through the action of another primase or by some alternative mechanism. Several models can be considered. First, the host DnaG primase might substitute for gp61. However, origin plasmid replication still occurs when a T4 *61* deletion mutant infects a host *dnaG* temperature-sensitive mutant under non-permissive conditions, arguing against an involvement of the host primase (14,19). Second, T4 may encode another DNA primase, perhaps a late gene product, to explain the replication delay in primase-deficient infections. Third, Mosig *et al.* (19) proposed that endonuclease VII (gp49) cleaves recombinational junctions in T4 DNA in *61* mutant infections and thereby provides primers for recombination-dependent replication. While this model could explain phage genomic replication, we cannot imagine how it could apply to origin plasmid replication. Fourth, RNA polymerase-generated transcripts that are able to hybridize to the single-stranded template may act as primers for lagging strand synthesis when gp61 is absent. We are currently defining the T4 gene products required for growth in the absence of gp61 by analyzing mutations that are synthetically lethal with the gene *61* deletion. With these and other experiments, we hope to solve the mystery of DNA replication in the absence of the gp61 primase.

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Appendix III

Guidance of circumferentially growing axons by netrin-dependent and -independent floor plate chemotropism

My contribution to this publication was the generation of the 293-EBNA$^{netrin\text{-}1}$ stable cell line used in the co-culture experiments of figure 3.

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Guidance of Circumferentially Growing Axons by Netrin-Dependent and -Independent Floor Plate Chemotropism in the Vertebrate Brain

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Summary

Netrin-1, a diffusible signal secreted by floor plate cells at the ventral midline of the vertebrate CNS, can attract ventrally migrating axons and repel a subset of dorsally migrating axons in the spinal cord and rostral hindbrain in vitro. Whether netrin-1 can act as a global cue to guide all circumferentially migrating axons is, however, unknown. Here, we show that netrin-1 can attract alar plate axons that cross the floor plate along its entire rostrocaudal axis. Dorsally directed axons forming the posterior commissure are, however, repelled by the floor plate by a netrin-independent mechanism. These results suggest that netrin-1 functions as a global guidance cue for attraction to the midline. Moreover, floor plate–mediated chemorepulsion may also operate generally to direct dorsal migrations, but its molecular basis may involve both netrin-dependent and -independent mechanisms.

Introduction

During development of the nervous system, growing axons navigate toward their targets in response to a variety of guidance signals in their surrounding environment. These cues include diffusible attractive or repellent molecules secreted by the intermediate or final cellular targets of the axons (for reviews, see Tessier-Lavigne, 1994; Goodman, 1996). One example of chemotropism is provided by floor plate cells at the ventral midline of the neural tube, which secrete chemotropic guidance signals that attract some ventrally directed axons from a distance, while repelling others away from it (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Shirasaki et al., 1995; Tamada et al., 1995). One molecule that appears to contribute to some of the activities of floor plate cells is netrin-1, a diffusible laminin-related protein expressed in the floor plate (Serafini et al., 1994; Kennedy et al., 1994). Two of the classes of axons attracted by floor plate cells, spinal commissural axons and cerebellar plate axons, are known to be attracted by netrin-1 (Kennedy et al., 1994; Shirasaki et al., 1995). In addition, one of the classes of axons repelled by floor plate cells, trochlear motor axons, is known to be repelled by netrin-1 (Colamarino and Tessier-Lavigne, 1995).

Netrin-1 is a vertebrate homolog of the UNC-6 protein of Caenorhabditis elegans, which is expressed in the ventral-most cells of the body wall of the nematode (Ishii et al., 1992; Wadsworth et al., 1996). UNC-6 is required to guide both ventrally and dorsally directed axons in the nematode, indicating that it too is a bifunctional guidance cue (Hedgecock et al., 1990; McIntire et al., 1992). A striking feature of UNC-6 function is that in *unc-6* loss-of-function mutants, circumferential axon migrations are affected throughout most of the animal, suggesting that UNC-6 functions as a global, rather than a regional, guidance cue (Hedgecock et al., 1990). The finding that netrin-1 mRNA is expressed in the floor plate at all axial levels (i.e., from the spinal cord through to the caudal diencephalon) (Kennedy et al., 1994) raises the question of whether netrin-1 also plays a global function in the guidance of circumferentially migrating axons at all axial levels of the CNS where floor plate cells are found. To address this possibility, we have considered two issues.

First, we have examined whether other dorsally migrating axons are repelled by netrin-1. For this, we have focused on the dorsally directed axons that form the posterior commissure (PC axons), because in the zebrafish the formation of the posterior commissure is disturbed in *cyclops* mutants, which lack the floor plate (Hatta et al., 1994; Patel et al., 1994). PC axons extend circumferentially toward the dorsal midline at around the rostral end of the mesencephalon (e.g., Chédotal et al., 1995; Mastick and Easter, 1996), like trochlear motor axons in the metencephalon (Colamarino and Tessier-Lavigne, 1995). They decussate at the dorsal midline roof plate and then project contralaterally (Lauder et al., 1986; Edwards et al., 1989; Chitnis and Kuwada, 1990; Wilson et al., 1990; Easter et al., 1993; Macdonald et al., 1994; Mastick and Easter, 1996). Here, we show that PC axons, like trochlear motor axons, are also repelled by the floor plate, and we address whether this repulsion is mediated by netrin-1.

Next, we examined the role of netrin-1 in guiding ventrally directed axons at different axial levels. It was previously shown that the floor plate attracts alar plate axons from the spinal cord, metencephalon, and myelencephalon (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Shirasaki et al., 1995; Tamada et al., 1995), and that netrin-1 can attract at least those in the spinal cord and metencephalon (Kennedy et al., 1994; Shirasaki et al., 1995). Here, we examine whether the floor plate and netrin-1 can attract alar plate axons from the myelencephalon through to the mesencephalon.

Taken together, our findings suggest that netrin-1 functions as a common chemoattractant for ventrally directed axons decussating at the floor plate, but that a floor plate–derived chemorepellent other than netrin-1 may be involved in repulsion of at least one dorsally directed axonal population, PC axons.

