Growth Cone Guidance in Insects: Fasciclin II Is a Member of the Immunoglobulin Superfamily

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The cellular cues that guide neuronal growth cones toward their targets are highly conserved in such diverse organisms as insects and vertebrates. Evidence presented here suggests that the molecular mechanisms underlying these events may be equally conserved. This article describes the structure and function of fasciclin II, a glycoprotein expressed on a subset of fasciculating axons in the grasshopper embryo. Antibody perturbation experiments suggest that fasciclin II functions in mediating one form of neuronal recognition: selective fasciculation. Fasciclin II is a member of the immunoglobulin gene superfamily and is homologous in structure and function to the neural cell adhesion molecule N-CAM and to several other vertebrate cell adhesion molecules.

DURING THE PAST DECADE, INSECTS HAVE BECOME AN attractive model for the study of the cellular and molecular mechanisms of growth cone guidance and neuronal recognition. From studies on large insects with highly accessible embryonic neurons (for example, the grasshopper) to small ones with powerful genetics (*Drosophila*), insects offer the advantages of combined cellular, classical genetic, and molecular genetic approaches to these problems (*1–3*). Given this enormous potential, it has been heartening to learn that invertebrates have many of the same cellular and molecular mechanisms for growth cone guidance and neuronal recognition as vertebrates have (*4*). For example, laminin is a substrate adhesion molecule (SAM) that promotes neurite outgrowth in vertebrates (*5*). Laminin is also found in *Drosophila* (*6*) as are the surface integrin receptors for SAMs (*7*). These adhesion molecules are likely to play similar roles in vertebrates and invertebrates in promoting cell migration and neurite outgrowth (*8*).

Studies of growth cone guidance in insects have focused on the embryonic central nervous system (CNS) of the grasshopper and *Drosophila* (most notably the segmental neuromeres) (*1*) and on several particularly accessible parts of the peripheral nervous system, including sensory neurons and motoneurons in the appendages of the grasshopper embryo (most notably the limb bud) (*9, 10*), sensory neurons in the imaginal discs of the metamorphosing *Drosophila* (most notably the wing disc) (*11*), and sensory neurons in the head and body segments of the *Drosophila* embryo (*12, 13*). Parallel studies have also been carried out on moth embryos (*14*) and imaginal discs (*15*). Moreover, genetic analysis in *Drosophila* has revealed mutations in genes such as *disco* which selectively perturb some of the events of pathfinding and target recognition (*13*).

In contrast to the wealth of knowledge from various insect systems on the specificity of growth cone guidance, little is known about the mechanisms underlying the development of synaptic specificity. However, recent results suggest that, here too, invertebrates and vertebrates may use similar, evolutionarily conserved mechanisms. For example, the initial synaptic connections by sensory neurons projecting into the insect CNS are refined during development by competitive interactions in a manner reminiscent of similar mechanisms in higher organisms (*16*).

The studies reported here support the notion that the molecular mechanisms controlling growth cone guidance are highly conserved in insects and vertebrates. We first review the cellular studies on growth cone guidance in the developing insect CNS. We then briefly review the patterns of expression of fasciclins, glycoproteins expressed on subsets of fasciculating axons. Finally, we describe our recent studies on the structure and function of one of these molecules: fasciclin II. Molecular analysis reveals that fasciclin II is a member of the immunoglobulin superfamily and is structurally and functionally similar to several vertebrate neural cell adhesion molecules.

Growth Cone Guidance in the Developing Insect CNS

The insect CNS develops from the neuroepithelium along the ventral surface of the embryo. The ventral surface of this epithelium includes the proliferative zone where neuronal precursor cells (neuroblasts) divide to generate columns of neuronal progeny, with the oldest generally closest to the dorsal (inner) surface of the epithelium. Growth cones navigate and axon pathways form just beneath the basement membrane that covers this dorsal surface. Within each CNS neuromere there develops a scaffold of axon pathways, including a pair of bilaterally symmetric longitudinal axon tracts, a pair of commissural tracts (anterior and posterior) connecting the two sides, and a pair of nerve roots exiting the CNS on each side (the segmental and intersegmental nerve roots). Each of the major tracts is subdivided into an array of distinct axon bundles, or fascicles, and it is at the intersection of these longitudinal and commissural fascicles that the neuropil, the region of axonal and dendritic branches and synapses, forms and expands.

The first growth cones extend over both extracellular matrix and the surfaces of glial cells, and in so doing, establish the initial axon pathways (*17, 18*). In *Drosophila*, the growth cones that pioneer the longitudinal axon tracts and intersegmental nerve roots are guided by an array of special glial cells that arise from the lateral neuroepithelium and migrate toward their characteristic position under the

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inner basement membrane (*18*). In the much larger grasshopper embryo, the glial cells do not completely cover the basement membrane at this early stage, and thus the first growth cones extend in contact with both the inner basement membrane and the processes of glial cells (*17, 18*). When these glial cells are selectively eliminated, either by cell ablations in culture or genetic manipulations, the formation of specific axon pathways is perturbed (*17, 19, 20*). These special glial cells in the insect CNS express on their surfaces and secrete into the surrounding environment a variety of interesting molecules of potential importance for growth cone guidance and cell migration, including laminin (*21*).

As development proceeds, large numbers of neurons are born, and their growth cones find themselves in an environment increasingly dominated by other axons. Most of these later growth cones do not contact the basement membrane or glia at all, but rather only contact the growth cones and axons of other neurons. As the scaffold of axon pathways grows larger and more complex within the CNS, these later growth cones show remarkable selectivity in their ability to recognize and extend along specific axonal surfaces (called selective fasciculation) (*22, 23*); similar selectivity for specific axon pathways has been revealed in the developing vertebrate spinal cord (*24*). Experimental studies on the mechanisms of selective fasciculation in insects (*25, 26*) led to the prediction (the labeled pathways hypothesis) that neighboring axon pathways are differentially labeled by surface recognition molecules that allow growth cones to distinguish among them.

What surface molecules mediate the recognition of specific axon pathways? At this stage of development, the more general substrate and surface adhesion molecules [reviewed in (*27*)], which help in part to guide the early, pioneering growth cones, appear insufficient on their own to furnish growth cones with the necessary specificity for the task at hand—to recognize an individual axon fascicle within an array of many available neighboring pathways (*22–26*). Thus, it seems likely that many mechanisms and molecules act in concert to generate these local patterns of specificity in axon pathways and growth cone choices. These observations and interpretations led to the search for glycoproteins expressed on subsets of fasciculating axons, as described in the next section.

Fasciclin Glycoproteins Are Expressed on Subsets of Axons

Candidates for axonal recognition molecules were identified by generating monoclonal antibodies (MAbs) that recognize surface antigens expressed on subsets of axon fascicles in insect embryos. These MAbs were used initially to characterize and purify three different membrane-associated glycoproteins, fasciclin I and II in grasshopper (*28, 29*) and fasciclin III in *Drosophila* (*30*). The genes encoding all three proteins were cloned (*29–31*), and fasciclin I cDNAs were sequenced in both grasshopper and *Drosophila* (*31*).

