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Nectin-like molecules/SynCAMs are required for post-crossing commissural axon guidance

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SUMMARY

The Necl/SynCAM subgroup of immunoglobulin superfamily cell adhesion molecules has been implicated in late stages of neural circuit formation. They were shown to be sufficient for synaptogenesis by their trans-synaptic interactions. Additionally, they are involved in myelination, both in the central and the peripheral nervous system, by mediating adhesion between glia cells and axons. Here, we show that Necls/SynCAMs are also required for early stages of neural circuit formation. We demonstrate a role for Necls/SynCAMs in post-crossing commissural axon guidance in the developing spinal cord in vivo. Necl3/SynCAM2, the family member that has not been characterized functionally so far, plays a crucial role in this process. It is expressed by floorplate cells and interacts with Necls/SynCAMs expressed by commissural axons to mediate a turning response in post-crossing commissural axons.

KEY WORDS: Axon guidance, In ovo RNAi, Nectin-like, Nectin, Midline crossing, Chicken embryo, Spinal cord development

INTRODUCTION

The establishment of functional circuits in the developing nervous system relies on the correct pathfinding of axons to their target cells, where they form synapses. On their journey, axons are guided by attractant or repellent guidance cues that either act locally or over long distances. Long-range guidance cues are secreted and to some extent diffusible, whereas short-range guidance cues act in a contact-dependent manner (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). Intermediate targets are important sources for axon guidance cues (Kaprielian et al., 2001; Stoeckli and Landmesser, 1998). The floorplate, the ventral midline of the spinal cord, represents a well-characterized intermediate target for commissural axons. In vertebrates, axons of dorsal commissural neurons are initially drawn to the ventral midline by attractive forces represented by netrin 1 and sonic hedgehog (Shh), which are secreted from the floorplate (Charron et al., 2003; Kennedy et al., 1994). Cell adhesion molecules (CAMs) of the immunoglobulin (Ig)-superfamily provide vital contacts between commissural neurons and the floorplate (Stoeckli and Landmesser, 1998). The interaction between axonin 1/TAG1 expressed on the surface of commissural growth cones and NrCAM expressed by floorplate cells is required for axons to cross the midline. Commissural axons leave the floorplate on the contralateral side owing to a change in responsiveness to repulsive cues, mainly because of the expression of Robo receptors that recognize midline-associated repellents, the Slit proteins (Slits) (Dickson, 2002; Giger and Kolodkin, 2001). Thus, midline crossing by commissural axons is regulated by a balance between positive axonin 1/NrCAM and negative Robo/Slit signals.

After midline crossing, dorsal commissural axons turn rostrally into the longitudinal axis and extend along the floorplate border (Stoeckli, 2006). In mouse, this is mediated by an attractive rostral^{high} to caudal^{low} Wnt4 gradient in the floorplate (Lyuksyutova et al., 2003). By contrast, in chick, Shh expressed in a rostral^{low} to caudal^{high} gradient in floorplate cells has been identified as a repellent for post-crossing commissural axons in a subtractive hybridization screen (Bourikas et al., 2005). The same screen produced nectin-like protein 3 (Necl3)/SynCAM2 as a candidate gene for guidance of post-crossing commissural axons.

A role for Necls/SynCAMs in axon guidance was unexpected, as these molecules were found to play a role in late aspects of neural circuit formation. Necls/SynCAMs were initially identified as synapse-inducing molecules (Biederer et al., 2002). However, Necls/SynCAMs were also described based on their structural similarity to nectins, a family of IgCAMs involved in the formation of adherens and tight junctions (Takai et al., 2003). In contrast to nectins, Necls are not involved in junction formation (Takai et al., 2008a; Takai et al., 2008b). Furthermore, some Necl family members were identified as tumor suppressor genes, called Tslc (tumor suppressor in lung cancer) and Tsll (Tslc-like) (reviewed in Takai et al., 2003). More recently, Necl1/SynCAM3 and Necl4/SynCAM4 were shown to be required for myelination in the peripheral and the central nervous system (Maurel et al., 2007; Park et al., 2008; Spiegel et al., 2007). Very little is known about the role of Necl3/SynCAM2. A recent study localized it to neurons, in particular myelinated axons, but no functional studies have been reported (Pellissier et al., 2007).

Necls/SynCAMs are Ca^{2+}-independent IgCAMs that contain three Ig-loops in the extracellular domain, followed by a transmembrane domain and a short cytoplasmic tail (Biederer, 2006; Takai et al., 2008b). Necls/SynCAMs and nectins perform their function by forming homo- or heterophilic interactions within or between these two families. Nectins have been shown to be linked to the cytoskeleton via the actin-binding protein afadin (Takai et al., 2008a). By contrast, Necls/SynCAMs lack the ability to bind to afadin but bind to MAGUK family members instead (Biederer et al., 2002; Kakunaga et al., 2005; Shingai et al., 2003). Nectins are involved in the formation of the nervous system by mediating cell adhesion (Mizoguchi et al., 2002; Okabe et al., 2004; Takai et al., 2008b). In vitro experiments suggested the importance of nectin 1-

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nectin 3 interactions in axonal navigation of commissural axons in the rodent hindbrain (Okabe et al., 2004). Nectin 1 and nectin 3 mutant mice exhibit a virtually identical phenotype, microphthalmia, owing to a morphogenesis defect of the ciliary body (Inagaki et al., 2005), corroborating the vital interaction of nectin 1 with nectin 3.