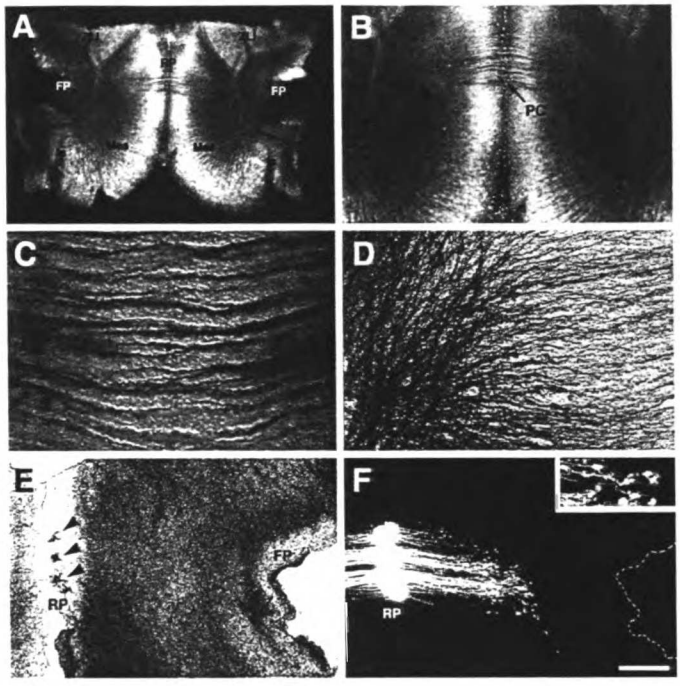

Figure 1. Trajectory of PC Axons in the Rat Embryo at E13

Staining with TAG-1 antibody (A–D) and DiI (E–F) in flat whole-mounted mesencephalic/diencephalic preparation. TAG-1 staining clearly demonstrates the presence of dorsal midline decussating axons at around the mesencephalon/diencephalon boundary.

(A) and (B) Photomicrograph showing TAG-1$^+$ axons in the flat whole-mounted preparation. Arrow in (B) denotes posterior commissure axons. PC axons gradually turn caudally as indicated by an arrowhead.

(C) Higher magnification view of posterior commissure at around the dorsal midline roof plate. Note that TAG-1 is continuously expressed at the midline.

(D) High-power micrograph of the area shown by an arrowhead in (B).

(E) Photomicrograph showing DiI injection site (arrowheads) at the dorsal midline region with bright-field illumination.

(F) Fluorescent micrograph of the area shown in (E), indicating retrogradely labeled axons and the origin of PC axons. Inset shows a higher magnification view of retrogradely labeled cells. An E13 rat brain was cut along the ventral midline, opened, and flattened. FP, floor plate; Is, isthmus; Mes, mesencephalon; PC, posterior commissure; RP, roof plate; ZLI, zona limitans intrathalamica. Rostral is up. (C) and (D): DIC micrographs. Bar, 800 μm (A); 400 μm (B, E, and F); 80 μm (C and D); 160 μm (inset in [F]).

Results

PC Axons in the Rat Mesencephalon Have a Dorsally Directed Trajectory Away from the Floor Plate In Vivo

PC axons express the axonal surface glycoprotein, TAG-1, during their early development in rodents (Yamamoto et al., 1986; Wolfer et al., 1994). This expression can be observed using a flat whole-mounted brain preparation, which enables ready recognition of axon trajectories, together with their relation to the circumferential and longitudinal axes of the developing brain (Shirasaki et al., 1995). Immunostaining of this preparation in embryonic day 13 (E13) rat brain using an antibody to TAG-1 reveals the trajectory of PC axons, which decussate dorsally at around of the rostral end of the mesencephalon (Figures 1A and 1B). PC axons express TAG-1 even at the dorsal midline roof plate, and tend to fasciculate with one another at around the dorsal midline (Figures 1B and 1C). After crossing the dorsal midline, they continue to express TAG-1 and extend ventrally, then make a gradual turn to project longitudinally in a caudal direction toward the metencephalon (Figures 1A, 1B, and 1D). TAG-1 does not label the cell bodies of origin of PC axons (as is the case for other neurons in the CNS that extend long axons [Yamamoto et al., 1986; Dodd et al., 1988; Wolfer et al., 1994]). To visualize these cell bodies, we therefore retrogradely labeled the cells by injecting the fluorescent carbocyanine dye DiI (for 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) into the dorsal midline region where PC axons decussate (Figure 1E, arrowheads). This resulted in labeling of PC cell bodies and their axons at around the rostral mesencephalon (Figure 1F). Thus, the trajectory of PC axons can be clearly recognized in flat whole-mounted preparations of the rat embryo: PC axons that originate from the ventral portion of the rostral mesencephalon (VRM) grow dorsally away from the floor plate, like trochlear motor axons in the metencephalon (Colamarino and Tessier-Lavigne, 1995).

The Floor Plate Repels and Reorients PC Axons at a Distance In Vitro

To test whether the floor plate can repel PC axons from a distance, we cocultured explants of the ventral half of the rostral mesencephalon (referred to hereafter as VRM explants) with floor plate explants, using collagen gel culture assays. When VRM explants dissected from flat whole-mounted preparations of E11/E12 rat embryos were cultured alone for 60–72 hr, axons grew from their cut edges in a radial fashion, except from the ventral cut edge that normally abuts the floor plate ($n = 22$; Figure 2A). In contrast, when a VRM explant was cultured with its cut dorsal edge placed at a distance from a metencephalic floor plate explant, axons growing from the dorsal side gradually turned away from the floor plate explants (48 of 48 explants tested; Figure 2B). The axons in the cocultures with floor plate explants appeared to be more extensively fasciculated than those from VRM explants cultured alone (compare Figure 2A with Figure 2B). Many of the axons that turned away from the floor plate did not contact floor plate explants or their processes (Figure 2B). Moreover, the turning of VRM axons could also be observed at an earlier time

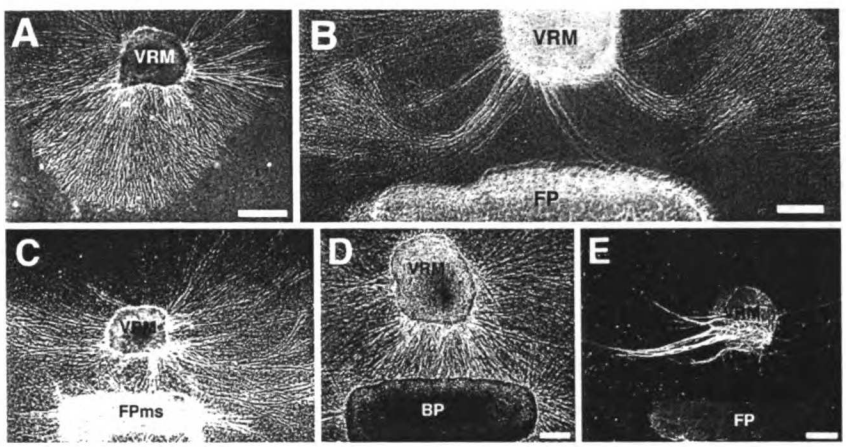

Figure 2. Chemorepulsion of PC Axons by the Floor Plate at a Distance In Vitro

(A) VRM explant cultured alone. Radial neurite outgrowth occurs from the dorsal cut edge of the VRM explant.