The fasciclin proteins have several features in common (*32*). These proteins are expressed during the period of axon outgrowth. Not only does the overall pattern of expression change during development, but in some cases, the expression of a fasciclin protein on an individual neuron is transient during a particular stage of axon outgrowth. For example, in grasshopper, the expression of fasciclin I on a subset of commissural pathways disappears by the time 70% of embryonic development is completed and never again appears on these commissural axons during embryonic or adult life (*28*).

The three fasciclin proteins are expressed on different but overlapping subsets of axon fascicles. For example, all of the axons in the intersegmental nerve root express fasciclin I and II (*28*), whereas only a subset of these axons express fasciclin III (*30, 33*). In contrast,

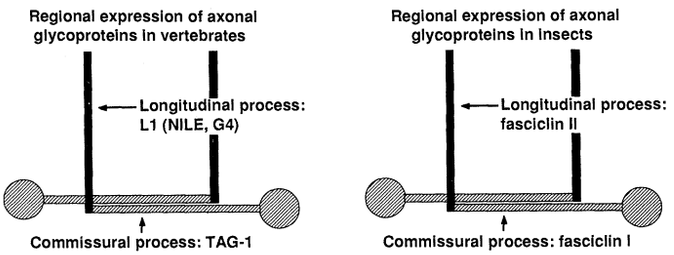

Fig. 1. In both insects and vertebrates, axonal glycoproteins are regionally expressed on particular portions of embryonic axons, often distinguishing between the commissural and longitudinal processes of an individual neuron. Schematic diagram showing the expression of TAG-1 on the commissural processes and L1 (same as NILE, G4, and Ng-CAM) on the longitudinal processes of developing interneurons in the rat embryo spinal cord [see (*36*)]; and the expression of fasciclin I on the commissural processes and fasciclin II on the longitudinal processes of developing interneurons in the grasshopper embryo segmental ganglia [see (*28*)].

some axon pathways express only one of these three proteins, and others express none at all. In addition to their expression on fasciculating axons, the proteins are also expressed on growth cones and their filopodia as they extend along these axon pathways. Thus, the expression of the fasciclin proteins on the surfaces of many neurons whose axons fasciculate together is consistent with their involvement in neuronal recognition and growth cone guidance.

Moreover, all three fasciclin proteins are regionally expressed on particular portions of embryonic neurons where their axons fasciculate together. On an individual axon, a particular fasciclin protein is regionally expressed on those portions that are part of fasciclin-positive bundles. For example, the expression of the fasciclins typically differentiates between the commissural and the longitudinal domain of a particular axon. Most projection interneurons have axons that cross in one of the commissures and then extend rostrally or caudally in one of the longitudinal pathways. Some of these interneurons transiently express fasciclin I on their commissural processes whereas there is maintained fasciclin II expression on their longitudinal axon segments (*28*).

Other glycoproteins with similar spatially restricted patterns of expression on subsets of axon pathways have been identified in both invertebrates (*34*) and vertebrates (*35, 36*) [reviewed in (*4, 27*)]. The discovery of the regional expression of both the fasciclin proteins in insects (*28, 30*) and of axonal glycoproteins such as TAG-1 and L1 (*36*) in vertebrates has led to the hypothesis that regionally expressed molecules of these kinds could help regulate the behavior of growth cones as they navigate through a changing environment (Fig. 1).

Fasciclin II Is Dynamically Expressed on Individual Neurons and Axon Fascicles

The fasciclin II protein is dynamically and regionally expressed on the growth cones and axons of a small subset of CNS neurons (Fig. 2), in a pattern that suggests an important function in the selective fasciculation of these neurons. Early in development, fasciclin II is uniformly expressed on the surface of all ectodermal cells in the grasshopper embryo. As neurogenesis begins, the level of fasciclin II expression decreases throughout the neuroepithelium relative to the body wall ectoderm, with two notable exceptions. First, the mesectodermal cells at the midline of the neuroepithelium express high levels of fasciclin II (Fig. 2A); these cells adhere tightly to one another and cluster separately from the other lateral neuroepithelial cells. Second, fasciclin II is expressed around 2 of the 30 neuroblasts (NBs) in each hemisegment at high levels (*37, 38*)—namely, NB 1-2 and NB 5-4 (the expression of fasciclin II is primarily on the

nonneuronal support cells that surround these NBs) (*38*).

Just before the initiation of the first neuronal growth cones, three neurons (MP1, dMP2, and vMP2) (*2, 22, 26, 39*) begin to express fasciclin II transiently on the surfaces of their cell bodies (Fig. 2A). These three neurons appear to be the only neurons in the grasshopper embryo whose cell bodies adhere tightly to the midline mesectodermal cells, and it is precisely where they contact these midline cells that they express their highest levels of fasciclin II. Subsequently, a few other neuronal cell bodies also transiently express fasciclin II.

The MP1, dMP2, and vMP2 neurons extend the first growth cones in the grasshopper CNS when around 30% of embryonic development is completed (*2, 22, 26, 39*); these growth cones initially extend along the inner basement membrane and the processes of the longitudinal glial cells as they pioneer the first two longitudinal axon pathways: the MP1/dMP2 fascicle and the vMP2 fascicle (Fig. 3A). As these three growth cones contact the basement membrane, and then turn either anteriorly (vMP2) or posteriorly (MP1, dMP2) along it, they do not express fasciclin II on the surface of their growth cones or axons (although their cell bodies continue to express the protein where they adhere to the midline cells) (Figs. 2A and 3B). The vMP2 does not express fasciclin II on either its growth cone or axon as it extends anteriorly into the next segment (Fig. 3C). Upon reaching the next segment, it switches from extending along nonneuronal surfaces to fasciculating selectively with the axon of its segmental homolog, forming a continuous fasciclin II–negative longitudinal axon pathway (the vMP2 fascicle) (Fig. 3, A and D).

