Use of Lipophilic Dyes in Studies of Axonal Pathfinding In Vivo

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ABSTRACT Fluorescent lipophilic dyes are an ideal tool to study axonal pathfinding. Because these dyes do not require active axonal transport for their spreading, they can be used in fixed tissue. Here, we describe the method we have used to study the molecular mechanisms of commissural axon pathfinding in the embryonic chicken spinal cord in vivo. Based on in vitro studies, different families of molecules had been suggested to play a role in the guidance of developing axons. In order to test their function in vivo, we used the commissural neurons that are located at the dorsolateral border of the chicken spinal cord as a model system [Stoeckli and Landmesser (1995) Neuron 14:1165–1179]. Axonin-1, NgCAM, and NrCAM, three members of the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs), were shown to be important for the correct growth pattern of commissural axons. We studied the effect of perturbations of specific CAM/CAM interactions by injection of function-blocking antibodies into the central canal of the spinal cord in ovo. After 2 days, the embryos were sacrificed and fluorescent tracers, such as Fast-Dil, were used to visualize commissural axons, and thus, to analyze their response to these perturbations in two different types of fixed preparations: transverse vibratome sections and whole-mount preparations of the spinal cord. Both pathfinding errors and defasciculation of axons were observed as a result of the perturbation of CAM/CAM interactions. Microsc. Res. Tech. 48:25–31, 2000. © 2000 Wiley-Liss, Inc.

INTRODUCTION

The correct wiring of the nervous system is a key issue in developmental neurobiology. During the last decade a lot of progress has been made towards an understanding of the molecular mechanisms underly–
ing the growth of individual axons toward their targets. This progress is on the one hand based on the elucidation of complex interaction patterns of molecules in vitro, and on the other hand, based on the development of in vivo assays for testing the role of such molecules in axonal pathfinding. Outgrowing axons in the developing nervous system are surrounded by a complex environment that changes over time. Therefore, pathfinding studies in relatively simple in vitro systems can only mimick isolated aspects of axonal behavior. Alternatively, complicated in vitro systems have to be used consisting mostly of explant or slice cultures that are far more difficult to control, but that provide a more physiological context for growing axons. Ideally, one sets out to study an axon’s behavior in vivo, in its natural environment. Common to both the explant cultures and the in vivo experiments is the need to visualize individual axons in order to monitor their behavior.

DISCOVERY OF LIPOPHILIC DYES AS A TOOL FOR AXON TRACING

More than 100 years ago, Santiago Ramón y Cajal (1995) used the silver staining method developed by Camillo Golgi to visualize individual neurons. Not only are his reports of outstanding quality and amazing detail, but he correctly predicted many of the dynamic processes involved in the development of the nervous system, although all was based on these static images. For instance, he correctly identified the tip of the axons as the site of growth. He also suggested that axons could be guided toward their target by chemotraction, a mechanism that could be demonstrated only much later (Lumsden and Davies, 1986; Tessier-Lavigne et al., 1988). The analysis of the static wiring pattern of the nervous system was tremendously facilitated by the use of tracers, substances that are injected, for example, into the target area of axons. They are taken up by axons and via axonal transport they end up in the cell somas of projection neurons. One of the most commonly used substances is horse radish peroxidase (HRP), which was used as a retrograde tracer starting in the early seventies. Later, it was shown that under certain conditions HRP could also be used as an anterograde tracer (Tosney and Landmesser, 1985, 1986). Because the use of HRP as a tracer depends on an intact axonal transport, it can only be used in living tissue. However, the visualization of HRP requires histochemical techniques that are not compatible with living tissue. Therefore, tissue is commonly fixed after HRP application and transport, but before processing for visualization of the tracer. This disadvantage has led to the search for new tracers with more convenient characteristics (Honig and Hume, 1986, 1989). The carbocyanine dye 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil) was found to fulfill the requirements very well. It has a high fluorescence intensity, is very resistant to photobleaching, and is not