All functions reported so far for Necls/SynCAMs affect late aspects of neural development. Here, we describe a function for Necls/SynCAMs in axonal pathfinding in the developing spinal cord in vivo providing compelling evidence that Necls/SynCAMs play a role in neural circuit development prior to synapse formation and myelination.

MATERIALS AND METHODS

In ovo RNAi

In ovo RNAi was performed as previously described (Bourikas et al., 2005; Pekarik et al., 2003). In brief, Hamburger and Hamilton stage 18-19 (HH18-19) (Hamburger and Hamilton, 1951) chicken embryos were electroporated (5 pulses of 25 V, 50 ms length, 1 second interpulse interval) with either 250 ng/μl dsRNA and 5 ng/μl β-actin-GFP as transfection control or an shRNA construct (details below). Depending on the position of the electrodes, it was possible to target one half of the spinal cord or either the floorplate or cells of the dorsal spinal cord (see Fig. 6 for electrode position for specific targeting). A plasmid encoding GFP under the control of the β-actin promoter was always co-electroporated to verify the specificity of targeting. In this case, only injection sites that were in the area with dsRNA or shRNA constructs targeted exclusively to the floorplate or commissural neurons, respectively, were included in the analysis. The following cDNA fragments were used for the generation of dsRNA: *Necl1* (3′UTR; ChEST478g10), *Necl2* (3′UTR; ChEST583g11), *Necl3* (two non-overlapping fragments of 3′UTR: ChEST96i3 and the original candidate clone identified in screen), nectin 1 (ORF; ChEST764m1), nectin 3 (ORF; ChEST294g23 bp167-406). For the downregulation of Necl2 and Necl3, 19 bp long shRNAs were expressed using the mouse U6 promoter: *Necl2* ACAGATAATGGTACATACC, *Necl3* AGGAACATATTTAACAAAC, control shRNA ATGCGTATAGTTCCGTGAA (random sequence; no similarity to any known chicken sequence). The loop sequence was TTCAAGAGA. The construct also contained a CMV-NLS-GFP-polyA cassette to visualize the transfected cells. Embryos were sacrificed at HH25-26 and spinal cords were prepared in the open-book configuration (Perrin and Stoeckli, 2000). Fast-DiI (5 mg/ml; Molecular Probes) was applied to dorsal commissural neurons. Labelled axons at the floorplate were documented by confocal microscopy (Leica SP2).

To demonstrate specificity and to quantify the downregulation of the targeted Necl, we co-injected and electroporated HH18-19 chicken embryos with a construct containing a destabilized GFP (Clontech) followed by the 3′UTR of *Necl3* (ChEST96i3; 500 ng/μl) or *Necl1* (ChEST478g10; 100 ng/μl), driven by the β-actin promoter, 10 ng/μl β-actin-*NLS-lacZ* [gift from Silvia Arber, University of Basel (Arber et al., 1999)], and 1 μg/μl dsRNA derived from either *Necl3* (3′UTR; ChEST96i3) or *Necl1* (3′UTR; ChEST478g10). As controls, 1 μg/μl dsRNA derived from *Necl2* (3′UTR; ChEST583g11) or *Necl3* (3′UTR; ChEST96i3) were used (see Fig. S1 in the supplementary material). Detection of *lacZ* activity was performed as described previously (Arber et al., 1999). From 5-10 sections per embryo, GFP intensity was divided by *lacZ* intensity. Intensities were measured using the analySIS Five Software from Olympus Soft Imaging System. For each condition, 3-6 embryos were analyzed.

Specificity and efficiency of the shRNA constructs were tested similarly. Myc-tagged *Necl2* or *Necl3* were co-electroporated with a construct containing a CMV-NLS-GFP cassette and a U6 promoter driving either shRNA control, shRNA *Necl2*, shRNA *Necl3* or no shRNA (mock). Myc expression intensities were divided by GFP intensity for 2-5 embryos per condition.

In situ hybridization and immunohistochemistry

For expression analysis, 20 μm cryosections were hybridized with digoxigenin-labeled probes as described previously (Mauti et al., 2006). Specific probes were prepared from the following cDNAs: *Necl1* (ChEST1028m10), *Necl2* (bp 369-746 of ORF), *Necl3* (ChEST96i3), nectin 1 (ChEST764m1), nectin 3 (bp 167-406 of ChEST294g23) and axonin 1/TAG1 (bp 1620-2298 of ORF) (Pekarik et al., 2003). Antibodies used in this study were: mouse anti-HNF3β (4C7), mouse anti-SC1/BEN (16.5H2), mouse anti-Myc (9E10; Developmental Studies Hybridoma Bank), rabbit anti-NrCAM (1:5000), rabbit anti-axonin 1 (1:1000), rabbit anti-6xHis (1:1000; 600-401-382; Rockland Immunochemicals), mouse anti-neurofilament (1:1500; 13-0700; RMO270, Invitrogen), rabbit anti-HA (1:1000; 600-401-384; Rockland Immunochemicals), FITC-conjugated goat anti-GFP (1:400; 600-102-215; Rockland Immunochemicals), donkey anti-mouse-Cy3 (1:250; 715-165-150; Jackson ImmunoResearch), goat anti-rabbit-Alexa488 (1:250; A11008; Molecular Probes).