Once the MP1 and dMP2 growth cones have extended about half the distance to the next posterior segment (34% of development), both neurons begin to express high levels of fasciclin II over their

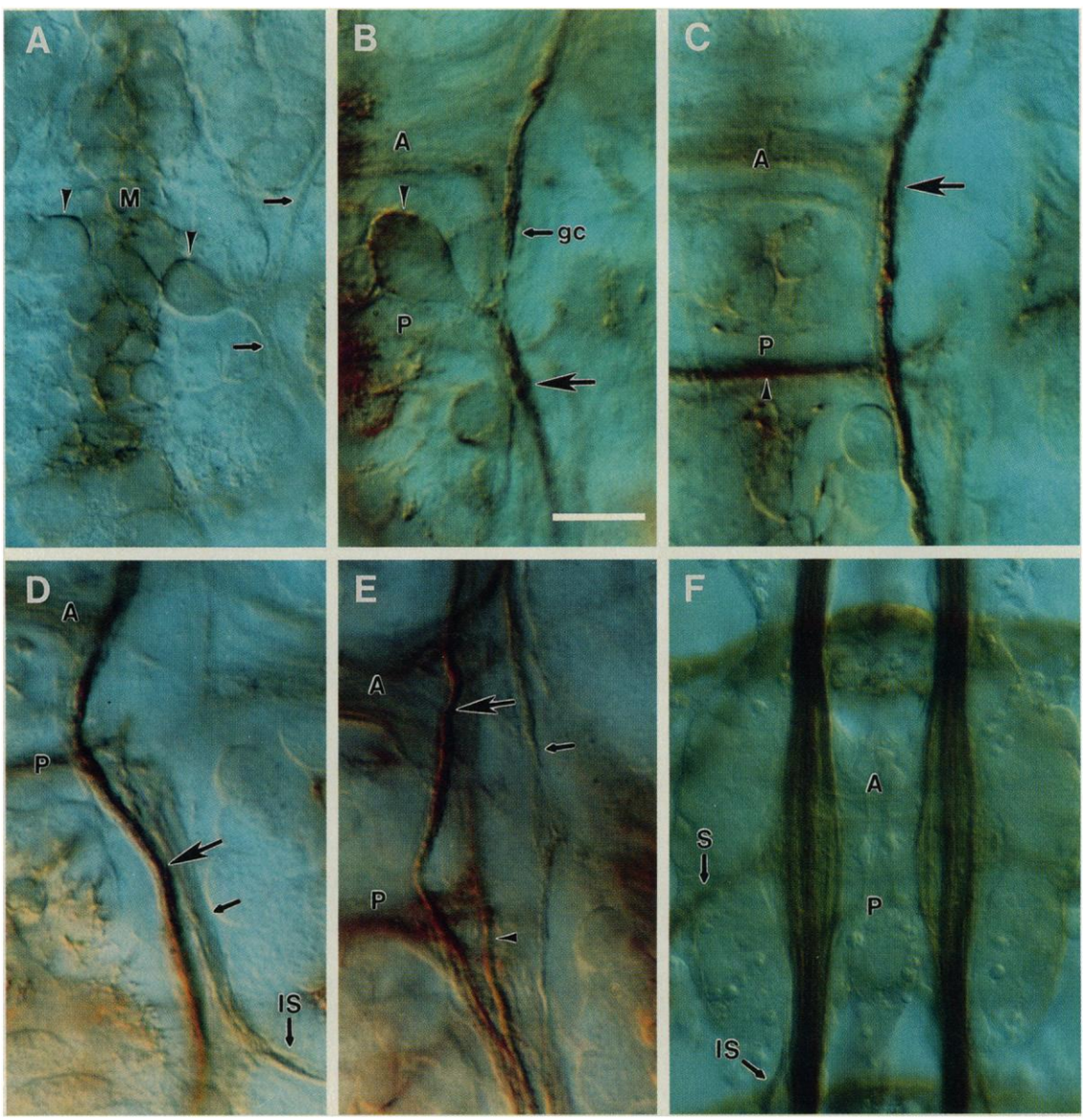

Fig. 2. Fasciclin II is dynamically expressed on a subset of axon pathways in the developing CNS of the grasshopper embryo. All photographs are dorsal views of the neuroepithelium of a single segment in the grasshopper embryo; anterior is up. (**A**) Early in neuronal development (31% embryo), fasciclin II is expressed on the surface of the midline neuroepithelial cells (M), and on the surface of the cell bodies of the MP1 (arrowhead), dMP2, and vMP2 neurons, where they contact the midline cells (dMP2 and vMP2 are out of the plane of focus). The initial anterior-extending growth cone of the vMP2 neuron (top arrow) and the posterior-extending growth cones of the MP1 and dMP2 neurons (bottom arrow) do not express fasciclin II at this time. (**B**) As the MP1 and dMP2 growth cones (gc) enter the next posterior segment and near the MP1/dMP2 axon fascicle (36% embryo) (large arrow), they express high levels of fasciclin II on their surface. (**C**) The selective fasciculation of the MP1 and dMP2 growth cones with the MP1/dMP2 axons from the next posterior segment establishes a continuous, fasciclin II–positive longitudinal axon pathway (37% embryo) (large arrow). Fasciclin II is transiently expressed on a single bundle in the posterior commissure: the L fascicle (arrowhead). (**D**) At 38% of development, the MP1/dMP2 fascicle expresses the protein at a high level (large arrow), whereas the U fascicle expresses the protein at a much lower level (small arrow). (**E**) At 39% of development, some axon pathways express fasciclin II at a high level [for example, the MP1/dMP2 fascicle (large arrow) and an unidentified fascicle (arrowhead)], whereas other pathways do not express fasciclin II at all [for example, the A/P fascicle (small arrow)]. (**F**) By 55% of development, most of the longitudinal axon pathways express fasciclin II at high levels, whereas none of the commissural pathways express the protein. A, anterior commissure; P, posterior commissure; S, segmental nerve root; IS, intersegmental nerve root. Scale bars: (A to E) 50 μm; (F) 125 μm.

entire surface, including their growth cones, filopodia, and axons (Figs. 2B and 3C). When the MP1 and dMP2 growth cones arrive at the next posterior segment, they too switch from extending along nonneuronal surfaces (the basement membrane and longitudinal glia) to extending along the axons of their segmental homologs, forming a continuous fasciclin II–positive longitudinal axon pathway (the MP1/dMP2 fascicle) (37% of development) (Fig. 2, B and C, and Fig. 3D).

At the time that the MP1 and dMP2 growth cones switch from guidance by nonneuronal substrates to guidance by selective fasciculation, these growth cones and the axons of their segmental homologs, for which they display a selective affinity, express the highest levels of fasciclin II in the developing CNS (Fig. 2). As described in the next section, application of antibodies against fasciclin II, just before this switch in affinity from nonneuronal guidance to selective fasciculation, appears to perturb selectively the ability of the MP1 and dMP2 growth cones to recognize the MP1/dMP2 fascicle.

As development proceeds and other longitudinal axon pathways are pioneered, some axons do and others do not express fasciclin II (Fig. 2, D and E). For example, the axons in the third longitudinal pathway, the U fascicle (pioneered by the two U axons and followed by the aCC growth cone) (*26*), express fasciclin II (although at a much lower level than the MP1/dMP2 fascicle) as they extend posteriorly and then turn laterally to pioneer the intersegmental nerve. In contrast, the axons in the A/P fascicle (pioneered by the two A and two P axons and followed by the G growth cone) (*23*) do not express fasciclin II (Fig. 2E).