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toxic for living cells (Godement et al., 1987; Honig and Hume, 1986). Its most important feature, however, is the fact that it can be used in fixed tissue, as its distribution is mainly by lateral diffusion in the plasma membrane rather than by axonal transport. Therefore, Dil can be used to trace axonal tracts over extremely long distances in fixed tissue that can be kept for weeks to allow sufficient time for the diffusion of the dye (Godement et al., 1987). Today, various derivatives of Dil have been synthesized. They differ in their diffusion rate and their fluorescence characteristics. As dyes are available in several colors, different neuronal populations can be labeled individually and their trajectories can be traced in the same slice or tissue. An impressive application of this was the use of lipophilic dyes in an automated analysis of a large screen for pathfinding mutants in the retinotectal system of zebrafish (Baier et al., 1996). Due to its non-toxicity and high fluorescence intensity, Dil is well suited for the tracing of extending axons in vivo (for a review see Fishell et al., 1995). Taking advantage of the transparency of zebrafish embryos Pike and Eisen (1990) demonstrated that individual motor axons could be followed as they navigated toward their target muscles. Kaethner and Stuermer (1992) used time lapse video microscopy to follow in vivo the terminal arbor formation of individual retinal ganglion cell axons in the tectum.

Carbocyanine dyes have also been used successfully for time-lapse recording of axonal behavior in higher vertebrates. However, as brain tissue from mammalian or chicken origin is not transparent, axonal tracing is more difficult and requires sectioning (Dailey et al., 1995). Taking advantage of the transparency of zebrafish embryos Pike and Eisen (1990) demonstrated that individual motor axons could be followed as they navigated toward their target muscles. Kaethner and Stuermer (1992) used time lapse video microscopy to follow in vivo the terminal arbor formation of individual retinal ganglion cell axons in the tectum.

COMMISSURAL AXONS OF THE SPINAL CORD AS A MODEL SYSTEM TO STUDY Molecular MECHANISMS OF AXON GUIDANCE

During the development of the nervous system, axons have to navigate through the preexisting tissue and to establish correct connections with their targets. For this purpose, the axon has a highly motile structure at its tip that acts as a sensor for guidance cues presented by the environment (Vogt et al., 1996). The growth cone guides the axon along its trajectory by constantly sampling and integrating the molecular signals derived from the interaction of its surface molecules with such guidance cues. The result of this process is translated into an intracellular signal that is transported back to the nucleus where it elicits the neuron’s response. We still have limited knowledge about the crosstalk of surface molecules with the cytoskeleton, the effect of the axon’s growth behavior. However, depending on the nature of the incoming signal the axon will continue to grow, stop, turn, or retract. Based on in vitro studies, a complex interaction pattern of surface molecules has been elucidated. Different families of molecules have been identified as candidates for molecular cues involved in the guidance of growing axons (Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Varela-Echavarria and Guthrie, 1997). Based on their effect, they are divided into two groups, chemoattractive and chemorepulsive guidance cues (Tessier-Lavigne and Goodman, 1996). Both groups can be further subdivided into short-range and long-range guidance cues. By definition, short-range cues are locally active, whereas long-range cues can act over some distance, because they are secreted and to some extent diffusible. A particular molecule can have different, even opposite effects on distinct neuronal populations. For example, netrin-1 was first identified as a chemoattractive molecule for commissural axons (Kennedy et al., 1994; Leonardo et al., 1997; Serafini et al., 1994), and subsequently, it was demonstrated to be a chemorepellent for trochlear motor axons (Colamarino and Tessier-Lavigne, 1995). As both some short- and long-range guidance cues have been identified, the commissural axons of the developing spinal cord are one of the best characterized model systems for pathfinding at the molecular level (for reviews see Stoeckli, 1998; Stoeckli and Landmesser, 1998). Commissural axon pathfinding has been studied at the molecular level in Drosophila (Kid et al., 1998, 1999; Seeger et al., 1993), in Caenorhabditis elegans (Zallen et al., 1998), and in chick (Stoeckli and Landmesser, 1995; Stoeckli, 1997).