(B) Coculture of a VRM explant with a floor plate explant, with a dorsal cut edge of a VRM explant facing the floor plate. The floor plate repels and reorients VRM axons at a distance. Some of the axons turn away from the floor plate without contacting it, indicating that the repulsion is mediated by a diffusible factor. In about 60% of cocultures with metencephalic floor plate explants, virtually all axons extending from the dorsal cut edge gradually turned away from the floor plate as shown in this panel, while in the remaining cocultures some axons grew toward the floor plate (see [C]).

(C) Coculture of a VRM explant with a floor plate explant from the mesencephalon (FPms). VRM axons are also repelled by the FPms.

(D) Coculture of a VRM explant with a basal plate (BP) explant from brachial region of the spinal cord. VRM axons show radial growth pattern.

(E) Most of the repelled axons express TAG-1 in vitro. Bars, 500 μm (A and C); 200 μm (B and D); 250 μm (E).

(45 hr in vitro) before any VRM axons had contacted floor plate explants (data not shown). These results suggest that the turning of VRM axons is mediated by a diffusible factor. VRM axons were also repelled by explants of mesencephalic floor plate (16 of 16 explants; Figure 2C) and spinal cord floor plate (10 of 10 explants; data not shown). In contrast, explants of basal plate taken from the brachial spinal cord (10 of 10 explants; Figure 2D) or the metencephalon (6 of 6 explants; data not shown) of E13 rats did not repel these axons. Thus, a chemorepellent activity for VRM axons is secreted by ventral midline floor plate cells at all axial levels, but is not secreted by adjacent basal plate cells.

We next examined the identity of VRM axons that are repelled by the floor plate. The VRM region at this stage contains several classes of early differentiating neurons, including the cells that give rise to PC axons (Lauder et al., 1986; Mastick and Easter, 1996; see Figure 1F), medial longitudinal fasciculus (Easter et al., 1993; Mastick and Easter, 1996; data not shown), tyrosine hydroxylase–expressing dopaminergic axons (e.g., Altman and Bayer, 1981; Specht et al., 1981; Hynes et al., 1995; data not shown), and oculomotor axons (Puelles and Privat; 1977; Altman and Bayer, 1981; Easter et al., 1993; Mastick and Easter, 1996; data not shown). Of these, PC axons and oculomotor axons express TAG-1 at early stages of their development (Yamamoto et al., 1986; Wolfer et al., 1994). We found that most of the VRM axons repelled by the floor plate also expressed TAG-1 in vitro (Figure 2E). Axons extending from the dorsal cut edge of the VRM explant (including the corners) either cultured alone or with the floor plate, however, did not express F84.1 (data not shown), a marker for cranial motor axons, including oculomotor axons (Prince et al., 1992). Thus, it is likely that these TAG-1-positive (TAG-1$^-$) VRM axons do not include oculomotor axons and that the major population of the VRM axons repelled by the floor plate are PC axons. Together, these results indicate that the floor plate secretes a diffusible factor(s) that repels and reorients PC axons.

Heterologous Cells Secreting Netrin-1 Do Not Repel PC Axons

The long-range repellent effect of the floor plate on dorsally directed PC axons is reminiscent of its effect on trochlear motor axons, which have been shown to be repelled by a floor plate–derived chemotropic molecule, netrin-1 (Colamarino and Tessier-Lavigne, 1995). This raises the question of whether netrin-1 can repel PC axons. To test this, we monitored the effect of aggregates of COS cells secreting recombinant netrin-1 on PC axons, under conditions where these aggregates can attract cerebellar plate axons (Shirasaki et al., 1995) and repel trochlear motor axons (Colamarino and Tessier-Lavigne, 1995).

In a first set of cultures, a COS cell aggregate was placed between cerebellar plate and VRM explant, with the lateral side of the cerebellar plate explant and the dorsal cut edge of the VRM explant facing the aggregate (Figure 3A). Whereas COS cell aggregates elicited extensive neurite outgrowth from the lateral side of cerebellar plate explants (as previously reported; Shirasaki et al., 1995), they had no effect on axons growing from VRM explants (15 of 15 explants tested; compare Figure 3A with Figure 2A).

In a second set of cultures, we compared the ability of

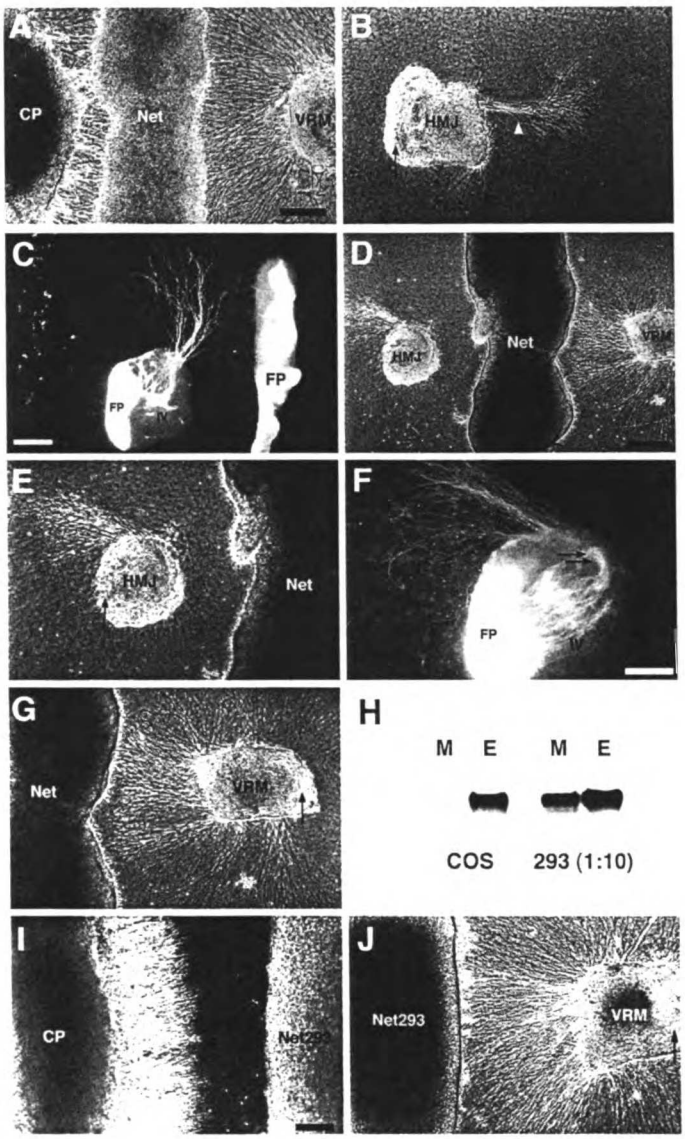

Figure 3. Netrin-1-Independent Chemorepulsion of PC Axons by the Floor Plate

(A) Triplet culture of a cerebellar plate (CP) explant, a VRM explant, and an aggregate of netrin-1-secreting COS cells (Net). While cerebellar plate axons show directed growth toward the COS cell aggregate, VRM axons are not repelled by the aggregate. No neurite outgrowth was observed from the lateral side of cerebellar plate explants grown alone (see Shirasaki et al., 1995).