There is also transient expression of fasciclin II in a few commissural axon fascicles. For example, in the posterior commissure, the axons in the Q fascicle (*22*) do not express fasciclin II, whereas the axons in the L fascicle do so transiently. The anterior commissure is divided into two regions, A and B, each containing many axon fascicles. None of the fascicles in the A commissure express fasciclin II, whereas a few axon fascicles in the B commissure do so transiently. All of the motor axons in the intersegmental nerve root express fasciclin II, whereas only a few of the many motor axon fascicles in the segmental nerve root transiently express the protein.

At around 45% to 50% of embryonic development, there is a dramatic transition in the expression of fasciclin II. By this stage, the transient expression in a few commissural fascicles has disappeared, even though new axons continue to add to these commissures. However, during this transition, the axons in most (although not all) of the longitudinal axon fascicles that did not previously express fasciclin II now begin to express the protein at relatively high levels (Fig. 2F), particularly between the developing ganglia where the different fascicles (although spatially separate in the middle of the segmental ganglia) come together and tightly bundle into one large longitudinal tract called the connective (*40*).

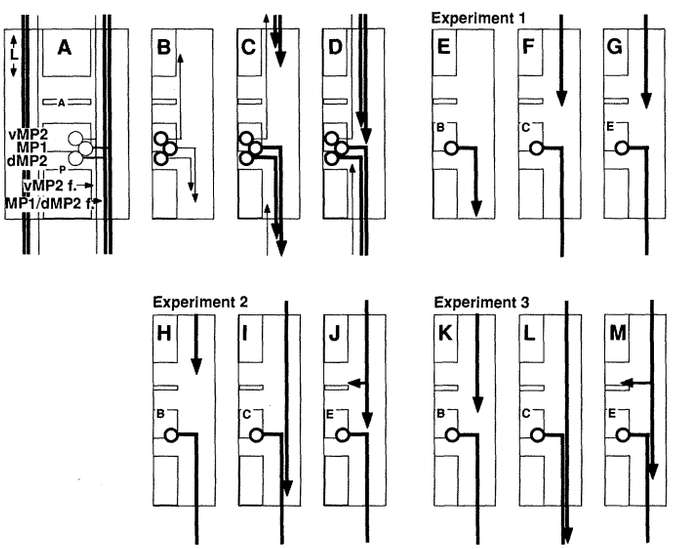

Fig. 3. Fasciclin II is dynamically expressed on individual neurons and axon fascicles and functions in the selective fasciculation of these neurons. Schematic diagrams showing (A to D) the dynamic expression of fasciclin II on the surfaces of the MP1 and dMP2 neurons in the developing CNS of the grasshopper embryo, and (E to M) the effects of antibody perturbation experiments on the MP1 growth cone's specific recognition of and selective fasciculation with the MP1/dMP2 fascicle. (**A**) Diagram of the differential expression of fasciclin II on the first two longitudinal axon fascicles in the grasshopper embryo: the fasciclin II–positive MP1/dMP2 fascicle, as pioneered by the MP1 and dMP2 axons, and the fasciclin II–negative vMP2 fascicle, as pioneered by the vMP2 axon. Diagram shows a single segment with A and P denoting the anterior and posterior commissures, respectively (at this stage, the A commissure is divided into two parts), and L, the longitudinal axon fascicles. (**B** to **D**) All three neurons transiently express fasciclin II on their cell bodies. The initial MP1, dMP2, and vMP2 growth cones and axons do not express fasciclin II (B), but as they cross the segment border, the posteriorly extending MP1 and dMP2 neurons begin to express fasciclin II at high levels along their growth cones and axons (C and D). See text for further discussion of dynamic expression. (**E** to **M**) Results of antibody perturbation experiments 1, 2, and 3, as described in detail in the text. For each experiment, B denotes the location of the MP1 growth cone at the beginning of the experiment (33%, 35%, and 36% of development, respectively), C denotes the posterior extension of the MP1 growth cone in control embryos cultured in antibodies against other surface antigens, and E denotes experimental embryos cultured for 24 hours in antibodies to fasciclin II. Antibodies to fasciclin II selectively perturb the ability of the MP1 growth cone to recognize and extend along the MP1/dMP2 fascicle and often lead to an extra abnormal branch which extends across in the anterior commissure.

Fasciclin II Plays a Role in Selective Fasciculation by the MP1 Growth Cone

Given the existence of antibodies against the fasciclin II protein in grasshopper, and a fairly robust grasshopper embryo culture system for experimental manipulations in vitro (*41*), we began a preliminary test of fasciclin II function during neuronal development by using antibody perturbation experiments (*42*).

We used a single choice point for our initial experimental analysis: the MP1 growth cone in the middle of the next posterior segment as it switches from nonneuronal surfaces to selective fasciculation with the MP1/dMP2 fascicle. At this choice point (between 35% and 37% of development), the MP1 growth cone has filopodial access to about ten longitudinal axon fascicles (including the vMP2 fascicle and the U fascicle) (*26*), many other transverse axon fascicles in the posterior commissure and segmental nerve root, and many of the same longitudinal nonneuronal cues that initially guided it posteriorly in its own segment (*43*). As described in the previous section, at this stage the MP1 and dMP2 growth cones, and the axons in the MP1/dMP2 fascicle with which they are about to fasciculate, express the highest levels of fasciclin II in the developing CNS.

In experiment 1 (Fig. 3, E to G) [see (*44*) for experimental methods], embryos were placed in culture at 33% of development, when the growth cone from the MP1 neuron in the T3 segment [MP1(T3)] is still extending posteriorly within its own segment and navigating along nonneuronal surfaces. In all experiments, the embryos were assayed 24 hours later—after approximately 3% to 4% of growth in culture—by intracellular injection of the fluorescent dye Lucifer yellow. In both the experimental embryos cultured with the polyclonal serum antibody (SAb) to fasciclin II ($n = 3$) (Fig. 3G) and control embryos cultured with the SAb to fasciclin I ($n = 3$) (Fig. 3F), the MP1 growth cone extended down into the A1 segment and had not yet reached the MP1/dMP2 fascicle. We conclude from this experiment that neither antibody perturbs the extension of the MP1 growth cone along nonneuronal substrates.

In experiment 2 (Fig. 3, H to J), embryos were placed in culture at 35% of development, when the MP1(T3) growth cone is just at the anterior commissure of the next posterior (A1) segment and not yet in extensive contact with the MP1/dMP2 fascicle (Fig. 3H).

In control embryos cultured with either the anti–fasciclin I SAb (n = 4), the anti–fasciclin I MAb (n = 2), or with a polyclonal antiserum against horseradish peroxidase [anti-HRP (*44, 45*)] (n = 3), the MP1 growth cone had selectively fasciculated with the MP1/dMP2 fascicle and had extended posteriorly about half the distance to the next segment (Fig. 3I). The shape of the growth cone was characteristic of a fasciculating neuron in that it was long and thin with several prominent terminal filopodia directed posteriorly along the fascicle.

