Interference with Axonin-1 and NrCAM Interactions Unmasks a Floor-Plate Activity Inhibitory for Commissural Axons

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Summary

Axonin-1 and NrCAM were previously shown to be involved in the in vivo guidance of commissural growth cones across the floor plate of the embryonic chicken spinal cord. To further characterize their role in axon pathfinding, we developed a two-dimensional coculture system of commissural and floor-plate explants in which it was possible to study the behavior of growth cones upon floor-plate contact. Although commissural axons readily entered the floor plate under control conditions, perturbations of either axonin-1 or NrCAM interactions prevented the growth cones from entering the floor-plate explants. The presence of anti-axonin-1 resulted in the collapse of commissural growth cones upon contact with the floor plate. The perturbation of NrCAM interactions also resulted in an avoidance of the floor plate, but without inducing growth-cone collapse. Therefore, axonin-1 and NrCAM are crucial for the contact-mediated interaction between commissural growth cones and the floor plate, which in turn is required for the proper guidance of the axons across the ventral midline and their subsequent rostral turn into the longitudinal axis.

Introduction

The establishment of connections between neurons and their target cells is a crucial step in the development of the nervous system. The precise mechanisms by which growth cones find their way through the preexisting tissue are not yet fully understood. However, based on their temporal and spatial expression pattern, cell adhesion molecules (CAMs) have been purported to play an important role in the pathfinding of developing axons (Goodman and Shatz, 1993; Burden-Gulley and Lemmon, 1995; Goodman, 1996). Extensive in vitro studies have revealed an increasingly complex pattern of potential interactions between the various cell adhesion mole-
Results

The Chemoattraction of the Commissural Axons toward the Floor Plate Occurs in a Two-Dimensional Culture System

To visualize individual growth cones before and during contact with the floor plate, we established a culture system wherein floor-plate explants were placed between 150 and 400 μm away from commissural neuron explants on a laminin substratum in a serum-free medium (see Experimental Procedures for details). Since it has been shown that the roof plate can interfere with commissural axon outgrowth (Placzek et al., 1990; Serafini et al., 1994), we made roof plate–free commissural explants by removing only small portions of dorsolateral spinal cord from transverse vibratome sections of the lumbosacral level of stage 20–21 spinal cords. At this stage of development, the region removed contains primarily commissural neuron cell bodies, and while some will have initiated axons, few, if any, will have contacted the floor plate (Stoeckli and Landmesser, 1995). Thus, our observations in culture were made on naive commissural axons that had not yet made contact with floor-plate cells. This is important, since one of the models to explain commissural growth-cone behavior at the midline postulates a floor plate–mediated alteration in the molecular properties of the commissural axons (Stoeckli and Landmesser, 1995). To ascertain that we were in fact removing the commissural cell bodies by this procedure, we cultured some of the slices used for an additional 24 hr. Figure 1a shows that during this culture period commissural neurons on the control side (left) had extended axons (arrowheads) to the floor plate, along the characteristic commissural trajectory. However, this trajectory was completely absent on the right side, from which the region containing the commissural cell bodies had been removed.

When cultured alone in floor plate–conditioned medium for 18 to 21 hr on a laminin substratum, commissural explants extended axons in a radial fashion (Figure 1b). The explant shown in Figure 1b was stained with an anti-neurofilament Mab, 1E9, which we routinely used to visualize the trajectory of the axons. However, we also confirmed that the commissural axons in our culture conditions expressed axonin-1 and NgCAM (not shown) as they do in vivo (Shiga and Oppenheim, 1991; Stoeckli before fixation to allow commissural axon growth. During this time, the neurons left in place produced axons (arrowheads), which reached the floor plate. Note the absence of commissural fibers from the right half of the spinal cord. An anti-neurofilament antibody, 1E9, was used to stain all neurites. Motoneurons are marked with asterisks. Although the commissural explants were capable of radial neurite extension in the absence of floor-plate explants (not shown) or in floor plate–conditioned medium (b), axons extended almost exclusively toward the floor-plate explants when commissural and floor-plate explants were cocultured (c). Note that the presence of a second commissural explant (b) has no effect on the number or direction of commissural neurites extending from the neighboring explant. Commissural neurite growth in the absence of floor-plate explants and without floor plate–conditioned medium was sparse, and the length of the neurites was considerably shorter (data not shown). Scale bar: 250 μm in (a), 160 μm in (b) and (c).