Binding assay

The ectodomains of *Necl2*, *Necl3*, nectin 1, and nectin 3 were fused to the AP-myc-6xHis tag of the *pAPtag5* vector (GenHunter). Fusion proteins were produced and used for binding assays as described previously (Cheng and Flanagan, 2001; Flanagan and Cheng, 2000). Transient transfection of HEK293T cells with full-length HA-tagged human *NECL1* (BC033819), chick *Necl2* [bp 14-403 of ChEST861m1 combined with a cDNA fragment obtained by PCR on a λ ZAP library prepared from E14 chicken brains (Zuellig et al., 1992)], chick *Necl3* (obtained by PCR on the λ ZAP library mentioned above), mouse nectin 1 (NM_021424), mouse nectin 3 (BC125588) or the empty vector as a negative control was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). All cDNAs, except *Necl3*, were cloned into *pcDNA3.1-myc/hisA* (Invitrogen). For *Necl3*, we used a β-actin promoter because of its low expression level.

Choice assay

COS7 cells were transfected with *pcDNA3-MARCKS-GFP* (kind gift of Silvia Arber) or *pCAGGs-Necl3-myc-His* using Lipofectamine 2000. After 24 hours, cells were plated at a density of 30,000 cells per well on 8-well LabTek slides (Nunc) coated with polylysine (10 μg/ml; Sigma) and laminin (10 μg/ml; Invitrogen). Two to three hours later, commissural neurons dissected from HH25-26 embryos were added. Per well, 8750 cells were cultured on the COS7 cell layer in MEM with GlutaMAX-I (Invitrogen), supplemented with N3 (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; all from Sigma) supplemented with 1 mM sodium pyruvate and 4 mg/ml Albumax (Invitrogen) for 42 hours. After fixation in 4% paraformaldehyde for 60 minutes at room temperature, cultures were stained with mouse anti-neurofilament (RMO270) and goat anti-GFP or rabbit anti-6xHis. Data of two independent experiments were pooled. Per condition, 25-30 pictures were randomly taken. The ratio of neurite tips terminating on a transfected cell was calculated as follows: (number of neurite tips on transfected cells / total number of neurite tips) / transfected area (measured with ImageJ).

Statistical analysis

For statistical analyses, all *P*-values were calculated with Microsoft Excel XP using a two-tailed Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant. Error bars shown in figures represent standard error of the mean (s.e.m.).

RESULTS

Necl3/SynCAM2 is necessary for commissural axon pathfinding at the midline

We identified *Necl3* in a subtractive hybridization screen for genes that are differentially expressed in HH26 versus HH20 floorplates and were therefore considered as candidate guidance molecules for commissural axons. To test for a role of Necl3 in commissural axon guidance we used in ovo RNAi to perturb its expression in the spinal cord (Fig. 1). Injection and subsequent electroporation of dsRNA derived from the candidate clone into one half of the spinal cord resulted in erroneous pathfinding of commissural axons at the midline. Mainly, axons failed to turn rostrally after crossing the

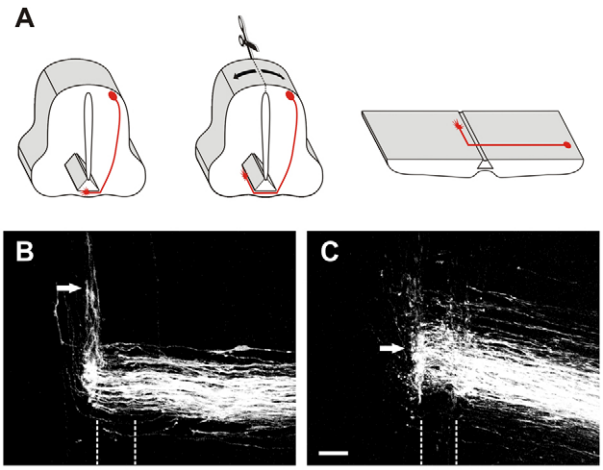

Fig. 1. Lack of Necl3/SynCAM2 causes pathfinding errors of commissural axons. (**A**) At the lumbosacral level of the chicken spinal cord, axons of dorsal commissural neurons extend ventromedially towards the midline at HH19, cross the floorplate at HH22-23, and then turn rostrally along the contralateral floorplate border at HH24. (**B**) At HH26, when visualized by injections of the lipophilic dye DiI into the area of the cell bodies, commissural axons have crossed the floorplate and extend along the contralateral border of the floorplate in untreated (data not shown) and control-injected embryos (arrow). (**C**) Silencing *Necl3* by in ovo RNAi resulted in aberrant navigation of commissural axons. Instead of turning into the longitudinal axis after crossing the floorplate, commissural axons lingered at the floorplate exit site and failed to extend along the longitudinal axis (arrow). Rostral is to the top in B and C. The floorplate is indicated by dashed lines. Scale bar: 50 μm.