(B) Phase-contrast micrograph of a hindbrain/midbrain junction (HMJ) explant extending a straight dorsal bundle characteristic of trochlear motor axons (arrowhead) (see also Colamarino and Tessier-Lavigne, 1995). Floor plate (FP) is to the left in this and subsequent panels with trochlear motor axons, except in (G) and (J), and indicated by a vertical arrow. Axons in the dorsal bundle expressed the motor axon marker, F84.1 (Prince et al., 1992) (data not shown).

(C) Epifluorescent micrograph of a coculture of an HMJ explant with a floor plate explant, showing that the dorsal bundle of trochlear motor axons from the HMJ is repelled by the floor plate. Trochlear motor axons (IV) are visualized by F84.1 immunolabeling. Floor plate cells also express F84.1, as previously described (Prince et al., 1992).

(D) Triplet culture of an HMJ explant, an aggregate of netrin-expressing COS cells, and a VRM explant. While axons from the HMJ explant that contains trochlear motor neurons are repelled, those from the VRM show radial growth as observed for VRM explants cultured alone (see Figure 2A).

(E) Higher magnification of the left side in (D), showing axons growing away from the netrin-1-secreting COS cell aggregate.

(F) Fluorescent micrograph of the HMJ explant shown in (E). The HMJ explant was stained for expression of F84.1. Note that F84.1$^+$ trochlear motor axons are completely deflected away from the COS cell aggregate. Double arrows indicate axons turning in the explant.

(G) High-power view of the right side in (D), showing that there is no indication that VRM axons are repelled by netrin-secreting COS cells.

(H) Recombinant netrin-1 is secreted into the medium at ~50-fold higher levels from stably transfected 293 cells than from transiently transfected COS cells. Conditioned medium [M] and 1 M NaCl extracts [E] were collected from transiently transfected COS cells expressing netrin-1 and from 293 cells stably expressing netrin-1, subjected to high speed centrifugation, TCA precipitated, resuspended in equal volumes of sample buffer, subjected to SDS–polyacrylamide electrophoresis, and immunoblotted using the 9E10 monoclonal antibody to detect the expressed tagged netrin protein. A 10-fold lower volume of each of the samples from the 293 cells was loaded onto the gel. The total amount of netrin-1, as well as the fraction of netrin-1 present in the medium, is higher from 293 cells than from COS cells.

(I) Coculture of a netrin-secreting 293 cell aggregate (Net293) with a cerebellar plate explant. Extensive neurite outgrowth occurs from the lateral side of the cerebellar plate toward the 293 cell aggregate (n = 16). In cocultures of control 293 cell aggregates with cerebellar plate explants, no neurite outgrowth from the lateral side of the cerebellar plate was observed (n = 6; data not shown).

(J) Coculture of a VRM explant with an aggregate of 293 cells stably expressing netrin-1. Axons from a VRM explant grow radially toward the aggregate. Repellent activity of 293 cell aggregates was monitored by an HMJ explant in the same culture. Similar radial growth patterns were also observed when cocultured with control 293 cell aggregates (n = 13; data not shown). In about 20% of cocultures with either netrin-secreting or control 293 cell aggregates, a small population of VRM axons showed mild deflections that did not qualify as "repulsion" by the criteria in the Experimental Procedures (data not shown). Bars, 300 μm (A, B, E, G, and J); 300 μm (C); 400 μm (D); 150 μm (F and I).

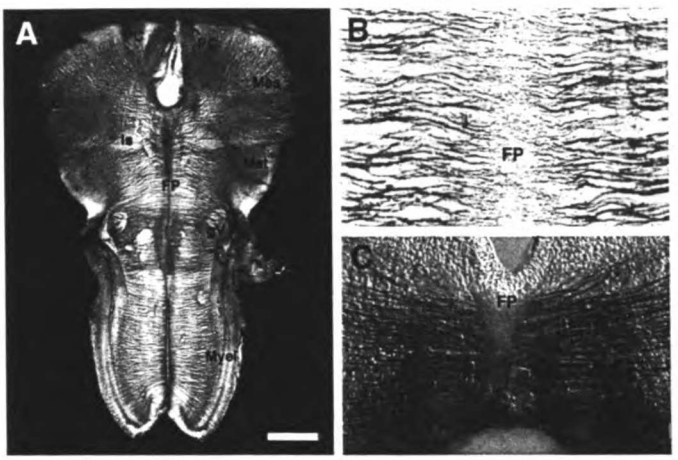

Figure 4. TAG-1 Expression in a Flat Whole-Mounted Embryonic Rat Brain Preparation

(A) and (B) Dark-field light micrographs of ventral view of a flat whole-mounted embryonic rat brain preparation stained for expression of TAG-1. This preparation includes the region extending from the myelencephalon (Myel) through to the mesencephalon. Rostral is up. An E14 rat brain was cut along the dorsal midline and opened. Note that a variety of midline decussating axons express TAG-1. These axonal populations may include efferent fibers of the cochlear nucleus in the myelencephalon (Kandler and Friauf, 1993), cerebellofugal axons in the metencephalon (Cholley et al., 1989; Shirasaki et al., 1995), and tectospinal axons in the mesencephalon (Shepherd and Taylor, 1995). (B) is a higher magnification of the metencephalic ventral midline region.

(C) DIC micrograph showing a TAG-1 stained coronal section of the ventral midline region at the metencephalon (Met). Note that the staining is interrupted at around the floor plate. A similar expression pattern was also observed in adjacent sections. gV, trigeminal ganglion. Bar, 800 μm (A); 80 μm (B and C).

COS cell aggregates to repel trochlear and VRM axons. Trochlear motor axon repulsion was studied in cultures of explants of the ventral portion of the hindbrain-midbrain junction from E11–12 rats (referred to hereafter as ventral HMJ explants). As described previously (Colamarino and Tessier-Lavigne, 1995), when ventral HMJ explants were cultured alone, a characteristic loose bundle of trochlear motor axons, which expressed F84.1 (data not shown), was observed projecting from the dorsal aspect of the explant (19 of 20 explants with dorsal bundles; Figure 3B). However, the direction of growth and formation of F84.1-positive (F84.1$^+$) dorsal bundles were perturbed when ventral HMJ explants were cocultured with floor plate explants (20 of 20 explants tested; Figure 3C). When an aggregate of COS cells secreting recombinant netrin-1 was placed between a ventral HMJ explant and a VRM explant, trochlear motor axons projecting from the former were repelled, whereas VRM axons were unaffected and grew in a radial pattern showing no sign of repulsion (14 of 14 explants; Figures 3D–3G).