Figure 1. The Chemoattractive Effect on Commissural Axons Emerging from Floor-Plate Explants Is Also Found in a Two-Dimensional Culture System

Commissural explants were taken from transverse vibratome sections of embryos from stages 20–21 prior to floor-plate contact of commissural axons (see Experimental Procedures for details). In the slice seen in (a), commissural neurons were taken from the right half of the spinal cord only. The slice was kept in culture for 24 hr
and Landmesser, 1995). When cultured without floor plate and in the absence of floor plate–conditioned medium, the commissural explants extended much shorter and fewer axons (not shown). In striking contrast to the radial outgrowth of commissural explants cultured alone, when cultured with floor plate, most of the axons showed a strong directional preference to grow toward the floor plate. In the example shown (Figure 1c), almost all the axons can be seen emerging from the side of the commissural explant facing the three floor-plate explants (asterisks). This data is presented quantitatively in Table 1. In the absence of a floor-plate explant, axons grew randomly from the commissural explant with the expected approximate 25% in each randomly chosen quadrant. In contrast, when cultured with floor plate, >80% of the axons emerged from the explant in the quadrant facing the floor plate. We conclude that even without a collagen gel matrix (Tessier-Lavigne et al., 1988), the floor plate is able to exert a chemoattractive effect on the commissural axons. Thus, similarly to the situation in vivo (Yaginuma and Oppenheim, 1991), the floor plate is able to exert a chemoattractive effect on the commissural axons. This temporary decrease in growth rate, however, is not due to the presence of soluble axonin-1 antibodies, as shown in Figure 2h, where the floor-plate explant comprises the entire field of view. This observation is consistent with previous in vivo observations, where injections of anti-NgCAM did not interfere with the ability of commissural axons to cross the midline by growing through the floor plate (Stoeckli and Landmesser, 1995).

In sharp contrast, commissural axons did not grow into floor-plate explants in the presence of either anti-axonin-1 (Figures 2b and 2f) or anti-NrCAM antibodies (Figures 2c and 2g). They were found to either stall on the laminin substratum near the floor-plate boundary or to grow around the floor-plate explants (asterisks indicate the border of the floor plate in Figures 2f and 2g). The same effect was seen when purified soluble axonin-1 was added to the culture medium (Figure 2d). Soluble axonin-1 would be expected to bind to and saturate any receptors for axonin-1 on the floor-plate cells, and thus would prevent axonin-1 on the growth cones from interacting with molecules on the floor-plate surface (Stoeckli et al., 1991; Stoeckli and Landmesser, 1995). Thus, consistent with the in vivo perturbations (Stoeckli and Landmesser, 1995), interfering with either axonin-1 or the commissural axons with NrCAM on the floor plate prevented the axons from entering the floor plate. The quantitative data, shown in Table 2, indicate that this effect was close to 100%. This contrasts with the in vivo study (Stoeckli and Landmesser, 1995), in which we never observed >50% of the commissural axons failing to enter the floor plate and making an erroneous turn on the ipsilateral side of the spinal cord. Although the commissural axons did not grow into the floor-plate explants in the presence of anti-axonin-1, anti-NrCAM, or soluble axonin-1, the chemotropic effect of the floor plate was not altered (Table 1); >80% of the axons still showed directional growth toward the floor-plate explant.

### Table 1. Proportion of Commissural Neurites Extending toward the Floor-Plate Explant

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Neurites toward Floor Plate (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commissural explants only</td>
<td>24.9 ± 2.6 (n = 7)</td>
</tr>
<tr>
<td>Commissural explants with explants other than floor plate</td>
<td>26.9 ± 4.9 (n = 7)</td>
</tr>
<tr>
<td>Commissural explants with floor plate explants in absence of antibodies</td>
<td>83.7 ± 2.1 (n = 22)</td>
</tr>
<tr>
<td>Commissural and floor plate explants in presence of anti-NgCAM antibodies</td>
<td>73.9 ± 7.3 (n = 4)</td>
</tr>
<tr>
<td>Commissural and floor plate explants in presence of anti-axonin-1 or anti-NrCAM antibodies</td>
<td>87.3 ± 3.1 (n = 5)</td>
</tr>
<tr>
<td>Commissural and floor plate explants in presence of soluble axonin-1</td>
<td>88.0 ± 2.7 (n = 6)</td>
</tr>
</tbody>
</table>

*In control wells that did not contain floor-plate explants, one quadrant was chosen randomly. The percentage of neurites in this quadrant was calculated as a proportion of all neurites.*
Figure 2. Perturbation of Axonin-1 and NcCAM Interactions Prevent Commissural Growth Cones from Entering the Floor Plate

In control cultures of commissural (C) and floor-plate explants (FP), axons were attracted toward the floor-plate explants and entered them (a and e). If anti-axonin-1 (b and f) or anti-NcCAM antibodies (c and g) were added to the culture medium, commissural axons no longer entered the floor-plate explants, but were found to stall and pile up, or to grow around the explants. The same effect was seen if soluble axonin-1 (d) was added to the culture medium. Anti-NgCAM antibodies (h) did not prevent the commissural growth cones from entering the floor-plate explants. The asterisks in (f) and (g) mark the floor-plate border. Floor-plate cells fill the entire view field in (e) and (h). In (g), two floor-plate explants are in close proximity (one from the upper right corner, the other from the lower left corner of the micrograph). Note that the commissural axons navigate around the floor-plate explants without entering either one of them. Scale bar: 100 μm in (a)–(d), 20 μm in (e)–(h).

had lamellipodia, but mostly they tended to be more filopodial in shape (Figure 3b). This is consistent with reports of commissural growth cone morphology in vivo by Yaginuma et al. (1991), but differs from observations made in the rat spinal cord (Bovolenta and Dodd, 1990), where growth cones extending toward the floor plate had only few or no filopodia. We found in our cultures that in addition to the filopodia emerging from the growth cone body, the axons sent out long filopodial extensions along their entire shafts. Individual axons from the same