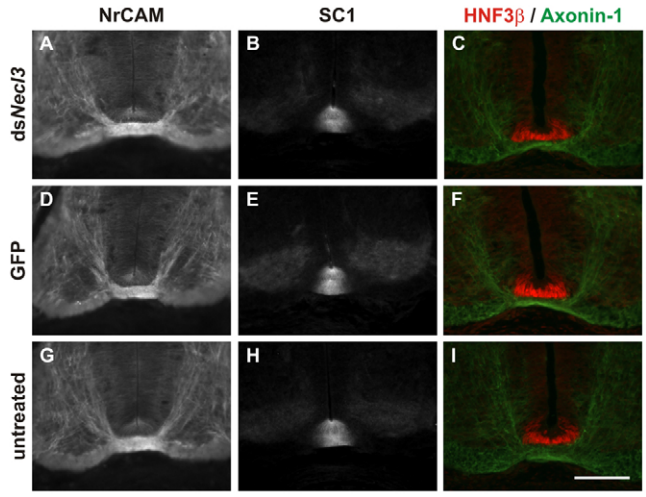

Fig. 2. Necl3/SynCAM2 is not required for the maintenance of cell-cell adhesion in the floorplate. (**A-I**) We did not observe any changes in floorplate morphology after downregulation of *Necl3* (A-C) compared with control-injected (D-F) and untreated control embryos (G-I). Floorplate cells were visualized by staining for NrCAM (A,D,G), SC1 (B,E,H) or HNF3β (red in C,F,I). The overlay of the HNF3β and an axonin 1 staining for commissural axons is shown in C,F,I and reflects no change in axon-floorplate contact. Scale bar: 100 μm.

floorplate. Instead, they stalled or made random turns once they had reached the contralateral side. This phenotype was seen at approximately two thirds of all DiI injection sites (see Materials and methods).

First, we ruled out the possibility that the observed pathfinding errors resulted from a disturbance of floorplate induction or morphology due to the lack of cell-cell adhesion mediated by Necl3. To this end, we stained the floorplate with antibodies against the IgCAMs NrCAM and SC1, or HNF3β, a marker for floorplate cells. Silencing *Necl3* by in ovo RNAi did not lead to any detectable change in floorplate morphology compared with GFP-expressing or untreated embryos (Fig. 2). These results suggested that Necl3 was not required for formation or maintenance of the floorplate structure. Similarly, *Necl3* was not required for axon-floorplate contact, as concluded from sections stained with anti-axonin 1 antibodies to visualize commissural axons. Rather, *Necl3* seemed to be required directly as guidance information for commissural axons.

Identification of three Necls/SynCAMs and two nectins in the chicken embryo

Mammalian Necls/SynCAMs have been demonstrated to interact homophilically or heterophilically with family members and with nectins (Fogel et al., 2007; Takai et al., 2003; Takai et al., 2008b). In order to identify the binding partner(s) of floorplate Necl3 on commissural neurons we analyzed the expression patterns of all Necl and nectin genes during the time of commissural axon pathfinding. Database searches revealed that chicken embryos express three Necl family members, *Necl1*, *Necl2*, and *Necl3*, in contrast to four in the mouse, and maybe five in human (Biederer, 2006; Takai et al., 2008b). As they had not been described previously, we named the chicken Necls according to the sequence similarity to their mammalian orthologs. Furthermore, the chicken genome contains only two rather than three nectins, which we called nectin 1 and nectin 3, corresponding to their mammalian orthologs. No chick ortholog could be identified for nectin 2.

Necl1 and *Necl3* were both found in the floorplate of the chicken spinal cord during the time of commissural axon navigation towards and across the midline (Fig. 3). The expression level of *Necl1* mRNA was already prominent at HH20 (Hamburger and Hamilton, 1951) (Fig. 3A) and persisted until after commissural axons had crossed the midline (Fig. 3C). By contrast, *Necl3* was expressed very weakly, if at all, at HH20 (Fig. 3G) but was upregulated later during development (Fig. 3H,I), consistent with its detection in our subtractive hybridization screen (Bourikas et al., 2005). Similarly, nectin 3 was detected in the floorplate at HH20 (Fig. 3M), HH23 (Fig. 3N) and HH26 (Fig. 3O). *Necl2* (Fig. 3D-F), as well as nectin 1 (Fig. 3J-L), were not expressed in the floorplate during the time window of interest. Notably, all members of the Necl/SynCAM family and nectin 1, but not nectin 3, were expressed in commissural neurons between HH23 and HH26, the time when commissural axons have reached the floorplate and turned rostrally into the longitudinal axis.