Thus, VRM axons are not repelled by concentrations of netrin-1 that are sufficient to attract cerebellar plate axons and repel trochlear motor axons. These experiments did not, however, exclude the possibility that higher concentrations of netrin-1 might repel these axons. To address this, we derived a stable 293 cell line that secretes about fifty times more netrin-1 protein in soluble form than do transfected COS cells (Figure 3H). Aggregates of 293 cells attracted axons from cerebellar plate explants (Figure 3I), and repelled trochlear motor axons (data not shown), but did not repel axons from VRM explants (40 of 40 explants tested; Figure 3J). Taken together, these results suggest that chemorepulsion of PC axons by floor plate cells involves a long-range chemorepellent(s) other than netrin-1.

Netrin-1 Attracts Ventrally Decussating Axons from the Myelencephalon through to the Mesencephalon

We next examined whether netrin-1 can attract ventrally migrating axons at different axial levels. We found that a marker for these axons is provided by TAG-1, which is expressed by ventrally migrating commissural axons that project to the floor plate in the spinal cord (Yamamoto et al., 1986; Dodd et al., 1988; Phelps et al., 1993; Wolfer et al., 1994), and which also labels ventrally decussating axons that are organized in a "consecutive stripe" pattern in the myelencephalon, metencephalon, and mesencephalon (Figure 4A; see also Yamamoto et al., 1986; Wolfer et al., 1994). TAG-1 expression tends to be reduced around the border of the floor plate and most TAG-1$^+$ axons cannot be followed continuously across the floor plate in the metencephalon (Figures 4B and 4C; see also Wolfer et al., 1994).

We examined whether netrin-1 can function as a chemoattractant for ventrally projecting axons at all these axial levels. Previous studies have shown that the floor plate can attract axons from alar plate (AP) explants of spinal cord, myelencephalon, and metencephalon (cerebellar plate) (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Shirasaki et al., 1995; Tamada et al., 1995), and that netrin-1 can mimic this effect at least on AP explants from spinal cord and metencephalon (Serafini et al., 1994; Kennedy et al., 1994; Shirasaki et al., 1995). Moreover, axons from spinal cord explants that are attracted by floor plate cells or netrin-1 were shown to express TAG-1 (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Kennedy et al., 1994). Extending these studies, we found that floor plate explants can attract axons from AP explants from mesencephalon (n = 13; Figure 5I), and that COS cells secreting recombinant netrin-1 can attract axons from AP explants of both myelencephalon (n = 14; Figure 5E) and mesencephalon (n = 7; Figure 5K). In addition, AP axons that were attracted by the floor plate or netrin-1 all expressed TAG-1, whether they were derived from the metencephalon (Figures 5B and 5D), the myelencephalon (Figure 5F and data not shown), or the mesencephalon (Figures 5J and 5L). Control COS cells transfected with vector alone had no such effect on explants of AP from metencephalon (n = 9; data not shown), myelencephalon (n = 3; Figure 5G), or mesencephalon (n = 6; Figure 5H). When the aggregates

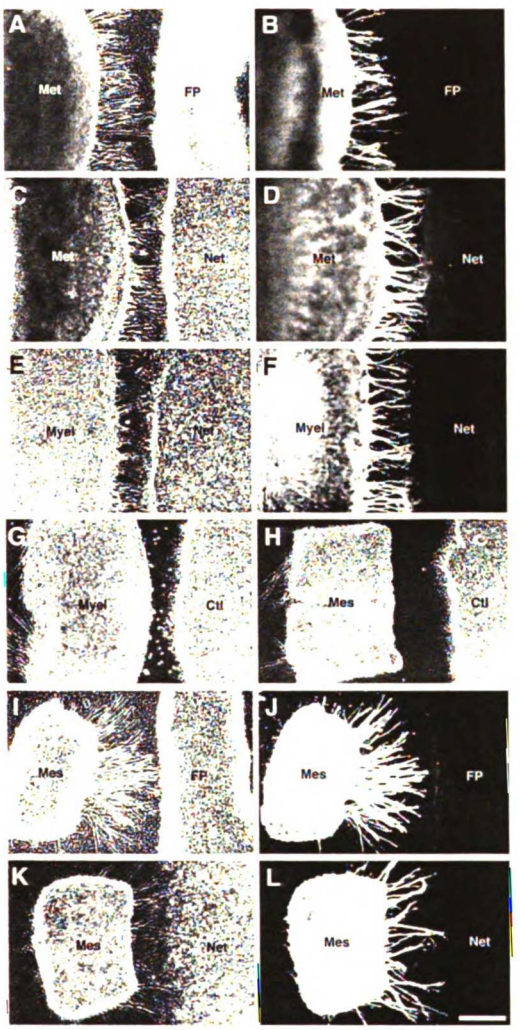

Figure 5. Chemoattraction of TAG-1-Positive Alar Plate Axons by Floor Plate Explants and Aggregates of Netrin-1-Secreting COS Cells

Axons from metencephalic (A–D), myelencephalic (E–F), and mesencephalic (I–L) AP explants are all attracted by floor plate explants (A, B, I, and J) and netrin-1-expressing COS cell aggregates (C, D, E, F, K, and L). Floor plate explants were in each case taken from the corresponding axial level of the AP explants. Growth of AP axons in the metencephalon and myelencephalon occurs ventrally in vivo (Shirasaki et al., 1995; data not shown). Thus, AP explants were oriented so that the dorsal side (lateral surface in whole-mount preparation) of the explants faces the floor plate explants or netrin-secreting COS cell aggregates to assay attraction of axons. When the AP explants were cultured alone or with aggregates of control COS cells (Ctl), axons emanated from the ventral cut edge but not from the dorsal side ([G] and data not shown; see also Shirasaki et al., 1995). Similarly, mesencephalic AP explants, when cultured alone (n = 7; data not shown) or with aggregates of control COS cells (H), showed some neurite outgrowth from the ventral cut edge, but little from the dorsal cut edge, as expected from ventrally directed growth of mesencephalic AP axons in vivo (Shepherd and Taylor, 1995; data not shown). Thus, mesencephalic AP explants were placed so that the dorsal side of the AP explants faces floor plate explants or netrin-expressing COS cell aggregates. Phase-contrast micrographs (A, C, E, I, and K) and corresponding immunofluorescence micrographs after staining with TAG-1 antibody (B, D, F, J, and L) demonstrate that the axons attracted by both the floor plate and netrin-expressing COS cells are TAG-1$^+$. Bar, 200 μm.

were juxtaposed aside AP explants of myelencephalon, metencephalon, and mesencephalon, turning and reorientation of AP axons toward aggregates of netrin-secreting heterologous cells were also observed within these AP explants (data not shown). Taken together, our data and previous studies show that both the floor plate and netrin-1 can promote the outgrowth of and attract ventrally decussating AP axons at all axial levels from the spinal cord to the mesencephalon.