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>% entering</th>
<th>% stalling</th>
<th>% being deflected</th>
<th>% avoidance (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38</td>
<td>96.1 ± 0.7</td>
<td>2.5 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>Anti-NgCAM</td>
<td>6</td>
<td>82.9 ± 9.8</td>
<td>5.9 ± 3.3</td>
<td>11.2 ± 7.2</td>
<td>17.1 ± 9.8</td>
</tr>
<tr>
<td>Anti-NcCAM</td>
<td>10</td>
<td>3.3 ± 1.7</td>
<td>58.5 ± 6.3</td>
<td>38.2 ± 5.9</td>
<td>96.7 ± 1.7</td>
</tr>
<tr>
<td>Anti-axonin-1</td>
<td>49</td>
<td>5.8 ± 1.0</td>
<td>64.5 ± 3.0</td>
<td>29.7 ± 2.8</td>
<td>94.2 ± 1.0</td>
</tr>
<tr>
<td>Axonin-1</td>
<td>9</td>
<td>6.3 ± 2.3</td>
<td>75.1 ± 2.9</td>
<td>18.6 ± 4.1</td>
<td>93.7 ± 2.3</td>
</tr>
</tbody>
</table>

The table summarizes the percentage of axons readily entering the floor-plate explants or avoiding them upon contact. The percentage of axons that either stalled upon floor-plate contact or that deflected and grew around the explants are given separately. The last column, % of total avoidance, combines both of these values. Percentages are given with standard errors of the means. n indicates the number of explants included in the quantification.
The Floor Plate Is Inhibitory in Absence of Axonin-1/NrCAM

Figure 4. In Control Cultures, Commissural Axons Rapidly Enter the Floor-Plate Explants upon Contact

Within minutes of initial contact between a filopodium and a floor-plate cell, the growth cone grew onto the floor-plate cell surface. In the sequence shown here, the initial contact (at time 0') was stabilized within 4–5 minutes. After 22', the growth cone was growing on the floor-plate cell surface. Numbers in the upper left corner of each frame represent minutes after first contact (negative numbers represent time before contact). The arrowhead in the frame labeled 0' marks the contact site of the filopodium with the floor-plate cell in the upper right corner. The arrowhead in the frame taken after 38' marks the axon extending across a floor-plate cell. The growth cones cannot be visualized clearly with video resolution once they have entered the floor plate. The view field was left unchanged throughout the observation period.

Figure 3. Growth Cones Stall and Pile Up at the Ipsilateral Border under Conditions, Which Were Found to Induce Pathfinding Errors In Vivo

In embryos injected in ovo with purified soluble axonin-1, anti-axonin-1, or anti-NrCAM, respectively, axons often were found to stall and pile up at the ipsilateral floor-plate border or to turn erroneously on the ipsilateral border. The open-book preparation of an embryo that was repeatedly injected with axonin-1 and sacrificed at stage 25 shows numerous growth cones lined up at the ipsilateral floor-plate border (a). This is not observed in control embryos. The width of the floor plate is indicated by the two vertical lines at the top of the panel. In the focal plane shown, no ipsilateral turns are found, which makes it easier to see the stalled growth cones piled up at the ipsilateral border (arrows).

b) Commissural growth cones cultured on a laminin substratum tend to be filopodial in shape. In addition to the long filopodia extending from the growth cones, commissural axons produced long lateral filopodia along their entire shaft. Scale bar: 50 µm.

Anti-Axonin-1 Antibodies Induce a Collapse of Commissural Growth Cones upon Filopodial Contact with the Floor-Plate Explant

If anti-axonin-1 antibodies were added to the cocultures, commissural axons were prevented from entering the floor-plate explants. Time-lapse recording revealed that growth cones collapsed after their filopodia made contact with the floor plate. In contrast to control cultures, filopodial contacts were never stabilized in the presence of anti-axonin-1 antibodies. Instead, as shown in Figure 5 (0'–18'), filopodia were retracted rapidly after touching
Figure 5. Anti-Axonin-1 Antibodies Induce the Collapse of Commissural Growth Cones upon Contact with the Floor Plate

In contrast to control cultures, initial filopodial contacts were not stabilized in the presence of anti-axonin-1 antibodies. Therefore, the time between first filopodial contact (0') and the time when the growth-cone body (63') was in contact with the floor plate was much longer. For instance, the filopodium touching the floor-plate cell at 0' was retracted at 10', and another filopodium transiently made contact with the floor-plate cell at 18'. Such a contact was eventually maintained for a few minutes, resulting in a partial (39') or full collapse of the growth cone. A full collapse of the same growth cone is shown at 154', followed by the retraction of the axons at 162'.

Note that the last frame is shifted with respect to the others, the diamond marking the same position in the last two frames. Before the growth cone collapsed fully and the neurite retracted as a result of a more intense floor plate contact, several partial collapses without neurite retraction were observed. One example is shown in frames 36' and 39'. After partial collapse, the growth cone recovered within a few minutes (41.5') and resumed its exploratory function. Recovery from a full collapse usually took longer. Arrowheads mark filopodial contacts with the floor plate, whereas arrows point to collapsed growth cones.