In chick, at the lumbosacral level, commissural neurons located in the dorsal half of the embryonic chicken spinal cord, close to the dorsal root entry zone, start to extend axons at stage 19 (Hamburger and Hamilton, 1951). The first fibers reach the floor plate at about stage 21. After crossing the midline, they turn rostrally along the contralateral border of the floor plate. Tessier-Lavigne and colleagues showed that floor-plate explants co-cultured with commissural neurons in a three-dimensional collagen matrix had a chemoattractive effect on commissural axons (Tessier-Lavigne et al., 1998). Subsequently, this in vitro assay system was used to purify the chemoattractive activity derived from the floor plate. Netrin-1, a 78 kDa protein expressed and secreted by the floor plate, was the first long-range guidance cue identified at the molecular level (Kennedy et al., 1994; Serafini et al., 1994). Based on their on their neurite outgrowth-promoting activity, cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily were suggested to play a role in axonal pathfinding (Brummendorf and Rathjen, 1996; Burden-Gulley and Lemmon, 1995; Doherty and Walsh, 1994). Such a role was corroborated, for instance, in vivo studies in chicken embryos (Stoeckli and Landmesser, 1995; Tang et al., 1992, 1994).

In this paper, we will focus on the functional studies done in chick and describe the use of carbocyanine dyes as axonal tracers in an in vivo pathfinding study, where FastDiI was used to trace commissural axons in the embryonic chicken spinal cord after perturbation of various interactions between cell adhesion molecules (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997).

MATERIALS AND METHODS

In Vivo Injections

Fertilized eggs were obtained from a local supplier. They were incubated at 38.5–39°C, before they were opened on the third day of incubation for in ovo injections. To have access to the embryo, a window was cut into the eggshell. In order to facilitate repeated
opening and closing, we sealed the window with a coverslip using melted paraffin. Proper sealing was crucial to avoid dehydration of the embryo. Chicken embryos were staged according to Hamburger and Hamilton (1951) at the time of injection. For injections into the spinal cord, the extraembryonic membranes were cut with spring scissors and removed from the lumbosacral region. The injections were made just rostral of the hindlimb, directly into the central canal of the spinal cord, using glass electrodes attached to a piece of teflon tubing. We controlled the injection volume by mouth and did not use a mechanical device. The electrodes were pulled on a Narishige PC-10 electrode puller to a tip diameter of 5 µm. In order to control the localization of the injection site, the quality of the injections, and the injected volume, 0.04 % Trypan Blue was added to the injected solutions. In order to assure the presence of function-blocking antibodies throughout the period of axonal pathfinding in sufficient quantity, high concentrations had to be injected to make up for the dilution due to diffusion and development-dependent increase in volume of the spinal cord. The injections were repeated every 8 hours. Thus, embryos were injected 4 to 5 times between stages 18 and 24. Because the injectable volume was very small (between 0.05 and 0.1 µl for early stages), the antibody concentrations used were 10 mg/ml or higher. The anti-axonin-1 antibody was used, as described in Ruegg et al. (1989); the rabbit antibody against NgCAM was described previously in Landmesser et al. (1988). The antibodies against NrCAM were raised in rabbit against affinity-purified NrCAM as described by de la Rosa et al. (1990).

The embryos were sacrificed and dissected between stages 25 and 26. After stage 26, the associational neurons that are localized close to the commissural neurons would have confused the results, as they project their axons ipsilaterally. Embryos injected with nonimmune serum were not different from non-injected embryos with respect to both development and commissural axon pathfinding. The pathfinding behavior of commissural axons was compared in two different types of spinal cord preparations. In transverse vibratome sections, the ipsilateral growth pattern was compared between control and experimental embryos. The ipsilateral turns and the defasciculation along the contralateral border were more obvious in whole-mount preparations, the so-called open-book preparations. For transverse vibratome sections, embryos were decapitated, dissected, and pinned down in a Sylgard dish for fixation in 4% paraformaldehyde in PBS for at least 3 hours at room temperature or at 4°C overnight. The embryos were rinsed in PBS and embedded in 7.5% ultra low-melting agarose (Sigma, Buchs, Switzerland). The blocks were trimmed and mounted such that transverse sections of 250 µm could be cut with an OTS-3000-04 oscillating tissue slicer (Electron Microscopy Sciences, Fort Washington, PA). For open-book preparations, the spinal cord was removed from the embryo after a ventral laminectomy using tungsten needles. The roof plate was cut and the cord was flipped open, flattened, and pinned down in a Sylgard dish for fixation. Fixation was as described for transverse sections.