Homophilic and heterophilic trans-interactions of Necls/SynCAMs and nectins

To determine the interaction patterns of Necls/SynCAMs and nectins, we generated fusion proteins by combining their ectodomains with human alkaline phosphatase (AP) and a myc-6×His tag. The conditioned medium containing the fusion proteins was collected and added to HEK293T cells expressing the potential binding partner. The binding of fusion proteins with Necls or nectins expressed in HEK293T cells was assessed using the phosphatase activity (data not shown) or by visualizing the myc-tag with

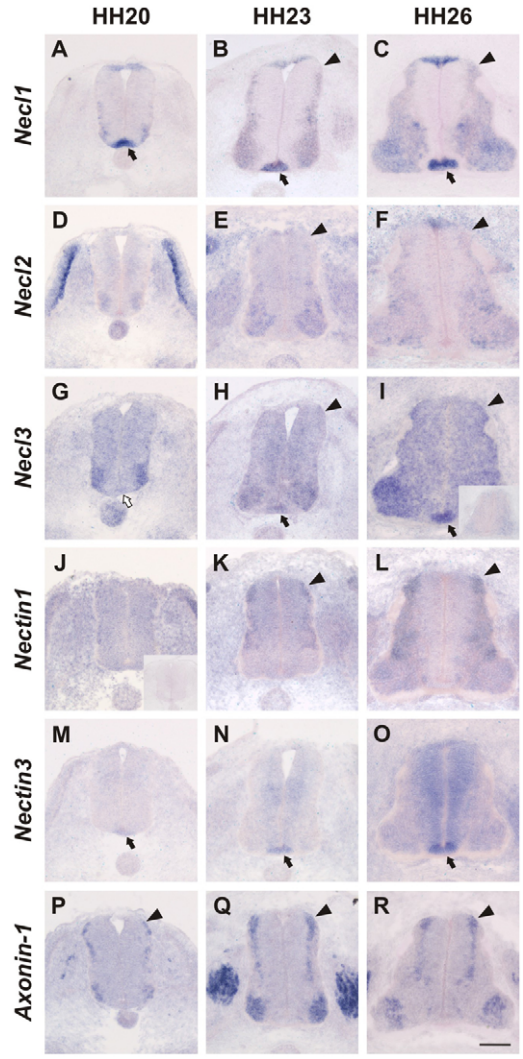

Fig. 3. Three Necls/SynCAMs and two nectins are expressed in the embryonic chicken spinal cord. (**A-R**) Expression patterns of Necls and nectins were analyzed by in situ hybridization on transverse sections of spinal cords at HH20 (A,D,G,J,M), HH23 (B,E,H,K,N) and HH26 (C,F,I,L,O). *Necl1* was expressed in the floorplate throughout this time window (A-C, arrows). Commissural neurons expressed *Necl1* between HH23 (B, arrowhead) and HH26 (C, arrowhead). In addition, *Necl1* was found in the roofplate, and in motoneurons as well as interneurons. By contrast, *Necl2* was not found in the floorplate (D-F). *Necl2* was upregulated in commissural neurons at the time when they had crossed the floorplate and were about to turn into the longitudinal axis. Levels were still low at HH23 (E, arrowhead) but increased afterwards. Expression in commissural neurons persisted at HH26 (F, arrowhead). *Necl3* was expressed in the floorplate at very low levels, if at all, at HH20 (G, white arrow), but subsequently increased (H,I, arrow). Expression of *Necl3* in commissural neurons was found with the same temporal pattern as *Necl2* (H,I, arrowheads). Nectins, a structurally related family of IgCAMs, were found in a complementary pattern in the developing chicken spinal cord. Nectin 1 was expressed in mature neurons, including dorsal commissural neurons (K,L, arrowhead), whereas nectin 3 mRNA was found in the ventricular zone at all stages that we examined. In contrast to nectin 1, nectin 3 was expressed in the floorplate (M-O, arrows). Axonin 1 expression was used to indicate the location of commissural neurons (P-R, arrowheads). Insets in I and J show sections processed with the sense probes of *Necl3* and nectin 1, respectively. Scale bar: 100 μm.

fluorescent antibodies (Fig. 4). We found strong binding of Necl2-AP-myc to Necl3-expressing cells (Fig. 4C). This interaction was confirmed by the converse experiment, adding Necl3-AP-myc to Necl2-expressing cells (Fig. 4H). Furthermore, nectin-3-AP-myc exhibited very robust binding to nectin 1 (Fig. 4V). Weak heterophilic interactions of Necl1 with Necl2 (Fig. 4A) and Necl3 (Fig. 4G) could be detected in addition to weak homophilic interactions of Necl2 (Fig. 4B) and nectin 1 (Fig. 4P).