In contrast, in embryos cultured with the anti–fasciclin II SAb (n = 7) or MAb (n = 2), the MP1 growth cone had not selectively fasciculated with the MP1/dMP2 fascicle, but rather was at the level between the two commissures or just about to get onto the pathway (Fig. 3J); in three of these experimental embryos, dMP2 was also assayed and showed the same stall in its behavior. The shape of the MP1 growth cone was more characteristic of a growth cone at a choice point in that it was broad and complex and had many long filopodia directed both posteriorly along the longitudinal fascicles (and often in extensive contact with the MP1/dMP2 fascicle) and laterally along the posterior commissure and segmental nerve root. In contrast, in these same experimental embryos, the vMP2 growth cone (normally fasciclin II–negative) continued to extend anteriorly along its normal pathway at its normal rate (n = 3).

We wondered whether this stall in the MP1 growth cone at its choice point was permanent or temporary, so we conducted a third experiment (Fig. 3, K to M) in which embryos were placed in culture at 36% of development, when the MP1(T3) growth cone is between the two commissures of the next posterior (A1) segment and in contact with the MP1/dMP2 fascicle (Fig. 3K).

In control embryos incubated with anti–fasciclin I SAb (n = 2), anti–fasciclin I MAb (n = 2), or anti-HRP (n = 1), the MP1(T3) growth cone had extended down along the MP1/dMP2 fascicle from the A1 segment into the A2 segment (Figs. 3L and 4A). In experimental embryos incubated with anti–fasciclin II SAb (n = 4) or MAb (n = 1), the MP1(T3) growth cone had begun to extend posteriorly from the A1 segment down toward the A2 segment, but not nearly as far as in the controls (Figs. 3M and 4B). In one of these embryos, the MP1(T3) growth cone was clearly not fasciculated with the MP1/dMP2 fascicle but rather was growing several micrometers lateral to it and presumably extending along nonneuronal surfaces. This growth cone was complex in shape and had extensive filopodial contact with the MP1/dMP2 fascicle, as well as with other surfaces in its environment. In the other four embryos, the MP1(T3) growth cone was growing along the MP1/dMP2 fascicle, although its growth cone had not grown as far as the controls (and lagged behind them by about 1% to 2% of development), was more complex in shape than the controls, and radiated numerous filopodia in extensive contact with both the MP1/dMP2 fascicle and with other surfaces in front and to the side of it.

We noted one other difference in the morphology of the MP1 neuron between experimental and control embryos. In 10 of the 14 experiment 2 and experiment 3 embryos incubated with antibodies to fasciclin II, the MP1 axon initiated a second growth cone, which in all 10 cases extended across the anterior commissure in a specific fascicle (Fig. 3, J and M, and Fig. 4C); MP1 normally does not extend a second growth cone and never extends across any commissural pathway. This extra branch was seen in only 1 of the 14 control embryos incubated with either anti–fasciclin I or anti-HRP. One possibility is that this extra branch in the experimental embryos indicates (i) that the MP1 growth cone has a hierarchy of affinities for different pathways; (ii) that normally its affinity for this specific commissural fascicle is considerably lower than its affinity for the MP1/dMP2 pathway; and (iii) that in the presence of antibodies against fasciclin II, the bias of affinities is shifted such that in many embryos MP1 now extends a secondary growth cone along this commissural fascicle.

These experiments suggest that at 35% to 37% of development, fasciclin II plays a role in the ability of the MP1 (and dMP2) growth cone to recognize, selectively fasciculate with, and continue to extend along the MP1/dMP2 fascicle. The ability of the antibodies to fasciclin II to cause the MP1 growth to stall at its choice point is a consistent result. However, after this temporary stall, the MP1 growth cone extends posteriorly, usually but not always along the MP1/dMP2 fascicle. The extension of the MP1 growth cone along nonneuronal surfaces is not surprising, since this was previously seen in cell ablation experiments (*26, 43*). The eventual extension of the MP1 growth cone along the MP1/dMP2 fascicle in many experimental embryos suggests an incomplete block in the recognition of this pathway and leads to two alternative explanations. First, the antibodies might only partially block the protein's function, perhaps because the serum and monoclonal antibodies attach to epitopes on the protein away from the active binding site.

The second and more likely alternative is that there are multiple (and to some extent redundant) adhesion or recognition molecules on the surface of the axons in the MP1/dMP2 fascicle at this stage of development and that blocking only one of them (fasciclin II) only partially interferes with the ability of the MP1 growth cone to recognize and extend along this axon pathway. In this model, blocking only fasciclin II leads to a temporary stall and then an impaired extension, rather than a permanent block in the posterior extension of the MP1 growth cone.

These results are consistent with results obtained in experiments

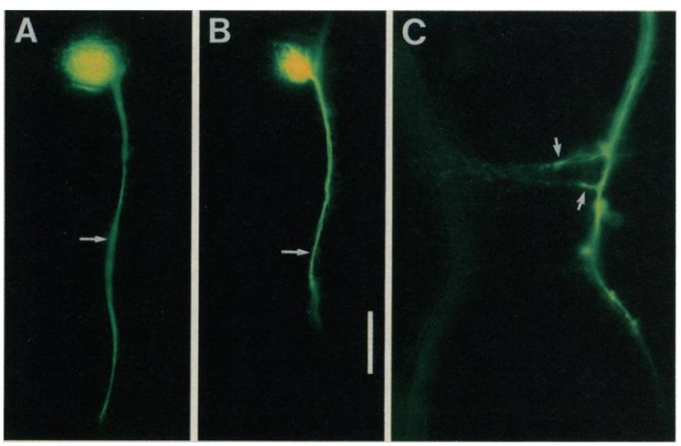

Fig. 4. Antibodies to fasciclin II perturb the growth of the MP1 neuron. Fluorescent photomicrographs of MP1 neurons filled with the dye Lucifer yellow in embryos placed into culture at 36% of development and assayed 24 hours later, after incubation in serum antibodies to fasciclin I [(**A**) control embryo] or in serum antibodies to fasciclin II [(**B** and **C**) experimental embryos]. The MP1 growth cone in the experimental embryos did not extend as far posterior as in the control embryo, and in 10 of 14 cases (C), initiated a second, abnormal growth cone that radiated profuse filopodia and extended across in a specific fascicle of the anterior commissure [arrowheads in (C)]. The arrowheads in (A) and (B) mark the posterior extent of the anterior commissure; this is the approximate location of the MP1 growth cone when the embryos went into culture 24 hours earlier. The fluorescent shadow on the left of (C) is the out-of-focus axon of the vMP2 neuron, which was filled in the next posterior segment. The normally fasciclin II–negative vMP2 neuron extends anteriorly at a normal rate in the experimental embryos incubated with the antibodies to fasciclin II. See text for further discussion. Scale bars: (A and B) 100 μm; (C) 50 μm.

on axonal adhesion molecules in vertebrates. In chick, at least three different axonal adhesion molecules, G4, F11, and neurofascin, are all expressed on fasciculating axons in the longitudinal axon tracts of the spinal cord (*35*). In an in vitro assay system (*46*), antibodies against any one of these axonal adhesion molecules alone only partly slows the rate of extension of neuronal growth cones along axon bundles to 60% to 80% of control values (*46*).

