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Intermediate targets and segmental pathfinding

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Abstract. Neurons must often extend axons over fairly long distances, making multiple changes in their trajectory of growth before arriving at their final target. It has become clear that as growth cones navigate these complex projections, they typically extend toward a number of intermediate targets before they contact their final target. Recent work from a variety of systems has identified intermediate targets that seem to play similar roles in vertebrate and invertebrate nervous system development. From these examples it appears that a general model of axon guidance can be proposed whereby neurons are guided to their targets segmentally. Within each segment, an intermediate target appears to be the primary target for growth cone recognition and thus the completion of the journey to the final target is determined by a series of successful segmental pathfinding decisions.

Key words. Intermediate targets; segmental pathfinding; guidepost; midline; pioneer neurons; grasshopper limb bud.

Introduction

A number of models have been proposed to explain how neuronal growth cones accurately pathfind to their correct targets. Over the last decade it has become increasingly apparent that many of these models do not adequately describe how many of the complex pathways are established in the developing nervous system. Although we have accumulated a wealth of data about the spatial and temporal distribution and nature of guidance cues, little progress has been made in describing new models of axon pathfinding. One of the reasons for this lack of progress is that the majority of systems are not amenable to examination of all stages of axon pathfinding, from axon initiation to target selection. However, recent observations from a variety of relatively simple projections in insects and vertebrates have started to provide a framework from which models of axon pathfinding can be proposed. The focus of this review will be to examine the role of a few intermediate targets in well-described systems and to discuss a simple model of axonal pathfinding that may apply to most developing nervous systems.

Pathfinding in the absence of intermediate targets

Roger Sperry's [1] now classical experiments on amphibian behavior after a variety of retinal and tectal manipulations provided some of the first ideas about how neurons establish connections with their targets. One of the outcomes of these experiments was the chemoaffinity hypothesis, an elegant model that suggested positional information within a target field could be detected by individual axons projecting to that target. It is important to realize that this model was mainly proposed as a mechanism by which growth cones identify their final targets. As the majority of these experiments were of regenerating retinal ganglion cells, little attention was paid to how the retinal ganglion cells navigated their pathway to the tectum. However, using a similar model system, Harris [2] was able to demonstrate that the tectum alone could provide sufficient guidance information to retinal ganglion neurons. In a series of informative experiments, Harris showed that ectopic eyes implanted into a variety of regions of the developing *Xenopus* brain were able to establish projections to the tectum [2]. These experiments were surprising as they suggested that there was information in most regions of the brain that could guide the developing retinal ganglion cells to the tectum. Thus, retinal ganglion cells did not appear to use intermediate targets to establish projections to the tectum. In these experiments however, the retinal ganglion cells from the ectopic eye always projected to the ipsilateral tectum. Assuming that donor eyes were not always taken from the side contralateral to the transplant (the majority of the frog retinotectal projection is crossed), this would

suggest that while they did project to the tectum, in many cases they were projecting to the incorrect tectum. While this is a trivial point in comparison to the profound implications of the experiments, it is interesting to note that intermediate targets may play an important role in directing which neurons cross the midline in the optic chiasm (see below).

Other experimental paradigms have suggested that the final target may provide sufficient guidance information to direct neurons [see refs 3 and 4 for examples]. The majority of these experiments, however, used in vitro paradigms and it is unclear whether the final targets in these examples provide sufficient guidance information to direct the entire pathway in vivo. However, it is certain that some neurons are able to respond to diffusible guidance cues secreted from their final targets at the very earliest stage of axon outgrowth. Whether this is common amongst other neuronal populations is still an actively investigated question. From these experiments then, it is clear that there are some projections where the final target provides sufficient information to guide neurons. However, recently it has become apparent that for many of the pathways established in the developing nervous system, axons typically respond and interact with a number of intermediate targets prior to contacting their final target.