Anti-NrCAM Antibodies Prevent Commissural Growth Cones from Entering the Floor-Plate Explants, but Do Not Induce Their Collapse

Although the addition of anti-NrCAM antibodies also prevented the commissural axons from entering the floor plate, the detailed cellular mechanism differed from that seen in the presence of anti-axonin-1 antibodies. In general, growth cones were found to stall upon filopodial contact with floor-plate cells. The growth cones constantly retracted old and extended new filopodia; however, collapse of the growth cones was not observed. Instead, the growth cones continued to actively explore the floor-plate cells with several filopodia, but failed to grow into the floor-plate explant (Figure 6). They appeared to walk along the floor-plate border, but they never actually entered the explant. Thus, although the end result was the same, namely, failure to enter the floor plate, interfering with axonin-1 and NrCAM produced differences in the detailed behavior of the growth cones upon floor-plate contact (see Discussion).
The Floor Plate Is Inhibitory in Absence of Axonin-1/NrCAM

Figure 6. The Presence of Anti-NrCAM Antibodies Did Not Induce the Collapse of Commissural Growth Cones

In the presence of anti-NrCAM antibodies, no stable filopodial contacts were established. The growth cones remained actively searching the floor-plate surface with their filopodia, but never succeeded in growing onto the floor-plate cells. In contrast to the addition of anti-axonin-1 antibodies, anti-NrCAM antibodies never induced the collapse of commissural growth cones. Arrowheads mark filopodial contacts with the floor-plate cells. The field of view was constant during the observation period of 11 hr.

Anti-NgCAM Antibodies Have No Effect on
Interactions between Commissural Growth
Cones and Floor-Plate Explants

Although the injection of anti-NgCAM into the spinal cord of chicken embryos in ovo caused the commissural axons to grow in a defasciculated manner (Stoeckli and Landmesser, 1995), it did not result in pathfinding errors. Therefore, we concluded that NgCAM expressed on the commissural growth cones was not crucial for the entry of commissural growth cones into the floor plate. In accordance with these results, the addition of anti-NgCAM antibodies to the culture medium did not prevent commissural growth cones from entering floor-plate explants. After 18–22 hr in culture, the commissural axons growing in the presence of anti-NgCAM were indistinguishable from control cultures. This was also
confirmed the hypothesis that interactions mediated by axonin-1 and NrCAM were required for the normal behavior of growth cones upon contacting the floor plate. In control cultures, commissural axons were strongly attracted toward the floor-plate explants, and readily entered them. When axonin-1 or NrCAM interactions were perturbed by the addition of soluble axonin-1 or antibodies against axonin-1 or NrCAM, respectively, commissural axons did not enter floor-plate explants, although they were still attracted toward them (Table 1). In these cases, they either stalled on the laminin substratum and piled up at the border of the floor-plate explant, or avoided the explants by growing around them.

These observations lend support to one of the hypotheses that we had previously proposed for the in vivo guidance of commissural growth cones (Stoeckli and Landmesser, 1995). We had suggested that, due to interactions mediated by axonin-1 and NrCAM, the floor plate would normally be more attractive to commissural growth cones than adjacent spinal cord regions. Growth cones would thus enter the floor plate and cross the midline, but upon reaching the contralateral side of the floor plate, they would turn to maintain contact with the floor plate, rather than switch onto less attractive tissue. However, when the function of either axonin-1 or NrCAM was blocked, the floor plate would become more inhibitory than adjacent cord tissue; axons would thus fail to cross the midline and would turn prematurely on the ipsilateral side of the cord. Our observations in culture clearly show that interfering with either axonin-1 or NrCAM causes the floor plate to become inhibitory for commissural growth cones. In culture, growth cones were found to take alternative paths around the explants, which would be analogous to our in vivo observations of commissural growth cones turning into the longitudinal axis along the ipsilateral floor-plate border, rather than crossing the midline.

The floor plate has been shown to be the source of inhibitory cues in other species as well. For example, in zebrafish, the absence of a floor plate allows axons that are normally constrained to the ipsilateral side to cross the midline (Hatta, 1992). In Drosophila, several genes have been identified that affect guidance at the midline. Furthermore, the behavior of axons in these mutants suggests that guidance at the midline involves inhibitory as well as attractive signals (Seeger et al., 1993). In the absence of the commissureless (comm) gene product, a transmembrane protein produced by midline glial cells, commissural axons are unable to cross the midline, even though they are attracted to it. In the comm mutants, the midline cells appear to be inhibitory (Tear et al., 1996). The existence of midline-inhibitory signals can also be inferred by the behavior of axons when the roundabout (robo) gene product is mutated. In this case, axons that would normally be repelled by the midline are now able to cross (Seeger et al., 1993). Thus, robo would appear to encode either an inhibitory midline molecule or its receptor. Normally, this inhibitory signal can be overcome by a positive signal derived from comm.