Discussion

Floor Plate Repulsion Can Guide PC Axons Dorsally, but in a Netrin-Independent Manner

We have shown that PC axons originating from the VRM region are repelled by the floor plate but not the adjacent basal plate in vitro. This observation is consistent with the finding that the formation of the posterior commissure is disrupted in the floor plate–deficient zebrafish mutant *cyclops* (Hatta et al., 1994; Patel et al., 1994), and suggests that the disruption in this mutant might be due to loss of the chemorepellent. Thus, together these studies suggest that a floor plate–derived chemorepellent plays an important role in guiding PC axons dorsally in vivo.

Our studies also indicate that netrin-1, which can repel trochlear motor axons, does not repel PC axons, implying that the repellent effect of floor plate cells on PC axons is mediated by a distinct factor. This finding is consistent with the observation that the posterior commissure in vivo develops apparently normally in mice deficient in *netrin-1* function (Serafini et al., submitted). PC axons were repelled by floor plate explants from all axial levels tested, indicating that, like netrin-1, the chemorepellent for PC axons is secreted by the floor plate at all axial levels. Although our studies have not identified the repellent, they suggest that it is likely to be functionally distinct from collapsin-1/semaphorin III, a soluble protein that can cause collapse of sensory growth cones (Luo et al., 1993) and can repel specific populations of sensory afferents in vitro (Messersmith et al., 1995; Püschel et al., 1995). Indeed, mRNA for this molecule is expressed in the basal plate in the spinal cord (Messersmith et al., 1995; Püschel et al., 1995; Shepherd et al., 1996; Wright et al., 1995), hindbrain, and midbrain (Shepherd et al., 1996), and basal plate explants from spinal cord secrete a collapsin-1/semaphorin III–like activity in vitro (Messersmith et al., 1995; Püschel et al., 1995), yet such explants do not repel PC axons in vitro.

Interestingly, the existence of a floor plate–derived chemorepellent activity distinct from netrin-1 was also recently demonstrated through studies on trochlear motor axons. Although these axons are repelled by netrin-1 in vitro (Colamarino and Tessier-Lavigne, 1995), they are also repelled by floor plate explants dissected from mice

deficient in *netrin-1* function, and their trajectory is largely normal in the mutant mice (Serafini et al., submitted). Recent studies have also demonstrated that floor plate cells secrete a chemorepellent for olfactory interneuron precursors that is distinct from netrin-1 and collapsin-1/semaphorin III (Hu and Rutishauser, 1996). It remains to be determined whether the same factor is responsible for all of these netrin-independent repulsive effects.

Netrin-1 as a Common Attractant for Alar Plate–Derived Ventrally Decussating Axons

Our results have extended previous studies (Tessier-Lavigne et al., 1988; Placzek et al., 1990, Shirasaki et al., 1995; Tamada et al., 1995) to show that TAG-1$^+$ AP axons, which project in vivo to the floor plate, are attracted by floor plate cells and by netrin-1 at all axial levels from the spinal cord to the mesencephalon. Since netrin-1 mRNA is expressed along the ventral midline up to the level of the caudal diencephalon (Kennedy et al., 1994), the present findings suggest that AP axons from the spinal cord up to the level of the mesencephalon are guided toward the ventral midline by a common netrin-1-mediated chemoattractive mechanism. In mice deficient in *netrin-1* function, the projection of spinal commissural axons to the floor plate is severely perturbed (Serafini et al., submitted), predicting that the normal trajectory of TAG-1$^+$ commissural axons at higher axial levels in vivo might also depend critically on *netrin-1* function.

Our results show interesting parallels and differences with results on the netrin-1 homolog UNC-6 in C. elegans. In the nematode, UNC-6 appears to function as a global guidance cue for almost all circumferentially migrating axons, and both ventrally and dorsally directed migrations are perturbed in *unc-6* mutants. A few populations of circumferentially migrating axons in the head region of the nematode, however, are not affected (Hedgecock et al., 1990). In vertebrates, our results suggest that netrin-1 might likewise function as a global guidance cue for ventrally directed circumferential migrations at all axial levels where the floor plate is found. In addition, our results and those of previous studies (Colamarino and Tessier-Lavigne, 1995; Tamada et al., 1995; Guthrie and Pini, 1995) suggest that the floor plate might play a global guidance role in repelling axons away from the floor plate. However, the findings reported here and in Serafini et al. (submitted) indicate that this presumed global guidance role of the floor plate is likely to be mediated by both netrin-dependent and -independent mechanisms.

Expression of TAG-1 as a Common Denominator of Axon Responsiveness to Floor Plate–Derived Guidance Signals

TAG-1, an immunoglobulin gene superfamily member (Furley et al., 1990), is expressed on ventrally decussating axons in a consecutive-striped manner throughout the hindbrain and midbrain. Our findings and those of previous studies indicate an interesting correlation between expression of TAG-1 by axons in the CNS and responsiveness to floor plate–derived diffusible guidance cues. Thus, as discussed above, TAG-1$^+$ AP axons from the spinal cord through to the mesencephalon are all attracted by the floor plate. In addition, PC axons, which also express TAG-1 during their early development (Yamamoto et al., 1986; Wolfer et al., 1994; this study), respond to a floor plate–derived chemorepellent. Recent studies have also demonstrated that motor axons in the hindbrain and the spinal cord, which also express TAG-1 during the period of initial axon extension (Yamamoto et al., 1986; Dodd et al., 1988; Wolfer et al., 1994), are repelled by the floor plate (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995). This correlation raises the possibility that the TAG-1 molecule itself might in some way be involved in reception or signal transduction of floor plate–derived signals. Consistent with this is the finding in *netrin-1*-knockout mice that the anterior commissure, hippocampal commissure, and corpus callosum are absent (Serafini et al., submitted), all of which express TAG-1 during their development (Yamamoto et al., 1986; Wolfer et al., 1994). It should be noted, however, that such a role for TAG-1 has not yet been demonstrated.

Mechanisms of PC Axon Guidance

Since PC axons grow dorsally toward the dorsal midline roof plate, it is possible that the roof plate secretes chemoattractive factors that guide these dorsally decussating axons, just as the ventral midline floor plate does for ventrally decussating axons. In our preliminary coculture experiments using collagen gels, however, we have been unable to demonstrate any chemoattractive activity of roof plate cells toward PC axons (R. S. and F. M., unpublished data).