**Dye Injections**

We used mainly FastDiI (1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine 4-chlorobenzensulfonate) obtained from Molecular Probes, Eugene, OR, dissolved at 5 mg/ml in methanol. The dye was injected with glass electrodes pulled to a tip diameter of up to 3 µm into the area of the somas of commissural neurons in either tissue slices or whole-mount preparations. After incubation for 36–48 hours, the preparations were mounted in PBS between coverslips and viewed with conventional fluorescence or confocal microscopy. As the fluorescence intensity of the lipophilic dyes is high, but the thickness of the preparations is rather big, objectives with a large focal depth may sometimes give better results than objectives with higher numerical aperture, but small focal depth.

**RESULTS**

**Use of Fluorescent Tracers in the Analysis of the Role of Guidance Molecules in Commissural Growth Cone Guidance and Fasciculation**

The Ig superfamily CAMs, axonin-1 and NgCAM, are expressed on commissural axons during initial stages of axon extension (Shiga et al., 1990; Shiga and Oppenheim, 1991; Stoeckli and Landmesser, 1995), whereas NrCAM is expressed on floor-plate cells (Krushel et al., 1993; Stoeckli and Landmesser, 1995). In vitro, axonin-1 was shown to bind to NrCAM (Suter et al., 1995), and furthermore, axonin-1 was shown to interact with NgCAM in the plane of the same membrane (Buchstaller et al., 1996; Stoeckli et al., 1996; Sonderegger et al., 1998). To test for a role of these molecules in commissural axon guidance in vivo, perturbation experiments were carried out (Stoeckli and Landmesser, 1995; Stoeckli, 1997). Function-blocking antibodies against axonin-1, NgCAM, or NrCAM were injected into the central canal of the developing chicken spinal cord in ovo (Fig. 1A) (see Materials and Methods for details). These injections were repeated during the time window of commissural axon pathfinding. Between stages 25 and 26, after commissural axons had turned into the longitudinal axis to extend along the contralateral floor-plate border, the embryos were sacrificed and fixed for analysis. Making use of the capability of fluorescent tracers to diffuse in fixed tissue, we visualized the trajectories of commissural axons in control and experimental embryos. Axonal trajectories in two different types of spinal cord preparations were analyzed and compared. Transverse vibratome sections (250 µm) of the spinal cord were cut from the lumbosacral level to look at the ipsilateral pathfinding behavior of commissural axons (Fig. 1B). To assess pathfinding errors and the growth pattern of commissural axons in the longitudinal axis along the contralateral side of the floor plate, the “open-book” preparation was more useful. This whole-mount preparation was obtained by flattening the spinal cord after transection of the roof plate (Fig. 1C). Lipophilic dyes were then injected in the area of the cell bodies to visualize commissural axon trajectories.
In non-injected and control-injected embryos, commissural axons grew as a discrete bundle toward the floor plate (Fig. 2A–C; see Stoeckli and Landmesser, 1995, for more details). Similarly, in open-book preparations the DiI-labeled axons were found to cross the floor plate and to turn rostrally along the contralateral border of the floor plate in a fasciculated manner (Fig. 2C). After perturbation of axonin-1 interactions by injection of function-blocking anti-axonin-1 antibodies, axons grew in a defasciculated manner both before and after crossing the floor plate (Fig. 2D, compare the distance between arrowheads in control [Fig. 2A] and experimental embryos [Fig. 2D]). Even more strikingly, some axons committed pathfinding errors (arrows in Fig. 2E and F). They failed to cross the midline and joined the longitudinal tract along the ipsilateral border of the floor plate (Fig. 2F, arrow). Using the same approach, a defasciculation of commissural axons was also shown to result from the perturbation of NgCAM interactions. However, in contrast to anti-axonin-1 injected embryos, no pathfinding errors were found in anti-NgCAM-treated embryos. The perturbation of NrCAM interactions resulted in similar pathfinding errors as the ones found after perturbation of axonin-1 interactions. Together with the notion that axonin-1 and NrCAM were heterophilic binding partners in vitro (Suter et al., 1995), these findings strongly suggested that NrCAM and axonin-1 were binding partners in the guidance of commissural axons across the floor plate (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). Axonin-1 and NgCAM seemed to be responsible for the fasciculation of commissural axons both before and after crossing the floor plate. Thus, it was concluded that NgCAM was involved in the fasciculation of commissural axons but not in their pathfinding. Taken together, these results showed that the defasciculation of commissural axons was not responsible for their failure to cross the floor plate, rather that these axons were capable of reading the guidance cues presented by the floor-plate surface on an individual basis.
**DISCUSSION**