Table 3. Proportion of Pathfinding Errors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>&lt;1%</th>
<th>1–20%</th>
<th>&gt;20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble axonin-1</td>
<td>0/8</td>
<td>2/8</td>
<td>6/8</td>
</tr>
<tr>
<td>Anti-axonin-1</td>
<td>0/8</td>
<td>4/8</td>
<td>4/8</td>
</tr>
<tr>
<td>Anti-NrCAM</td>
<td>1/7</td>
<td>3/7</td>
<td>3/7</td>
</tr>
<tr>
<td>Sol. ax-1/anti-NrCAM</td>
<td>0/5</td>
<td>2/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Anti-ax-1/anti-NrCAM</td>
<td>0/7</td>
<td>5/7</td>
<td>2/7</td>
</tr>
</tbody>
</table>

Simultaneous Injections of Either Anti-NrCAM and Anti-Axonin-1 or Anti-NrCAM and Soluble Axonin-1 Do Not Produce More In Vivo Pathfinding Errors than Soluble Axonin-1 Alone

Axonin-1 and NrCAM were shown to interact with each other directly when coupled to Covasphere beads (Suter et al., 1995). A direct heterophilic interaction between axonin-1 and NrCAM is consistent with the results of our in vivo study (Stoeckli and Landmesser, 1995). However, interactions between axonin-1 on the growth cones and additional molecules on floor-plate cells could occur, as well as interactions of NrCAM on the floor plate with other molecules on commissural growth cones. In the previous in vivo study, interfering with a single molecule, either NrCAM or axonin-1, never caused >50% of the axons to fail to cross the floor plate. In an attempt to test for the involvement of additional heterophilic binding partners for axonin-1 and NrCAM in this system, we carried out a new series of in vivo experiments. In addition to the injection of either soluble axonin-1, anti-axonin-1, or anti-NrCAM, we injected a combination of anti-NrCAM antibodies with soluble axonin-1 or anti-axonin-1 antibodies, respectively. If NrCAM was interacting predominately with a different binding partner on the growth cone, or axonin-1 with another binding partner on the floor plate, blocking the interactions mediated by both NrCAM and axonin-1 would be expected to result in increased pathfinding errors. However, the ‘double injections’ did not result in an obvious increase in the number of axons committing pathfinding errors compared to the injection of soluble axonin-1 alone. In Table 3, embryos are divided into three classes according to the level of pathfinding errors found in open-book preparations of their spinal cords (see Experimental Procedures and Stoeckli and Landmesser, 1995, for additional details).

Discussion

The Ability of Commissural Growth Cones to Enter the Floor Plate Critically Depends on Axonin-1 and NrCAM

To test the hypothesis that axonin-1 and NrCAM are important in mediating interactions between commissural growth cones and the floor plate (Stoeckli and Landmesser, 1995), we studied such encounters in a two-dimensional culture system. These observations
Using confocal microscopy, Myers and Bastiani (1993) described the behavior of the Q1 growth cone at the grasshopper midline in vivo. They observed stalling and retraction of this particular growth cone upon contact with the midline in about 50% of the cases, indicating that the midline contained an inhibitory cue. However, in the other cases, the growth cones did not retract, but actually accelerated. This behavior was explained by the ability of the Q1 growth cone to contact the Q1 growth cone from the opposite side, and thereby the repulsive signal from the midline was overcome. Although differing in detail from our observations in the chick, this study also clearly shows that the pathfinding of commissural growth cones across the midline depends on a balance of positive and negative signals. In the chick, both the positive and negative signals are derived from interactions between molecules on commissural growth cones and the floor-plate surface, while in the grasshopper, the Q1 growth cone requires a positive cue from its contralateral homolog to override the negative cues from the midline.

In our cocultures, the inhibitory effect of the floor plate in the absence of axonin-1 and NrCAM interactions was very strong (close to 100%). The same polyclonal antibodies and purified soluble axonin-1, when used in our in vivo experiments, never caused more than about 50% of the commissural axons to commit pathfinding errors, namely, failing to cross the floor plate and turning erroneously along the ipsilateral floor-plate border (Stoeckli and Landmesser, 1995; present study). There are several possible explanations for this discrepancy. First, although the injections in ovo were repeated every 8 hr, fluctuations in antibody concentrations most likely occurred in the spinal cord owing to diffusion of the injected protein. Therefore, it is plausible that the concentrations were not sufficient at all times during the development of the commissural pathway to prevent all growth cones from interacting with the floor plate. In contrast, in vitro, a constant concentration of antibodies was present in the medium throughout the entire culture period. Second, we cannot exclude the possibility that only a subpopulation of commissural neurons is sensitive to perturbations of axonin-1 and NrCAM interactions. Although we consider it very unlikely (see Experimental Procedures for a detailed explanation), it is formally possible that only these commissural neurons extend axons in vitro. Likewise, it is possible that the in vivo perturbations actually induced pathfinding errors in all neurons belonging to this class, which would represent approximately 50% of commissural neurons. The remaining 50% of the axons, which did not turn ipsilaterally but managed to cross the midline and turn along the contralateral floor-plate border, would do so by using guidance cues other than axonin-1 and NrCAM. While this explanation is formally possible, there is no evidence for subpopulations of commissural axons that would be differentially sensitive to axonin-1–NrCAM interactions. For example, all axons express axonin-1 and NgCAM.