Intermediate targets

Intermediate targets are typically thought to occur in at least two forms: discrete and continuous [5]. Discrete intermediate targets are typically thought to act as contact-mediated guidance cues that provide discontinuous guidance information. For example, individual rocks that serve as stepping-stones across a flowing stream would be considered discrete intermediate targets for a hiker in the woods. In contrast, continuous intermediate targets provide continuous guidance information over long distances, much as a bridge would provide continuous guidance over a flowing river. However, mechanistically it is unclear whether there is any significant difference between these two types of intermediate targets, therefore, for simplicity, in this review a distinction will not be made between the forms of intermediate targets.

The classic intermediate target: guidepost cells in the grasshopper limb bud

One of the first described groups of intermediate targets were the guidepost cells identified in the developing grasshopper limb bud. These cells were originally described by Bate [6] as signposts possibly playing a role in guiding the first projection in the developing grasshopper limb. This projection, the tibial (Ti) pioneer projection, is the first projection established in the limb bud, providing a scaffold upon which later arising sensory neurons fasciculate with and extend axons into the central nervous system (CNS). The Ti pioneer projection is stereotyped, making a number of steering decisions at precise regions in the limb [7, 8]. Strikingly, at least two of the signpost cells are located in the area where abrupt turns are made in the Ti pioneer neuron pathway (Tr and Cx cells in fig. 1). It was suggested by Bate [6] that these cells may act as stepping stones for the Ti growth cones, thus playing an important role in guiding the Ti pioneer projection. Extensive examination of this pathway by the Bentley laboratory and others confirmed that the pioneer neurons showed a special affinity for the signpost cells (subsequently renamed guidepost cells) [7–10]. It was proposed that as 'stepping stones' these guidepost cells may provide sufficient guidance information to establish the Ti pioneer projection. With the introduction of antibodies that recognized the developing insect nervous system, the Bentley laboratory carefully examined the nature of the guidepost cells and their relationship to the developing Ti pioneer projection [7, 8]. From this work it was apparent that the guidepost cells were actually immature neurons (pre-axonogenesis) that did not differentiate until after the Ti pioneer projection had been established into the CNS. Once the Ti projection is established, each of the guidepost neurons then extends an axon along the pioneer projection, following it into the CNS. Thus the guidepost cells first act as discrete intermediate targets to help establish the Ti pioneer pathway and then use the pioneer axons as a continuous intermediate target to establish their projections into the CNS.

The pioneer neurons act as intermediate targets for other neurons in addition to the guidepost neurons [11, 12]. Many of the neurons born in the limb bud at later embryonic stages contact and grow along the pioneer projection in order to send a projection into the CNS. In the absence of the pioneer projection, a number of peripheral neurons do not establish their correct projection. Typically these neurons will initiate and correctly establish the first part of the pathway, but in the absence of the Ti pioneer neurons they fail to complete their correct projection [11, 12]. Thus, not only do the Ti pioneer neurons interact with intermediate targets as they establish their projection, they also serve as intermediate targets for later-arising neurons.

The mammalian thalamocortical projection: a model of grasshopper peripheral nervous system development?

One of the first descriptions of intermediate targets in the vertebrate nervous system was of the cortical subplate neurons in the developing cat cortex [13, 14]. They share many similarities with the Ti pioneer neurons. Positioned just below the developing cortex, they pioneer the first descending projections from the cortical plate through the internal capsule [14]. Similar to the Ti pioneer neurons, they provide a scaffold for ascending thalamic axons that project to the developing cortex. In addition, ablation of the subplate neurons results in incorrect pathfinding of the thalamic axons [13]. Interestingly, when subplate neuron ablations were localized to very precise cortical regions, the appropriate thalamic axons were still able to project along the remaining subplate axons; however, they typically by-passed their