These results suggested an interaction of Necl3 and nectin 3 expressed in the floorplate with Necl2 and nectin 1, respectively, expressed by commissural neurons. To confirm that Necl3 and nectin 3 expressed by floorplate cells could interact with crossing commissural axons in vivo, we incubated chick spinal cord sections with Necl3-AP- or nectin-3-AP-conditioned medium (Fig. 5). Indeed, both Necl3-AP and nectin-3-AP bound robustly to the ventral commissure, clearly demonstrating their interaction with molecules expressed by commissural axons while crossing the floorplate. In contrast to nectin-3-AP, Necl3-AP also bound to floorplate cells (arrowhead in Fig. 5A), most likely reflecting an interaction between Necl3 and Necl1 that was expressed at high levels in the floorplate throughout the time window analyzed in this study.

Taken together, these results suggest an interaction of Necl3 presented by floorplate cells with Necl2 expressed by commissural axons. Additionally, nectin 3 present on floorplate cells interacted with nectin 1 on commissural axons.

Necl3/SynCAM2 and nectin 3 from the floorplate are essential for commissural axon guidance

To assess the in vivo function of Necls/SynCAMs and nectins in commissural axon guidance, we used in ovo RNAi to interfere with the expression of these genes specifically either in the floorplate or in commissural neurons (Fig. 6). Silencing *Necl3* in the floorplate using long dsRNA resulted in an increase in aberrant projections of commissural neurons at the midline. On average, erroneous pathfinding behavior was found at 53.2±5.9% of the DiI injection sites per embryo (n=18 embryos, 154 injection sites). At the majority of these injection sites, commissural axons entered and crossed the floorplate normally but then failed to initiate their stereotypic rostral turn into the longitudinal axis, resulting in axons lingering at the floorplate exit site without any obvious bias in rostral direction. Notably, dsRNA derived from two independent cDNA fragments of *Necl3* resulted in the same phenotype (data not shown). In addition, we could phenocopy the abnormal pathfinding behavior by utilizing a shRNA construct that specifically affected the expression of *Necl3*, resulting in aberrant pathfinding at 59.7±11.3% of the injection sites (n=9 embryos, 64 injection sites). A control shRNA construct with a scrambled sequence had no effect; pathfinding errors were not more common than in GFP-expressing control embryos (see Table S1 and Fig. S1 in the supplementary material).

Downregulation of nectin 3 in the floorplate by in ovo RNAi using long dsRNA resulted in pathfinding errors similar to those observed after silencing *Necl3*: axons did not turn into the longitudinal axis after crossing the floorplate at 58.5±6.9% of the injection sites per embryo (n=25 embryos, 196 injection sites). By contrast, silencing *Necl1*, *Necl2* or nectin 1 in the floorplate had no effect on the trajectories of commissural axons compared with non-injected or GFP-expressing control embryos (Fig. 6I; see Table S1 in the supplementary material). *Necl2* and nectin 1 were used as negative controls, as neither one of them was expressed in the floorplate during the time of commissural axon pathfinding.