Another possibility is that floor-plate cells and/or commissural neurons in culture do not express their full set of recognition molecules/guidance cues. They might then be more susceptible to perturbation of axonin-1 and NrCAM interactions, since they cannot fall back on a redundant set of guidance cues. Finally, it should be noted that the laminin substratum used for our cocultures is not the usual substratum for most commissural neurites in vivo, even though the first commissural axons extend along the spinal cord margin in contact with the basal lamina containing laminin (Shiga and Oppenheim, 1991). It is possible that the use of laminin as a substratum altered the expression of some CAMs on the commissural growth cones. For instance, it has been shown that the expression of NrCAM on retinal ganglion cell axons is substratum dependent (de la Rosa et al., 1990), and the substratum has also been shown to alter the distribution of CAMs on the growth cones of cultured DRG neurons (Stoeckli et al., 1996). When grown on either an NgCAM or an axonin-1 substratum, there was a rapid redistribution of axonin-1 and NgCAM to the substratum-facing membrane of the growth cone. This did not occur on a laminin substratum (Stoeckli et al., 1996). Therefore, the use of a laminin substratum in vitro, as opposed to the much more complex ‘substratum’ provided to commissural axons in vivo, could result in differences in their behavior.

With the tools currently available, we cannot identify which of the possibilities noted above actually accounts for the quantitative differences observed in commissural axon behavior following perturbation of growth cone–floor plate interactions in vivo and in vitro. However, the in vitro studies clearly support our hypothesis that in the absence of axonin-1 or NrCAM interactions, the floor-plate cells become less attractive, or even inhibitory, for commissural growth cones. As a consequence, pathfinding errors would occur in vivo because the growth cones would stall at the ipsilateral floor-plate border, where they would respond prematurely to guidance cues directing them to turn into the longitudinal axis.

**Antibodies Against Axonin-1 and NrCAM Perturb the Growth Cone–Floor Plate Interactions by Different Mechanisms**

Interestingly, we observed that commissural growth cones behaved differently upon floor-plate contact in the presence of anti-axonin-1 as compared to anti-NrCAM. This would not be expected if the only positive signal causing growth cones to enter the floor plate was a direct transinteraction between axonin-1 on the growth cone and NrCAM on the floor plate, since either anti-axonin-1 or anti-NrCAM would perturb this equally. Taken in isolation, this finding might thus suggest that axonin-1 and NrCAM do not interact directly during commissural axon growth across the floor plate. However, several pieces of evidence suggest a direct axonin-1–NrCAM interaction. First, axonin-1 and NrCAM have been shown to bind to each other in vitro when coupled to polystyrene beads (Suter et al., 1995). Second, the injection of soluble axonin-1 into the chicken spinal cord in vivo induced the highest rate of pathfinding errors, presumably by binding to floor-plate NrCAM and therefore by competing for binding sites with growth-cone proteins. Since the concomitant injection of soluble axonin-1 and anti-NrCAM antibodies did not increase the...
number of fibers committing pathfinding errors above the effect of axonin-1 alone, both soluble axonin-1 and the injected antibodies must be affecting the same molecular interactions: interactions between growth-cone axonin-1 and floor-plate NrCAM.

So how can the difference in growth-cone behavior in the presence of anti-axonin-1 versus anti-NrCAM antibodies be explained? Several scenarios are possible. The basic feature of growth-cone guidance across the floor plate in the chick as well as in Drosophila (Seeger et al., 1993; Tear et al., 1996) seems to be a balance between positive (attractive) and negative (repellent) signals. In the chick, axonin-1 and NrCAM interactions contribute to the positive signals. If these interactions are perturbed, the collapse-inducing, negative signal is unmasked and dominates the behavior of the growth cones upon floor-plate contact. We would further postulate that in the presence of anti-axonin-1, the repulsive, collapse-inducing effect of floor plate would be dominant, whereas in the presence of anti-NrCAM antibodies the repulsive effect would be milder, preventing the commissural growth cones from entering the floor plate, but not strong enough to induce their collapse. Since the perturbation of axonin-1 interactions had a stronger effect on growth cones than the perturbation of NrCAM interactions, additional binding partners are most likely contributing to the resulting signal.

A simplified working model to explain pathfinding of commissural axons across the floor plate is depicted in Figure 7. In a control situation, the floor plate is an attractive substratum for commissural axons. Positive signals resulting from growth-cone axonin-1 interacting with NrCAM and additional unidentified floor-plate protein(s) cause the axons to grow into the floor plate. These positive signals would mask a latent collapse-inducing (negative) activity present on the floor-plate cells. In the presence of anti-axonin-1, the collapse-inducing activity would come to dominate, since both the positive signals overriding this negative signal would be eliminated. In contrast, anti-NrCAM would only block direct axonin-1–NrCAM interactions, but would not interfere with the interaction of axonin-1 with the postulated additional positive guidance molecule(s) on the floor plate. Thus, the collapse-inducing activity of the floor plate would partially be overcome, and while growth cones would not find the floor plate sufficiently attractive to enter, they would not be induced to collapse.