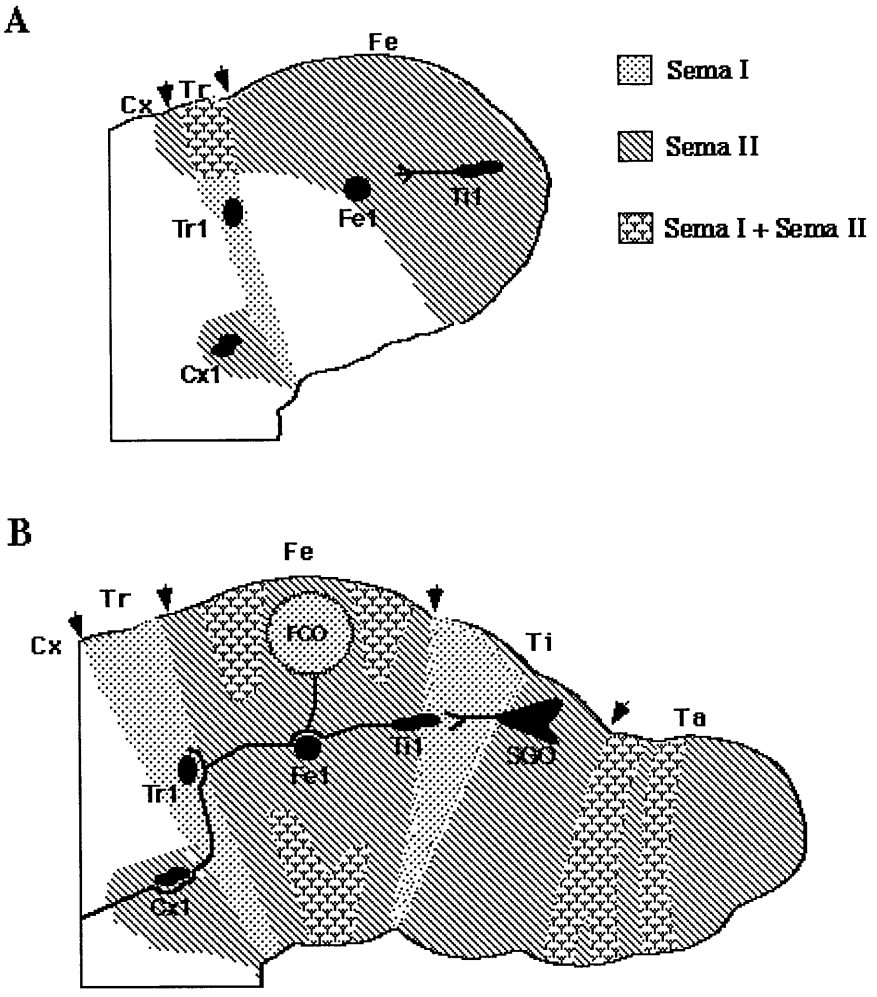

Figure 1. Segmental pathfinding in the developing grasshopper limb. This schematic shows the distribution of the guidance molecules Semaphorin (Sema) I and II and the location of the guidepost cells in the limb bud at approximately 32% (*A*) and (38%) (*B*) of embryonic development. (Cx, coxa; Tr, trochanter; Fe, femur; Ti, tibia; Ta, tarsus; arrows demarcate segment boundaries). (*A*) A hierarchy of cues guides the Ti pioneer growth cones. Sema II, a repulsive guidance cue, shows a graded distribution in the limb, with the highest levels found dorsally and distally. As the Ti growth cones extend down the gradient of Sema II, they typically contact the two intermediate target cells Fe1 and Tr1. At the Tr1 cell, the pioneer growth cones turn ventrally (possibly due in part to the dorsal expression of Sema II) and migrate along the Sema-I-expressing epithelium, a preferred substrate. Upon filopodial contact with the Cx cells, the pioneer neurons leave the Sema I band and continue proximally into the CNS. In the absence of the Cx cells, the pioneer growth cones do not leave the Sema I band. (*B*) Other neurons use the Ti pioneer pathway as an intermediate target. In addition to the guidepost cells, which all extend axons along the pioneer pathway (not shown), the femoral chordontonal organ (FCO) and subgenual organ (SGO) extend axons along the pioneer pathway. In the absence of the Ti pioneer neurons, the SGO neurons still extend axons into the Sema I band, but will not leave the band. Growth cones will often turn circumferentially within the band rather than extend onto the Sema-II-expressing epithelium [12]. Thus, both the Ti pioneer neurons and the Cx guidepost cells are situated at ideal locations to assist a growth cone to cross from a preferred substrate to a less preferred substrate.

normal targets, projecting into cortical areas adjacent to the ablated region. This may suggest that the subplate axons provide a permissive cue for thalamic growth, but the cell bodies provide the instructive information on when to defasciculate and invade the overlying cortex. Finally, similar to the Ti pioneer neurons, the subplate neurons die during development, soon after the establishment of the thalamic projections to the cortex.