These results are also consistent with results from in vitro experiments of growth cone extension along muscle (*47*) or glial (*48*) cell surfaces in which multiple adhesion molecules appear to participate. Antibodies that block a single adhesion molecule only partly impair growth cone extension, whereas simultaneous addition of antibodies that block multiple adhesion molecules has a much greater effect on blocking growth cone extension.

Fasciclin II Is a Member of the Immunoglobulin Superfamily

Using oligonucleotide probes based on protein microsequence data, we previously isolated fasciclin II cDNA clones from a grasshopper embryo cDNA library (*29*). Partial cDNA sequence analysis of one of these clones was sufficient to identify the same amino acid sequence as was derived from protein microsequencing and thus to confirm the cloning of fasciclin II (*29*).

Using standard cloning techniques (*49*), we isolated and sequenced cDNA clones from the grasshopper embryo cDNA library (*50*). Figure 5 presents the deduced amino acid sequence for the entire protein coding region of fasciclin II and compares the sequence of the extracellular region with the mouse neural cell adhesion molecule (N-CAM), as described below. The deduced amino acid sequence generates a mature protein with a molecular size of about 97 kD; the purified protein runs as a single band on reducing SDS-polyacrylamide gels with an apparent molecular size of 95 kD (glycosylated) or 87 kD (deglycosylated) (*29*). The deduced amino acid sequence of fasciclin II predicts a hydrophobic signal sequence of 22 amino acids, a mature extracellular region of 742 amino acids, a hydrophobic transmembrane domain of 25 amino acids, and a cytoplasmic region of 108 amino acids.

The extracellular region of fasciclin II contains two types of domains. Close to the amino terminus are five repeats of a common domain structure with two cysteines in each domain (separated by approximately 50 amino acids) and many other conserved amino acids, particularly around the cysteine residues (Fig. 6A). The structure of these domains is typical of C2 type immunoglobulin (Ig) domains (*51*). In addition to the five Ig-like repeats, near to the transmembrane domain, the extracellular region of fasciclin II also contains two copies of another repeat domain which is similar to fibronectin (Fn) type III domains (*52*) (Fig. 6C).

Among the different subclasses of the immunoglobulin superfamily, fasciclin II is most highly related to the vertebrate neural adhesion molecules with C2 type Ig domains (*53*), including N-CAM (*54*); myelin-associated glycoprotein (MAG), a glial adhesion molecule (*55*); and L1, an axonal adhesion molecule (also called NILE in mammals and G4 and Ng-CAM in chick) (*56*) (Fig. 6B). Moreover, in addition to multiple Ig repeats, two of these vertebrate neural cell adhesion molecules, N-CAM and L1, also contain Fn type III repeats near their transmembrane domains (*54, 56*), and the two Fn type III repeats in fasciclin II have significant amino acid homology with these domains (Fig. 6C).

Comparison of fasciclin II with this group of Ig superfamily vertebrate neural cell adhesion molecules shows that they are most alike in the third Ig-like domain (Fig. 6B). The third Ig-like domain of fasciclin II has greatest similarity to the third Ig-like domain of MAG (Dayhoff ALIGN score 11.1) (*57*), less to the L1 third domain (ALIGN score 9.15), and least to the N-CAM third domain (ALIGN score 8.77). The fibronectin-related domains in fasciclin II bear greater resemblance to those of L1 than to those of N-CAM (Fig. 6C).

Nevertheless, the greatest overall similarity occurs between fasciclin II and N-CAM (Fig. 5). Comparison of fasciclin II with mouse N-CAM by use of the FASTP alignment program (*58*) yields an optimized score of 586. Detailed analysis using the ALIGN program (*57*) indicates that the similarity between fasciclin II and N-CAM extends throughout the entire extracellular portions of both proteins; when fasciclin II and N-CAM domains are compared, the best match of the five Ig domains and two Fn type III domains of fasciclin II are with the same seven domains of N-CAM—namely, domain 2 with domain 2, domain 3 with domain 3, and so on. The aligned sequences from mouse or chicken N-CAM and fasciclin II show that these molecules have 25% amino acid identity over their extracellular domains. The conserved amino acids tend to be those characteristic of the immunoglobulin fold and the type III fibronectin domain (Fig. 6, B and C).

Although fasciclin II is more highly related to N-CAM than to L1 in its structure, it is more similar to L1 in its expression—both fasciclin II and L1 are expressed on a subset of longitudinal axon pathways, whereas N-CAM is expressed on all axons. This ambiguity makes it difficult to assign precise molecular homologies among these related insect and vertebrate molecules. The conserved amino acid sequence and seven homologous domains of fasciclin II and N-CAM (five Ig-like domains and two Fn type III domains) suggests that this structure may more closely reflect a primitive adhesion molecule from which these and some of the other neural adhesion molecules later diverged during arthropod and chordate evolution. On the other hand, in terms of expression and function, fasciclin II is more similar to L1 than to N-CAM.

Fasciclin II is not the only insect protein to be identified that contains Ig-related domains. Seeger and Kaufman (*59*) have shown that the *amalgam* gene in *Drosophila* encodes a protein that contains

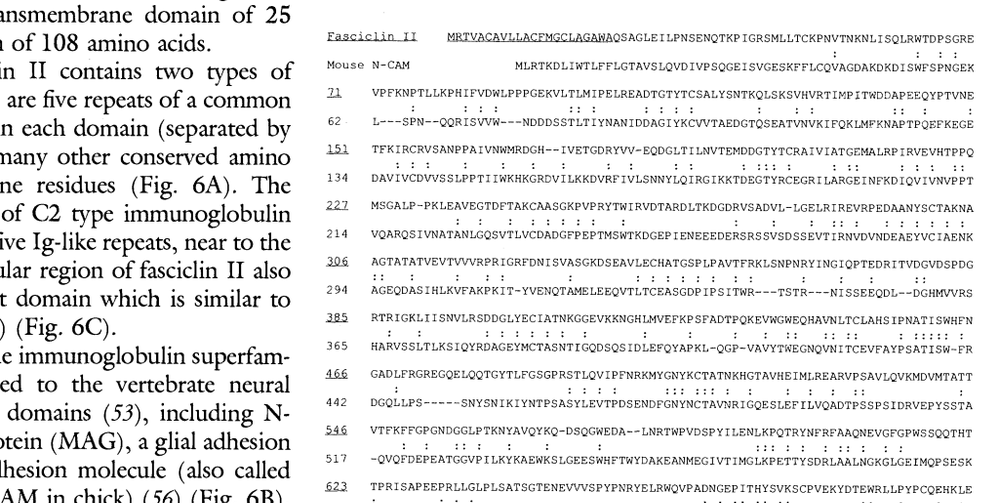

Fig. 5. Deduced amino acid sequence from fasciclin II cDNA clones and comparison of the predicted amino acid sequences in the extracellular domains of fasciclin II and mouse N-CAM (*54*), obtained by using the FAST-P program (*58*). Double dots indicate amino acid identities. The putative signal sequence and transmembrane domain are underlined.

three Ig domains (Fig. 7), and the Ig domains of fasciclin II and the amalgam protein are highly related (*58*) (Fig. 6B). Whereas fasciclin II is transiently expressed on a subset of axon fascicles in the developing grasshopper CNS, antibodies against the amalgam protein reveal that it is expressed on the surface of all axons in the developing *Drosophila* CNS (*59*), similar in some respects to N-CAM.