The mechanism that allows PC axons to cross the dorsal midline is unknown, but one intriguing feature that might be related to this mechanism is the fasciculation of PC axons at the dorsal midline. Axon–axon fasciculation at the midline has not been observed for ventrally decussating axons in the embryonic chick spinal cord (Yaginuma et al., 1990), but is reminiscent of the behavior of commissural growth cones in the grasshopper embryo, where successful growth-cone crossing of the midline requires contact and fasciculation with the contralateral homologue (Myers and Bastiani, 1993a, 1993b). It will therefore be of interest to examine whether PC axons can advance across the dorsal midline in the absence of their contralateral counterparts.

PC axons grow ventrally for some distance after crossing the roof plate, then gradually turn caudally at around the ventral mesencephalon. The gradual turning suggests that PC axons might retain responsiveness to a floor plate–derived chemorepellent(s). Thus, the situation with PC axons might be different from that of ventrally decussating axons. Cerebellar plate axons, for example, lose responsiveness to a floor plate–derived chemoattractant after once crossing the floor plate, as demonstrated in whole-mount culture preparations in which a second floor plate is implanted (Shirasaki et al., 1996). It is interesting in this light that PC axons do not lose expression of TAG-1 after crossing the midline, whereas ventrally decussating axons do (Dodd et al., 1988; Wolfer et al., 1994; this study). Thus, the correlation between expression of TAG-1 and responsiveness

to floor plate–derived diffusible cues might apply not just to different classes of axons, but also to different stages of development of a given class of axons.

Experimental Procedures

Immunostaining and DiI Labeling

All procedures followed those of Shirasaki et al. (1995), with some modifications. In brief, the midbrain and hindbrain of E13–E14 Wistar rat embryos (E0 is the day of vaginal plug) was cut along either the ventral or dorsal midlines, whole-mounted with the ventricular side down, and fixed with 4% paraformaldehyde. Small crystals of DiI (Godement et al., 1987; Honig and Hume, 1989) were placed into the dorsal midline near the mesencephalon/diencephalon boundary. At 1–5 days after DiI implantation, the preparations were observed by epifluorescence microscopy.

For immunostaining, whole-mount preparations were stored in PBS containing 0.1% NaN_3. The preparations were incubated in PBS containing 1% Triton X-100 (PBST) with gentle agitation for at least 30 min, incubated in PBST containing 0.3% H_2O_2 for 30 min to reduce the endogenous peroxidase-like activity of red blood cells. Subsequently, they were incubated in normal goat serum (NGS) (diluted 1:10 in PBST containing 0.1% bovine serum albumin) for 2 hr. The preparations were then incubated in anti-TAG-1 (4D7) antibody (undiluted supernatant, kindly provided by Dr. M. Yamamoto) as a primary antibody for 3 hr, washed at least six times for 30 min each in NGS (diluted 1:100 in PBST), and then biotinylated secondary anti-mouse IgM (Vector Labs, 1:200) for 3 hr, washed as before in PBS, incubated in avidin–biotin peroxidase complex (ABC) reagent (Vector Labs, Vectastain ABC kit, diluted 1:100 in PBS) for 3 hr, washed, and then in diaminobenzidine (DAB) (0.05% in TBS) with 0.01% H_2O_2 for visualization. Some preparations were embedded in a 10% gelatin-egg yolk mixture, which was then hardened for about 24 hr in a 1 cm-cube mold. We cut 60 μm transverse sections of fixed preparations out from the cube on a Microslicer (Dosaka EM) and immunostained them as described above, but with a shorter incubation period.

Staining procedures of cultured explants in collagen gels followed those of Tamada et al. (1995), with some modifications. In brief, following fixation, the explants were stained using anti-TAG-1 (diluted 1:1 in PBST) or F84.1 antibody (supernatant, gift from Dr. W. Stallcup; diluted 1:10 in PBST) as primary antibodies for 4 hr, biotinylated secondary anti-mouse IgM (Vector Labs, 1:200) or biotinylated anti-mouse IgG (Vector Labs, 1:200) for 4 hr, and then Cy3-conjugated Streptavidin (Jackson ImmunoResearch, 1:500) or Fluorescein Avidin D (Vector Labs, 1:200) for 3 hr, prior to mounting with glycerol in PBS. Washing at least four times for 1 hr each was performed after each incubation step. All immunohistochemical procedures were carried out at room temperature.

Explant Culture in Collagen Gels

Procedures for explant culture followed those of Shirasaki et al. (1995). In the chemoattraction assay, the brains of E12–E13 Wistar rat embryos were used. After we dissected AP explants of the metencephalon, myelencephalon, mesencephalon, and the floor plate at corresponding axial level from flat whole-mounted preparations, the explants were embedded into collagen gels, which enable detection of diffusible chemotropic activities (Ebendal and Jacobson, 1977; Lumsden and Davies, 1983, 1986). AP explants of the mesencephalon were taken from the ventral AP region so as not to include the dorsal-most AP of the mesencephalon, which contains the cells of origins of ipsilaterally growing axons repelled by the floor plate (Tamada et al., 1995). AP explants of the metencephalon, myelencephalon, and mesencephalon were cocultured for 24–30 hr with floor plate explants or for 30–35 hr with COS cell aggregates. For detection of chemorepellent activity, E11–E12 rat embryos were used, since PC axons appear to initiate growth dorsally at around this stage (Shirasaki and Murakami, unpublished data; see Lauder et al. [1986] and Mastick and Easter [1996]). E11–E12 rat embryos were dissected in cold DMEM/F-12 (Sigma) with 15% fetal bovine serum (HyClone) after treatment with 0.5% trypsin in Ca^{2+}- and Mg^{2+}-free Hanks' solution for 45 min on ice. VRM explants were dissected from the ventral half of entire explants between rostral mesencephalon and caudal diencephalon. HMJ explants were obtained as previously described (Colamarino and Tessier-Lavigne, 1995). Basal plate explants were dissected from the spinal cord in brachial regions and from the metencephalon, since the boundary between the basal plate and the floor plate was more clearly discernible in these regions compared to the mesencephalon. Explants were embedded in collagen gels and cultured for 60–72 hr as described previously (Shirasaki et al., 1995; Tamada et al., 1995), except that the culture medium was supplemented with 2 mM L-glutamine and 10 μg/ml streptomycin sulfate. In all cocultures, dorsal cut edge of VRM explants were faced with floor plate explants or with COS cell aggregates, separated less than 1 mm. Axons growing from the dorsal cut edge, including the corners, were monitored and analyzed for repellent activity. Aggregates of transfected or mock-transfected COS cells or 293 cells were prepared by the hanging drop culture method as described previously (Kennedy et al., 1994; Shirasaki et al., 1995).