Crossing the great divide: the midline

Recently, examples of intermediate targets have been described in homologous regions in widely divergent organisms: the ventral midline of the mammalian spinal cord and *Drosophila* nerve cord. A large number of neurons in the developing nervous system extend axons across the midline before extending along longitudinal tracts on the contralateral side. In the developing *Drosophila* ventral nerve cord, many neurons extend axons across the midline and then turn longitudinally along one of the developing longitudinal fascicles. Similar to the developing mammalian spinal cord (see below), an important intermediate target necessary for the correct establishment of the commissural pathways are cells in the ventral midline [11, 15–18]. Commissural formation is determined by a fine interplay between attractive and repulsive guidance cues expressed in the ventral midline (for a more thorough discussion of this topic see the article by Tear in this issue). Axons that express high levels of Roundabout, a new subfamily member of the Ig superfamily proteins, are repelled from the ventral midline. Axons that express low or no levels of Roundabout are attracted, at least in part, by netrin secreted from midline glial cells. Thus the regulation of Roundabout, a putative receptor for an inhibitory molecule expressed in the midline, is an important feature determining whether or not axons cross the midline. In addition, this regulation of Roundabout expression may be important for preventing axons from crossing the midline more than once. A similar interplay between attraction and repulsion may also occur at the *Caenorhabditis elegans* midline where the attractive properties of UNC-6 (the first identified netrin homologue [19]) are balanced by the repulsive effects of Sax-3 (Roundabout homologue) [20], suggesting that the function of midline cells as intermediate targets and the molecules they utilize may be conserved in a variety of organisms.

Similar to *Drosophila* nerve cord, midline structures act as intermediate targets for commissural axons in the developing mammalian spinal cord. The dorsal spinal commissural neurons extend circumferentially in the spinal cord toward the ventral midline structure, the floor plate, cross the midline and abruptly turn and ascend longitudinally in the spinal cord. In collagen gel assays, ventral spinal cord explants are able to attract dorsal commissural axons [21, 22]. Similar to the *Drosophila* midline, the chemotropic activity in the floor plate is the vertebrate form of netrin [21, 23, 24]. The floor plate therefore acts as an intermediate target, guiding dorsal commissural axons to the ventral midline of the spinal cord.

Midline intermediate targets also play a significant role in the establishment of crossed axonal projections in the developing mammalian brain. The development of the corpus callosum and the anterior commissure appear to rely on the preferred substrate provided by glial cells that span the midline [25–27]. Glial intermediate targets also seem to be important in the establishment of the retinogeniculate pathway. Unlike the amphibian retinotectal pathway where the majority of axons extend to the contralateral tectum, in mammals, retinal axons from each eye extend toward each other along the ventral diencephalon until they reach the midline where up to half of the axons turn away from it and extend to the ipsilateral geniculate body. In this region, the optic chiasm, growth cones are sorted and directed to either cross the midline or remain on the ipsilateral side of the diencephalon. The chiasm appears to act as an important intermediate target in two respects. First, cells in the optic chiasm, most notably glial cells that form a pallisade, act as contact-mediate repulsive cues, forcing axons from the ventrotemporal retina to turn away from the midline and establish an ipsilateral projection [28, 29]. Second, the chiasm also secretes a diffusible cue, which while not acting as a chemoattractant or chemorepellant, slows the growth of all retinal fibers [30]. This chemosuppression may force retinal growth cones to slow their growth, promote defasciculation and respond to local discrete cues. This slowed growth may also permit the change in expression of particular surface molecules. Similarly, other intermediate targets appear to slow or stop axonal progression in other systems. For example, subplate neurons in the developing cortex appear to act as temporary targets for thalamocortical projections [13], while inner hair cells act as a temporary target for cochlear axons that ultimately are sensory to outer hair cells [31]. Furthermore, guidepost cells in the developing grasshopper limb bud temporarily halt the progression of the Ti pioneer neurons [32].