The observation that insects have at least two different members of the immunoglobulin superfamily expressed on either a subset (fasciclin II) or all (amalgam) of the axon pathways in the developing CNS leads to three interesting hypotheses. First, just as their closest vertebrate relatives are all neural cell adhesion molecules, so fasciclin II and amalgam are likely to be neural cell adhesion molecules in insects. Second, given the fact that fasciclin II is expressed on only a restricted subset of axon pathways in the developing insect CNS, and primarily around 2 of the 30 NB families during neurogenesis, it is likely that many other Ig-like molecules exist in insects and are used for specific adhesion and recognition during neuronal development.

Third, the discovery of fasciclin II and amalgam shows that molecules with repeated Ig-like domains clearly evolved long ago, before the phyletic split of the arthropod and chordate lines, as previously suggested by Williams (*51*) (Fig. 7). The expression of Ig-like molecules during neuronal development in insects supports the notion that (i) a gene coding for Ig-like domains evolved first as a cell adhesion molecule; (ii) this gene, through duplication and divergence early in evolution, expanded into a gene family that functioned at least in part as neural cell adhesion and recognition molecules; and (iii) only later in chordate evolution some of these genes were selected for special functions in the immune system.

Growth Cone Guidance and Neuronal Recognition

What are the prospects for a detailed understanding of such complex processes as growth cone guidance and neuronal recognition in organisms with relatively simple nervous systems such as insects? Work at the cellular level has uncovered a rich network of cellular interactions and continues to guide the molecular studies. A molecular genetic analysis of these phenomena in insects is just beginning. We know only a few of the molecules that are likely to be involved (for example, fasciclin II) [see also (*6, 28–31*)], and have mutants in only a few *Drosophila* genes (*13*) that selectively perturb these events. Nevertheless, the enormous potential of insects for cellular and molecular genetic studies will undoubtedly lead to greater insights over the next decade into the mechanisms controlling these complex processes. But will these insights apply to higher organisms? The homology in cellular mechanisms, and the similarity in adhesion and recognition molecules, between invertebrates and vertebrates (*4*) encourages us to think that answers learned from simple systems will be directly applicable to more complex organisms and, conversely, that the insights gained from higher organisms will help guide the studies on insects.

At the cellular level, pioneering growth cones in both invertebrates and vertebrates use nonneuronal substrates, such as extracellular matrix and glial processes, whereas later growth cones rely more heavily on neuronal substrates, such as other axons, for their guidance. At the molecular level, invertebrates and vertebrates use the same substrate adhesion molecules (for example, laminin) (*6*) and surface receptors that bind to them (for example, integrins) (7).

In insects, axonal glycoproteins have been discovered (for example, fasciclin I, II, and III) (*28–31*), which are dynamically and regionally expressed on subsets of fasciculating axons; similarly, in vertebrates, axonal glycoproteins such as L1 (G4, NILE, Ng-CAM), F11, neurofascin, and TAG-1 (*4, 34, 35*) have been discovered that are dynamically and regionally expressed on subsets of axons (Fig. 1). How neurons regulate the regional expression of these axonal glycoproteins on portions of their surface (for example, commissural versus longitudinal processes) is poorly understood.

Some of these molecules (fasciclin II and L1) have remarkably similar patterns of expression (longitudinal axon tracts in the segmental ganglia and spinal cord, respectively) (*28, 35, 36*) (Fig. 1). Even more striking is their structural homology: fasciclin II and L1 are closely related members of the immunoglobulin superfamily and both contain multiple Ig-like domains and Fn type III domains (*56*) (Fig. 7). Moreover, the two molecules share functional homology: both appear to be axonal adhesion molecules, and both appear to

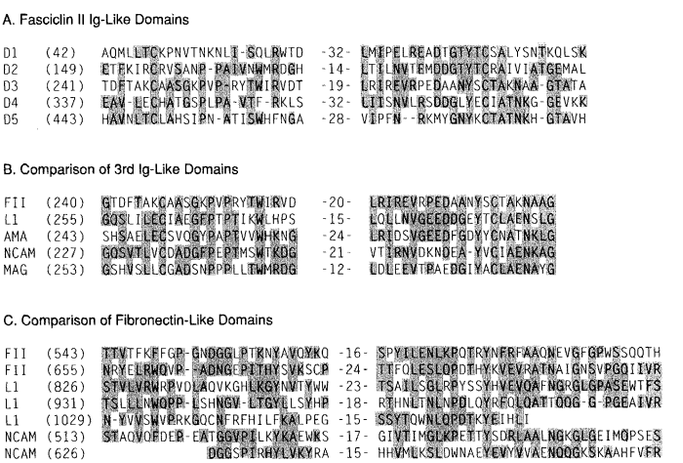

Fig. 6. Sequence similarities of (**A**) the five immunoglobulin (Ig)-like domains of fasciclin II, (**B**) the third Ig-like domain of fasciclin II compared with the third Ig-like domain in mouse N-CAM (*54*), rat MAG (*55*), rat L1 (*56*), and *Drosophila* amalgam (*59*), and (**C**) the two fibronectin type III domains in fasciclin II compared with the two domains in N-CAM (*55*) and the three domains in L1 (*56*). Sequences were visually aligned; see text for discussion of similarities.