The advantage of the method used for this pathfinding study is the possibility to follow the behavior of commissural axons in their natural environment. The perturbation of a specific guidance cue thus affects the interactions of this particular molecule with all its binding partners, whereas independent molecular interactions are left untouched. Although the intact spinal cord of a chicken embryo does not allow the optical access to follow living axons directly, the fact that the trajectory of commissural axons can be visualized after fixation provides a means to circumvent this problem and to dissect the role of individual guidance cues. We have successfully used this approach to study the role of CAMs (described here) and floor-plate derived extracellular matrix molecules (Burstyn-Cohen et al., 1999).

An advantage of the visualization of axons by dye injections rather than by immunohistochemistry is the independence from a particular marker protein. Even if such markers exist, their expression could be influenced by the perturbation of cell/cell contacts as a result of the absence of specific CAM/CAM interactions, and, thus, the analysis could be compromised. Because lipophilic dyes spread by diffusion in the entire cell membrane, axon tracing with FastDiI allows the visualization of fine structural details, such as filopodia on growth cones (Fig. 3). In a recently published study, Liljelund and Levine (1998) compared morphology and behavior of DiI-labeled growth cones in living slices with fixed preparations of early postnatal rat cerebellum. Similarly, lipophilic dyes were used in the retino-tectal system to correlate growth cone morphology with behavior at specific choice points (Godement et al., 1994; Mason and Wang, 1997). All these studies would not have been possible with the use of HRP or similar tracers that require tissue processing for their visualization.

Because labeling of axonal tracts with lipophilic dyes is compatible with subsequent immunohistochemistry, a combination of dye injections and staining with fluorescent antibodies is possible (Elberger and Honig, 1990). Thus, lipophilic dyes are an invaluable tool for many different types of studies. They have been used successfully in pathfinding studies in many species,

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**Fig. 2.** In the absence of axonin-1 interactions commissural axons commit pathfinding errors. Specific interactions between the candidate guidance cue axonin-1 and its binding partners were perturbed by the injection of function-blocking antibodies into the spinal cord of chicken embryos in ovo. The effect of these perturbations on commissural axon trajectories were analyzed in transverse sections (A, B, D, E) and whole-mount preparations (C, F) of the spinal cord. FastDiI was injected into the area of commissural neuron somas to trace their axon trajectories (see Fig.1). In control embryos (A–C), commissural axons project ventromedially toward the floor plate as a compact bundle (A and B; the bundle is outlined with arrowheads in A). All axons cross the midline and turn rostrally along the contralateral border of the floor plate (C). In contrast, in embryos treated with anti-axonin-1 antibodies (D–F), commissural axons grow defasciculated (D and E, compare width of the bundle in A and D). In the absence of axonin-1 interactions, some commissural axons commit pathfinding errors (marked with arrows in D through F). Instead of crossing the midline, they turn prematurely along the ipsilateral border of the floor plate (F). Arrowheads in A and D indicate the width of the commissural axon trajectory toward the floor plate. Arrows indicate axons committing pathfinding errors (D–F). Bar = 100 µm in A and D, 50 µm in B and E, and 40 µm in C and F.
both in living as well as fixed preparations. Their versatility will certainly make them the tracer of choice for many future applications.

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