Intermediate targets and segmental pathfinding

A number of examples have been presented of neurons that can be guided to their targets in the absence of any obvious intermediate targets. Why then would certain projections rely on intermediate targets when others do not? To explore some of the possible answers to this question, the specific roles of intermediate targets will be examined in two projections where they appear to be necessary for the establishment of correct neuronal projections.

The developing grasshopper peripheral nervous system: one segment at a time

Intermediate targets play an essential role in the establishment of the peripheral nerves in the developing grasshopper limb bud. However, not all of the intermediate target cells appear to be necessary. In the establishment of the Ti pioneer projection, the only intermediate target that appears to be necessary for correct navigation of the pioneer growth cones are the Cx cells [33] (see fig. 1). These cells are located just proximal to the trochanter limb segment. In the absence of the Cx cells, the pioneer growth cones do not cross over into the coxa segment, preferring to remain on the trochanter epithelium [33]. Similarly, in the absence of the Ti pioneer neurons, the distally arising subgenual organ neurons fail to extend axons across the tibia-femur segment boundary [12, 34]. Normally, the subgenual neurons contact and fasciculate with the pioneer neuron cell bodies that are located near the tibia-femur segment boundary (see fig. 1). In the absence of the pioneer neurons, the subgenual neurons extend circumferential branches along the proximal tibial epithelium [12]. We have recently proposed a hierarchy of cues that would explain the behavior of the pioneer and tibial growth cones and the necessity of the proximal guidepost neurons (fig. 1). Analysis of the expression pattern of two members of the semaphorin family of guidance molecules suggests that they may be important in guiding neurons in the developing limb bud [12, 35–37]. A secreted semaphorin, grasshopper Semaphorin II (gSema II), is typically expressed in the distal two-thirds of each limb segment [35]. In contrast, a transmembrane semaphorin, gSema I, is typically found along circumferential bands of epithelium at the proximal ends of each limb segment [12, 35, 37]. While there are small regions of overlap, there is a general pattern of expression where gSema II is located distally and gSema I proximally within limb segments. Experimental perturbation of gSema I and gSema II suggests a hierarchy of guidance cues, with gSema II the least preferred substrate, gSema I more preferred, and guidepost neurons the most preferred [12, 35]. For the pioneer or subgenual neurons to establish their correct projections they must travel from a region of gSema II expression into a region of gSema I expression before crossing over into another region of gSema II expression. In the absence of the intermediate targets (guidepost neurons), the growth cones will not extend from the more preferred gSema I substrate into the less preferred gSema II substrate. Thus in the developing limb bud, intermediate targets may provide convenient targets to assist growth cones to overcome a repulsive guidance cue. This type of pattern may be beneficial to the organism as the reiteration of the same molecular expression pattern within limb segments would be economically advantageous for the genome because fewer molecules would be required to establish projections that extend over long distances.

Crossing the midline: one segment down, more to go

In contrast to the reiteration of guidance cues observed in the developing limb bud, commissural axons in the developing nerve cord of *Drosophila* and the spinal cord of mammals pathfind through a series of segments with varied substrates and guidance cues. For example, the dorsal spinal commissural axons in the spinal cord are first attracted to the floor plate; then, as they reach the midline they make an abrupt turn and continue across the midline toward the developing longitudinal tracts and, finally, they turn again and extend along a longitudinal fascicle. One potential role then for the midline intermediate target is to ensure that commissural axons do not choose the longitudinal fascicles on the ipsilateral side of the cord as their target. This is particularly true of the *Drosophila* ventral nerve cord where many of the ipsilateral longitudinal fascicles are within filopodial reach of commissural growth cones. The ventral midline could accomplish this in two ways. First, it may regulate the responsiveness of commissural axons to guidance cues on the longitudinal fascicles and/or second, it may possess much stronger attractive cues than the ipsilateral longitudinal fascicles. The fact that the ventral midline can be such an attractive target for commissural axons raises some intriguing questions. For example, why do growth cones continue to extend beyond the midline once they have reached it? Why do they not remain there? In addition, why do growth cones suddenly have the ability to recognize cues that guide them to the correct longitudinal fascicle? While some of these questions remain unanswered, recent work has provided significant insight into the role played by intermediate targets in these pathfinding decisions.