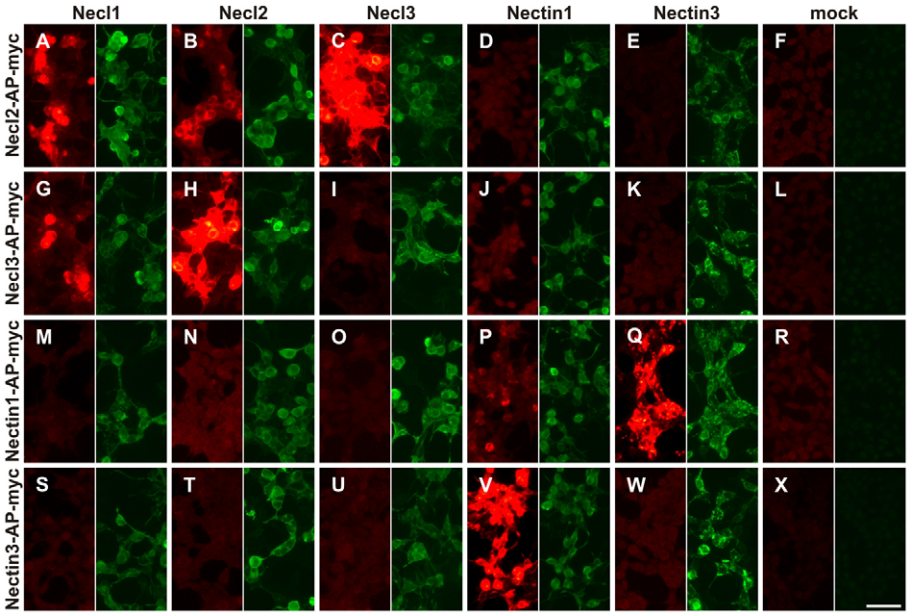

Fig. 4. Necls/SynCAMs and nectins interact mainly in a heterophilic manner. (**A-X**) HEK293T cells transfected with HA-tagged *Necl1* (A,G,M,S), *Necl2* (B,H,N,T), *Necl3* (C,I,O,U), nectin 1 (D,J,P,V), nectin 3 (E,K,Q,W) or the empty *pcDNA3.1-myc/His* vector (F,L,R,X) were incubated with conditioned medium containing Necl2-AP-myc (A-F), Necl3-AP-myc (G-L), nectin-1-AP-myc (M-R) or nectin-3-AP-myc (S-X). Similar transfection levels were obtained for all HA-tagged proteins, as visualized with an anti-HA antibody (shown in green on the right in each panel). Binding of the AP-myc fusion protein was visualized with an anti-myc antibody (shown in red on the left in each panel). Strong interactions could be detected between Necl2 and Necl3 (C,H), as well as between nectin 1 and nectin 3 (Q,V). Weaker interactions were found between Necl1 and Necl2 (A) and between Necl1 and Necl3 (G). Furthermore, Necl2 (B) and nectin 1 (P) were found to interact homophilically. All pictures were taken with the same exposure time, except for C,H,Q and V, where myc staining (red) was imaged with half the exposure time. Scale bar: 50 μm.

Together, these results demonstrate that floorplate-derived Necl3 and nectin 3 are essential for proper pathfinding of commissural axons at the midline. By contrast, downregulation of Necl1, Necl2 or nectin 1 does not affect axon guidance at the floorplate.

Necl2/SynCAM1, Necl3/SynCAM2, and nectin 1 on commissural axons are essential for their navigation at the floorplate

Next, we tested which of the Necl and nectin family members expressed by commissural neurons were involved in axonal navigation at the midline. Again, we turned to in ovo RNAi, but this time we specifically targeted commissural neurons (Fig. 6B). As expected based on our binding studies, we found Necl2 and nectin 1, the binding partners of Necl3 and nectin 3, respectively, to be required for midline crossing. Downregulation of Necl2 in commissural neurons using long dsRNA resulted in erroneous axonal navigation at 53.1±5.4% of the injection sites per embryo (n=17 embryos, 134 injection sites; Fig. 6F,J). Similar results were obtained with a shRNA construct specifically interfering with the expression of *Necl2*; pathfinding errors were seen at 63.3±8.2% of the injection sites (n=15 embryos, 156 injection sites; data not shown; see Table S1 in the supplementary material). The electroporation of a control shRNA did not significantly increase the number of injection sites with aberrant pathfinding. Silencing nectin 1 induced aberrant pathfinding at 65.9±11.3% of injection sites per embryo (n=10 embryos, 90 injection sites). Unexpectedly, silencing *Necl3* in commissural neurons also led to pathfinding errors at 58.5±8.5% of the injection sites per embryo (n=12 embryos, 72 injection sites; Fig. 6G,J). The observed errors were qualitatively indistinguishable from the ones seen after downregulation of Necl3 or nectin 3 in the floorplate. The majority of the axons stalled at the contralateral border of the floorplate and failed to turn rostrally. By contrast, silencing *Necl1* and nectin 3 in commissural neurons did not affect axonal pathfinding when compared with GFP-expressing or uninjected control embryos (Fig. 6J; see Table S1 in the supplementary material).

In summary, using in ovo RNAi to target genes specifically either in the floorplate or in commissural neurons, we demonstrated that interactions between Necls/SynCAMs and nectins were required for commissural axon pathfinding. We found Necl3 and nectin 3 to be required in the floorplate, whereas Necl2, Necl3 and nectin 1 were necessary in commissural neurons.

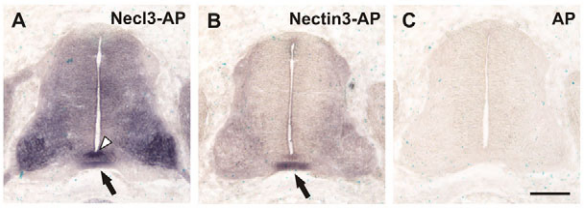

Fig. 5. Necl3/SynCAM2 and nectin 3 interact with proteins expressed in the ventral commissure. (**A-C**) Transverse sections of HH25 chicken spinal cords were incubated with conditioned medium containing Necl3-AP (A), nectin-3-AP (B) or AP (C). Necl3-AP (A) and nectin-3-AP (B) bound to commissural axons forming the ventral commissure (arrows). AP alone (C), used as control, showed no staining. Furthermore, Necl3, but not nectin 3, also bound to floorplate cells (A, arrowhead). Scale bar: 100 μm.