Conclusion

The guidance of commissural axons across the midline depends critically on the appropriate interaction of their growth cones with the floor plate. The interaction of axonin-1 on commissural growth cones with NrCAM and probably at least one additional heterophilic binding partner on the floor plate normally overrides a collapse-inducing signal from the floor plate. A perturbation of the interactions of axonin-1 or NrCAM shifts the balance of positive and negative cues and thus prevents the commissural growth cones from establishing stable contacts with the floor plate. Therefore, the proper balance between positive and negative signals provided by the midline, as hypothesized for accurate pathfinding in the Drosophila nervous system (Seeger et al., 1993; Tear et al., 1996), also seems to be required for the guidance of commissural axons across the floor plate in chicken embryos. Further in vivo and in vitro studies will be required to identify the repellent forces as well as the additional postulated binding partner for axonin-1.

Experimental Procedures

Cocultures of Commissural Neurons and Floor-Plate Explants

Cultures were grown on 8-well LabTek glass slides (Nunc, Naperville, IL) or on glass coverslips if they were used for video microscopy. The glass was coated with 0.2 mg/ml poly-ornithine (Sigma, St. Louis) and 12.5 μg/ml laminin (GIBCO, Gaithersburg, MD). We used a chemically defined, serum-free medium consisting of DME/F12 (Sigma) supplemented with 5 mg/ml Albumax I (GIBCO), 100 μg/ml transferrin, 10 μg/ml insulin, 20 ng/ml triiodothyronine, 40 nM progesterone, 200 ng/ml corticosterone, 200 μM putrescine, 60 nM sodium selenite, 1 mM sodium pyruvate, 2 mM glutamine, and Pen/Strep (all from Sigma).

Floor-plate explants were obtained from whole-mount (open-book) preparations of embryos. We routinely used spinal cords from stages 25½–26½. The whole mounts were prepared as described earlier (Stoeckli and Landmesser, 1995). The floor-plate explants were collected with a micropipette, which was pressed against the center of the floor plate. By applying slight suction, pieces of floor-plate tissue were cut out. With this method, we minimized contamination of the floor-plate explants with adjacent tissue. We were particularly concerned about contamination with motoneurons or PL cells, a population of neurons that extend axons along the floor plate (Yaginuma et al., 1990). It was suggested that commissural axons could be guided into the longitudinal axis by fasciculation with the PL neurites. We therefore discarded all floor-plate explants that contained neurite-producing cells. Unfortunately, there are no unique markers that would unequivocally prove that our explants consisted only of floor-plate cells. However, consistent with the expression pattern in vivo (Shiga et al., 1990; Shiga and Oppenheim, 1991; Stoeckli and Landmesser, 1995), we never found expression of axonin-1 or NgCAM in our floor-plate explants. Both proteins have been described on PL cells (Shiga and Oppenheim, 1991).

To identify the explants in the cocultures, we labeled floor-plate explants with 5- (and 6-) carboxy-2', 7'-dichlorofluorescein diacetate succinimidy l ester (CFSE, C-1165; Molecular Probes, Eugene, OR). For this purpose, spinal cords were incubated for 30 min in a 20 μM solution of CFSE in PBS before floor-plate explants were prepared. The explants were transferred into the LabTek wells or into the culture dishes containing the cover slips. After 2 to 3 hr, the explants of commissural neurons were positioned 150–400 μm away from the floor-plate explants. The explants containing the commissural neurons were punched out from transverse vibratome sections of the lumbosacral level of the spinal cord using the same method as described for the floor-plate explants.

The commissural explants were taken from embryos between stages 20 and 21. The neurons were dissected before they had established contact with the floor plate in vivo, and thus the axons grown in culture were for the most part the first axons initiated by the commissural neurons, and not regenerating axons. Furthermore, by using explants from stage 20–21 spinal cords, we avoided ipsilaterally projecting neurons in our explants, which could have interfered with the observation of commissural axons. At the lumbosacral level of the embryonic chick spinal cord, associational neurons do not develop before stage 26. In addition, in none of the hundreds of injections of Dil into the regions of control embryos (Stoeckli and Landmesser, 1995; present study), from which the ‘commissural’ explants were removed, did we observe any ipsilaterally projecting Dil-labeled fibers. Finally, the fact that >96% of the ‘commissural’ axons in control cultures were attracted at a distance, presumably in response to floor plate–generated chemoattractants, and subsequently entered the floor plate, strongly suggests that these would be commissural neurons in vivo. The fact that following the perturbation of axonin-1 or NrCAM interactions the same high proportion of
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axons were attracted up to the floor-plate explant, but then failed to enter, strongly suggests that these are also 'commissural' and not 'associational' neurons that normally might be repelled by the floor plate.

Where indicated, soluble axonin-1 (300–400 μg/ml), Fab or IgG from goat anti-axonin-1 antiserum (250 or 500 μg/ml), rabbit anti-NgCAM (250 μg/ml), or rabbit anti-NrCAM (250 μg/ml) were added to the culture medium 30 min after plating the commissural explants. The addition of antibodies to the culture medium did not change the commissural axons' attraction toward the floor plate or their morphology.