To assess chemorepulsion of VRM axons, we first defined an axis by a line drawn perpendicularly to the dorsal cut edge of the VRM explants. Axons emanating from this edge were considered repelled if their initial trajectory was within 90° of the defined axis, but then turned to project in a direction opposite to the defined axis. Cultures that contained any axons that showed such dramatic changes in trajectory were considered to exhibit repulsion. In most cases, in cultures that showed any repulsion, a major population of axons emanating from the cut edge were repelled. Axons that showed irregular trajectories and those that contacted the explants or cell aggregates were excluded from the analysis.

Generation of the Cell Line Stably Secreting Netrin-1

Netrin-1^{myc} from pGNET1^{myc} (Serafini et al., 1994) was first transferred to pcDNA3 (Invitrogen) by digestion with EcoRI and XhoI, then to pCEP4 (Invitrogen) by digestion with HindIII and XhoI. This construct was introduced into 293-EBNA cells (Invitrogen) using LipofectAMINE (GIBCO BRL) as directed and grown under selection (250 μg/ml Geneticin, 200 μg/ml Hygromycin B). To prevent loss of the extrachromosomal episomes, the transfected cells were continually maintained in selective media. Purified recombinant netrin-1 secreted by these cells had a similar specific activity to that of netrin-1 secreted by COS cells (data not shown).

Expression of Netrin-1 Protein

A COS-1 cell monolayer was transiently transfected in 35 mm wells with pGNET^{myc} as described previously (Kennedy et al., 1994). Upon removal of the transfection media, the monolayer was washed with PBS, then incubated for 3 days with 1.5 ml of OptiMEM I supplemented with GlutaMAX I (GIBCO BRL) and antibiotics. In parallel, netrin-1 stably transfected 293-EBNA cells were grown to confluence in 35 mm wells, washed with PBS, then incubated for 3 days with 1.5 ml of OptiMEM I, GlutaMAX I, and antibiotics. After harvesting the conditioned media from both cell types (to which were added 1X protease inhibitors [1 mg/ml of each leupeptin, aprotinin, pepstatin]), the cells were rinsed with PBS, then incubated for 30 min with 1.5 ml of extraction buffer (1 M NaCl, 20 mM NaPi [pH 7.5], 1X protease inhibitors). Media and extracts were centrifuged at 2000 × g for 10 min, then at 60,000 × g for 100 min. The supernatants were TCA precipitated as described (Kennedy et al., 1994), then resolubilized in SDS sample buffer. Half of the protein from transiently transfected COS-1 cells expressing netrin-1 and 1/20 of the protein from 293-EBNA cells stably expressing netrin-1 were subjected to Western blot analysis using 9E10 culture supernatant (directed to the myc epitope tag on netrin-1).

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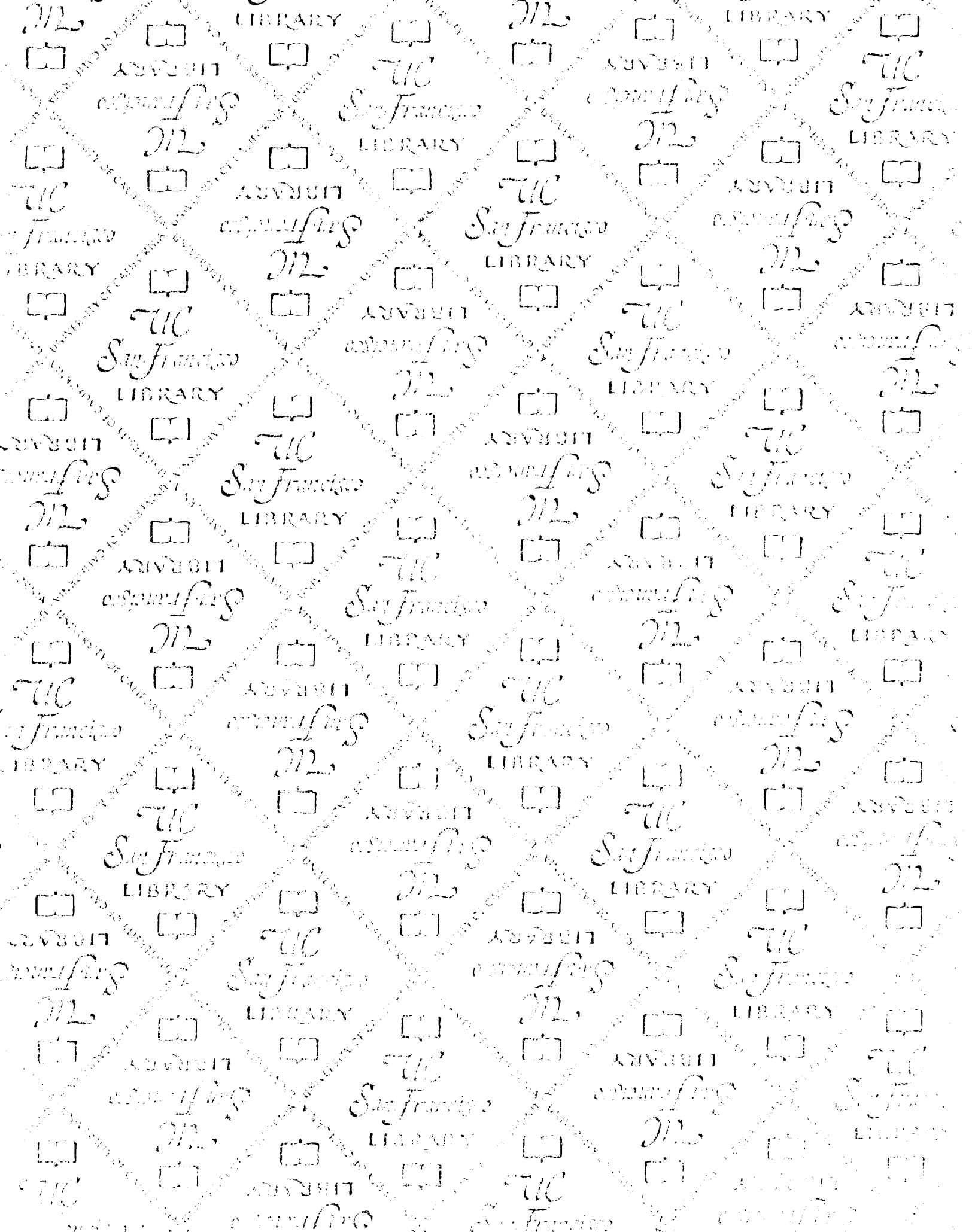
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