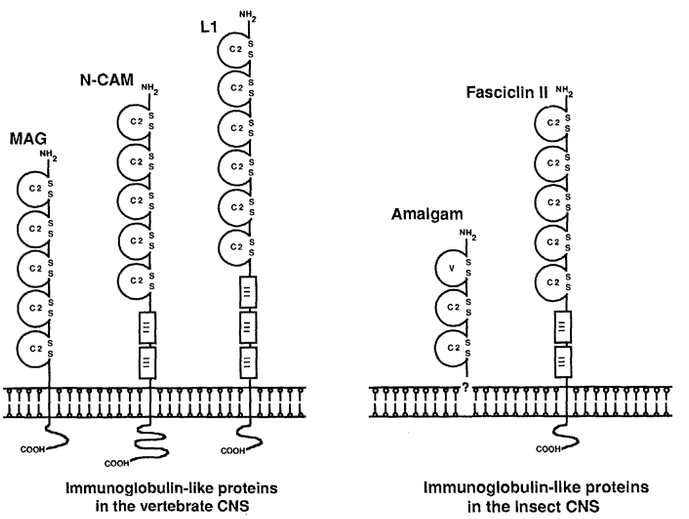

Fig. 7. Schematic diagram showing the domain structure of some related immunoglobulin superfamily molecules expressed in the central nervous system of vertebrates and insects. The C2 and V inside the circles indicate the domain type as defined by Williams (*51*). All of the molecules have C2 type domains except *Drosophila* amalgam which has one V type domain and two C2 type domains (*59*). The boxes with the III inside indicate the fibronectin type III domains.

function as part of a seemingly redundant set of axonal glycoproteins that promote neurite extension (*34, 44*). Fasciclin II helps mediate the recognition of specific axon pathways; the regional expression of L1 (*36*) suggests a similar function.

During the past several years, two additional guidance mechanisms, contact-mediated inhibition (*60–62*) and chemotropic guidance (*63*), have become increasingly well characterized in vertebrate systems, particularly through the use of cell and tissue culture experiments. With the notable exception of in vitro studies on inhibition by neurotransmitters in mollusks (*64*), these types of mechanisms have been largely ignored by studies on invertebrate systems, probably because of the lack of extensive cell culture studies. The discovery of these guidance mechanisms in vertebrates ought to be a catalyst to those working on simpler organisms to design in vitro assays or mutant screens that might uncover similar mechanisms.

There are many important questions for the future. For example, how many different mechanisms and molecules control the behavior of an individual growth cone at a single complex choice point? How does a growth cone regulate the expression of its surface proteins in response to and in preparation for its changing environment from one choice point to the next? How much redundancy is built into the system to ensure normal development in spite of the temporal variability and number and complexity of choices being made? And of course, how many different recognition and adhesion molecules are required to generate the specificity of growth cone guidance and neuronal recognition in the central nervous system? The expression of fasciclin II on a subset of fasciculating axons, and our limited knowledge about similar molecules specifying other axon pathways, suggests to us that what we now know about insect development represents just the beginning, and that there are likely to be many more molecules awaiting future discovery.

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42. These experiments have certain limitations. First, because the cultures and assays are tedious, only certain chosen growth cones were assayed. Second, we had only limited quantities of rat antisera, and, in particular, we did not have Fab' for monovalent blocking experiments.
43. From previous cell ablation experiments (*26*) we know that when the MP1 and dMP2 neurons are ablated, the MP1 growth cone from the next anterior segment does not permanently stop but rather continues to extend posteriorly through the experimental segment, presumably by using the same nonneuronal cues that it used in its own segment of origin.
44. Experimental embryos were incubated in either a 1:200 dilution of rat polyclonal serum antibody to fasciclin II or a 1:100 dilution of ascites fluid from the MAb to fasciclin II (8C6) in culture medium. Control embryos were incubated in either a 1:200 dilution of rat antiserum to fasciclin I, a 1:100 dilution of ascites fluid from the MAb to fasciclin I (3B11) (which labels a different subset of axon fascicles), or in a 1:100 dilution of polyclonal antisera against horseradish peroxidase (anti-HRP), which labels the surface of all axon fascicles by recognizing a nervous system–specific carbohydrate moiety (*45*). All embryos were assayed 24 hours later, after approximately 3% to 4% of growth in culture, by intracellular injection of the fluorescent dye Lucifer yellow into the cell body of the MP1 (and sometimes also the dMP2) neuron in the T3 segment [MP1(T3)]. As a control, in some embryos we also assayed the extension of the vMP2 growth cone (which normally does not express fasciclin II).
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Neuronal Cytomechanics: The Actin-Based Motility of Growth Cones

STEPHEN J SMITH

The patterns of synaptic connection that underlie brain function depend on the elaborate forms characteristic of neurons. It is therefore a central goal of neuroscience to understand the molecular basis for neuronal shape. Neuronal pathfinding during development is one major determinant of neuronal shape: growing nerve axons and dendrites must navigate, branch, and locate targets in response to extracellular cue molecules within the embryo. The leading tips of growing nerve processes, structures known as growth cones, contain especially high concentrations of the ubiquitous mechanochemical protein actin. Force generation involving this cytoskeletal molecule appears to be essential to the ability of growing nerve fibers to respond structurally to extracellular cues. New results from electronically enhanced light microscopy of living growth cones are helping to show how actin-based forces guide neurite growth and synapse formation.

CELL MOTILITY MECHANISMS ARE FUNDAMENTAL TO DEVELopment of the nervous system: they are expressed during neuronal and glial proliferation and migration, neurite growth, and the selection of pathways and synaptic partners. Later, functional plasticity of the mature nervous system may also involve motility and structural change. This article will focus on the mechanisms used by growing neurites to select pathways and synaptic partners: mechanisms based primarily on the mechanochemical protein actin. While the motility mechanisms of neurons are probably similar to those in other metazoan cells, major questions remain about even the simplest of actin-based motions (*1–4*). This article will provide an overview of recent progress on the mechanisms of actin-based neuronal motility. In the process, I shall illustrate how electronically enhanced light microscopy can be used to study the dynamic aspects of cell motility and neuronal development.

The enlarged terminal ending of a growing axon or dendrite is known as the growth cone. This structure exhibits striking locomotory motility (*5–8*). The abilities of the growth cone to crawl, to explore, and to exert force enable developing neurites to reach their proper targets (*8–11*).The growth cone also may be the site at which neurite elongation occurs (*12*). It is probably helpful, however, to distinguish between the motility involved in neurite guidance and the process of neurite elongation itself (*10*). While the two processes must interact, guidance and elongation may be distinguishable on the molecular level: guidance at the growth cone may be mainly the realm of actin, while elongation is more fundamentally dependent on microtubules, another cytoskeletal constituent, and their tubulin subunits. In this article, I will focus on actin-based motility mechanisms rather than on the neurite elongation process.

The precise and specific nature of synaptic connection bespeaks strong regulation and guidance of neuronal motility. This guidance probably reflects the responsiveness of growth cones to temporal and spatial patterns of extracellular cue molecules (*13, 14*). These molecules may be parts of the extracellular matrix, they may be on the surface of other cells, or they may be diffusible, like hormones or neurotransmitters. These extrinsic cue molecules are presumed to act by binding to specific receptors on the surface of the motile cell, where they may generate physical adhesive forces or act as regulatory signals, either directly or via intracellular second messengers. The exuberant motility characteristic of the growth cone (Fig. 1) allows it to explore relatively large areas of its environment as it migrates, often contacting and "tasting" many surfaces before choosing one for further migration or synapse formation (*9, 15*).

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