In both *Drosophila* and mammalian systems there is evidence for differential expression of molecules along

axons once they cross the midline. For example, dorsal spinal commissural axons express the adhesive molecule TAG-1 on their surface as they extend ventrally and across the midline [23, 38]. Axons extending along the longitudinal fascicle, however, do not express TAG-1, but instead express a second adhesive molecule L1 [23]. This would suggest that the midline intermediate target may induce differential expression of receptors that recognize longitudinal cues. In addition, encounters with an intermediate target may also suppress the responsiveness of commissural growth cones to the midline chemoattractant. Recent elegant experiments by Shirasaki et al. [38] have shown that commissural axons in the rodent metencephalon lose their responsiveness to the ventral midline chemoattractant once they cross the floor plate. Using a slice preparation, they were able to show that once commissural axons had crossed the floor plate they would not respond to a second ectopic floor plate closely applied to the contralateral side. In addition, commissural cells would not respond to an ectopic presentation of netrin once they had crossed the floor plate. In contrast, commissural neurons retained their chemoattractant responsiveness if they crossed the midline without contacting the floor plate. There are a number of possible mechanisms that may underlie this change in responsiveness to the midline chemoattractant. One is that contact with the floor plate may change the expression of the receptors that respond to the chemoattractant. Thus, the down-regulation of 'deleted in colorectal cancer' (DCC), the receptor for netrin [39], may change the responsiveness of the commissural axons. However, DCC expression does not appear to change on commissural axons once they cross the floor plate [38]. Alternatively, floor plate cells may alter the chemoattractant receptor responsiveness locally in the growth cone. For example, one possible scenario is that a second molecule, localized to the floor plate, modifies the responsiveness of the chemoattractant receptors in the growth cone. This could be mediated directly by interacting with the receptor or, alternatively, by indirectly changing the cyclic nucleotide levels in commissural growth cones. In support of this second scenario, responsiveness to a number of guidance cues, including netrin, has been shown to be sensitive to cyclic nucleotide levels in the growth cone [40].

While the above two examples show important roles for intermediate targets in directing neurons across the midline and assisting neurons to cross into fields of repulsive guidance cues, intermediate targets will certainly be necessary for other aspects of axon guidance. For example, given theoretical models of guidance gradients, it has been suggested that the maximal distance at which a diffusible gradient could provide guidance information is 1 mm [41]. Theoretically therefore, intermediate targets will be necessary for projections greater than 1 mm in length.

Summary and questions for future directions

It is now evident that intermediate targets play an essential role in neuronal pathfinding. By establishing a succession of intermediate targets during development, the daunting task of connecting up the nervous system is simplified to a series of successful segmental pathfinding decisions. While we have learned much about the nature and role of intermediate targets, there are several interesting and important questions still to be answered. For example, do growth cones possess the full complement of receptors necessary to interact with all their intermediate targets and final target at the time of axon outgrowth? It would be interesting to examine whether dorsal spinal commissural neurons can recognize their final targets prior to crossing the floor plate. In addition, do neurons need to contact their intermediate targets in order to recognize their final target? Certainly, in some examples, neurons can recognize their final targets soon after outgrowth, however, is contact with intermediate targets necessary for long complex pathways where several changes in trajectory are made? Many other questions remain. What are the molecular mechanisms that underlie the change in responsiveness of commissural axons to the midline? Are these similar to the change in responsiveness observed when Ti pioneer neurons leave a discrete intermediate target? Does a similar interplay between repulsive and attractive guidance cues observed in *Drosophila* play a role in directing dorsal spinal neurons across the midline? Finally, can segmental pathfinding by intermediate targets be considered a bona fide model of growth cone pathfinding? Answers to these and other questions should bring us one step closer to answering the well-used presentation query: 'How do neurons find their targets?'

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