Staining of Cultured Explants
The cultures were fixed after 18–22 hr in 2% paraformaldehyde, which was added directly to the culture medium as a concentrated solution prepared in 50 mM sodium cacodylate (pH 7.0). Fixation was at 37°C for 1 hr. The cultures were rinsed with PBS, incubated in 10% fetal bovine serum (FBS) in PBS to block unspecific antibody binding, and then incubated over night at 4°C in the primary antibody solution. Antibodies were used as hybridoma supernatants or purified IgG diluted in 10% FBS–PBS. After rinsing and blocking again, the cultures were incubated with the secondary antibodies diluted in 10% PBS at 4°C for 3–4 hr. The cells were rinsed with PBS and mounted in 80% glycerol–PBS containing 0.1% p-phenylenediamine (Sigma). Antibodies used were: goat anti-axonin-1 (Stoeckli et al., 1991), rabbit anti-NgCAM (Landmesser et al., 1988), rabbit anti-NrCAM (Stoeckli and Landmesser, 1995), and the anti-neurofilament antibodies RMO270 (Zymed, South San Francisco) and 1E9 (L. L.). The secondary antibodies were purchased from Sigma: rabbit anti-goat IgG-TRITC, goat anti-rabbit IgG-FITC, and goat anti-mouse IgG-TRITC, goat anti-mouse IgM-FITC. Goat anti-mouse IgG-TRITC, goat anti-mouse IgM-TRITC, and rabbit anti-mouse IgG-FITC were from Zymed.

Quantification of Neurite Growth in the Presence and Absence of Floor-Plate Explants
To test whether the two-dimensional culture system was able to mimic the chemotactic effect of the floor plate on the commissural axons, which was described earlier by M. Tessier-Lavigne et al. (1988) for the three-dimensional collagen gel system, we calculated the proportion of commissural axons extending toward the floor-plate explants. Micrographs of these explants were overlayed with two perpendicular lines creating four quadrants. The number of neurites in the quadrant facing the floor-plate explant was counted as a proportion of the neurites found in all four quadrants. These values in the absence and presence of antibodies or soluble
axonin-1 are given in Table 1. As a control, commissural explants in the absence of floor-plate explants, or cocultured with spinal cord explants other than floor plate, were included in the quantitation. The number of neurites extending from the explants depended primarily on the size of the explant. The average number of axons per explant in control cultures was 38 ±16. The average number of axons extending from an explant in the presence of antibodies was 34 ±13 and 43 ±16 in the presence of soluble axonin-1, respectively.

Quantification of Neurite Growth into the Floor Plate in the Presence and Absence of Antibodies and Soluble Axonin-1

For the quantification of axon behavior, we counted the number of axons entering the floor-plate explant, or stalling at the floor-plate explant border, or the number of axons growing around the floor-plate explant (being deflected). These numbers are given as the percentage of all axons leaving the commissural explant in Table 2. As noted above, the number of axons varied with the size of the explant, but was not altered by the addition of antibodies or soluble axonin-1. The presence of anti-axonin-1 or anti-NrCAM did, however, have a strong effect on the proportion of axons actually entering the floor-plate explants.

Time-Lapse Video Microscopy

Cultures used for time-lapse video microscopy were grown in the tissue-culture incubator for 6–8 hr. At this time, when the first growth cones were extending from the commissural explants, an appropriate explant pair was chosen and the culture dish was transferred to a heated microscope stage. To prevent evaporation of the medium, the culture was covered with a thin layer of mineral oil. The growth cones were videotaped using a 20× objective on a Nikon Diaphot microscope. We captured one frame every 6 s using a Toshiba Time-Lapse Video Recorder attached to an Argus-10 from Hamamatsu (Hamamatsu Photonics, Bridgewater, NJ). After 24–30 hr, the taping was stopped because by this time nonneuronal cells usually had migrated from the explants far enough to fill the view field or interfere with the growth cone–floor plate contact.

In Vivo Injections of Axonin-1, Anti-Axonin-1, and Anti-NrCAM Antibodies

The injections of soluble axonin-1 and antibodies against axonin-1 or NrCAM were injected into the embryonic chick spinal cord in ovo, as described in Stoeckli and Landmesser (1995). However, in addition to the separate injection of either the purified protein axonin-1 or antibodies against axonin-1 or NrCAM, respectively, we mixed axonin-1 and anti-NrCAM or the two types of antibodies before injection to test for an additive effect of axonin-1 and NrCAM in the pathfinding of commissural neurites. Embryos were injected 4 times every 8–9 hr before they were sacrificed at stage 25–25½. Whole-mount preparations in which commissural neurites had been labeled by FastDiI (1,1-dilinoleyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate obtained from Molecular Probes) injected into the area of their cell bodies were analyzed for pathfinding errors as described earlier (Stoeckli and Landmesser, 1995). Embryos were divided into three classes according to the proportions of fibers estimated to have failed crossing the floor plate: <1%, 1%–20%, and >20% (see Stoeckli and Landmesser, 1995, for more details).

Acknowledgments

We thank Dora Fitzli, Stefan Kunz, and Louise Milner for critically reading the manuscript. This work was supported by NIH grant NS 19640.

Received September 30, 1996; revised January 10, 1997.

References


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