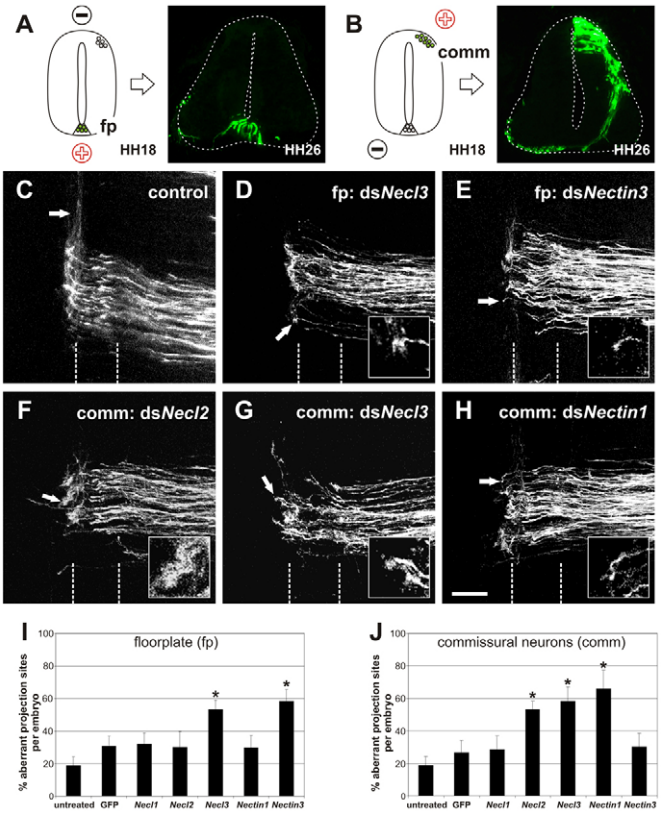

Fig. 6. Necls/SynCAMs and nectins are required for commissural axon pathfinding. (**A-H**) Specific electroporation of floorplate cells (A) or commissural neurons (B) was achieved by precise positioning of the electrodes. Co-electroporation of a plasmid encoding GFP was used to visualize the targeted area. (C) At HH26, commissural axons have crossed the midline and extend rostrally along the contralateral border of the floorplate (arrow) in control-injected embryos. (D) After downregulation of Necl3 in the floorplate, commissural axons failed to turn into the longitudinal axis. Instead, they lingered at the exit site and randomly pointed rostrally or caudally without extending along the anteroposterior axis (arrow in D; inset shows growth cone pointing caudally at higher magnification). (E) The same phenotype was observed (arrow) after downregulation of nectin 3 in the floorplate. Downregulation of Necl1, Necl2 or nectin 1 in the floorplate did not affect post-crossing commissural axon pathfinding (data not shown). By contrast, downregulation of Necl2 (F) and nectin 1 (H) in commissural neurons resulted in aberrant pathfinding at the floorplate exit site. As seen after downregulation of Necl3 and nectin 3 in the floorplate, lack of Necl2 or nectin 1 in commissural neurons resulted in axons lingering at the exit site and in random orientation of growth cones in anterior or posterior directions (arrow in F and H, respectively). (G) Downregulation of Necl3 in commissural axons mimicked the loss of *Necl3* function in the floorplate. (**I**,**J**) Quantification of the effects obtained after downregulation of target genes specifically in the floorplate (I) or in commissural neurons (J). Values are given in Table S1 in the supplementary material as mean±s.e.m.; *, $P \leq 0.05$. Rostral is to the top in C-H. Insets show growth cones pointing in the caudal direction, indicated by arrows in D-H, at higher magnification. Scale bar: 50 μm.

Necl3/SynCAM2 is required for commissural axon guidance, not growth

To rule out that the observed phenotype was due to a delay of midline crossing or a decrease in growth rate of commissural axons in the floorplate area, we also analyzed older embryos. When embryos were sacrificed one day later, at HH28, post-crossing commissural axons still lingered at the floorplate exit site and failed to extend along the longitudinal axis (see Fig. S2 in the supplementary material). The quantification of axon guidance defects at HH28 resulted in the same difference between embryos lacking Necl3 and controls as found at HH26 (21.3% compared with 22.6%; Fig. 6). Therefore, we excluded a decrease in growth rate as the underlying mechanism of the *Necl3* loss-of-function phenotype.

An alternative explanation for the observed phenotype could be the decrease of floorplate-derived repellents that would allow post-crossing growth cones to linger at the exit site. However, a repellent activity of Necl3 was unlikely in view of the known functions of Necls/SynCAMs as synaptic adhesion molecules and mediators of Schwann cell/axon interactions during myelination. In fact, we found that Necl3 has an attractive effect on commissural axons. In co-cultures of commissural axons and COS7 cells expressing Necl3, we found 3.3 times more growth cones on Necl3-positive cells compared with GFP-transfected cells (Fig. 7).

Based on our in vivo studies, the activity of floorplate Necl3 had to be mediated by a homophilic and/or a heterophilic interaction with Necl2 or nectin 1 expressed by commissural axons. Indeed, we found a strong trans-interaction between Necl3 and Necl2 in our binding assay (Fig. 4). However, there was no evidence for a homophilic interaction of Necl3 or a heterophilic interaction with nectin 1. Therefore, our finding that Necl3 was also required in commissural neurons was unexpected. The most probable explanation is that a cis-complex formed between Necl3 and either Necl2 or nectin 1 on commissural axons. As so far no cis-interactions between Necls and nectins have been found (Takai et al., 2003), we favor a cis-complex comprising Necl2 and Necl3 on growth cones as the binding partner for floorplate Necl3 in post-crossing commissural axon guidance (Fig. 8).

In summary, our in vitro and in vivo results support a model for post-crossing commissural axon guidance that suggests a functional interaction between floorplate Necl3/SynCAM2 with a cis-complex formed by Necl2 and Necl3 on commissural growth cones.

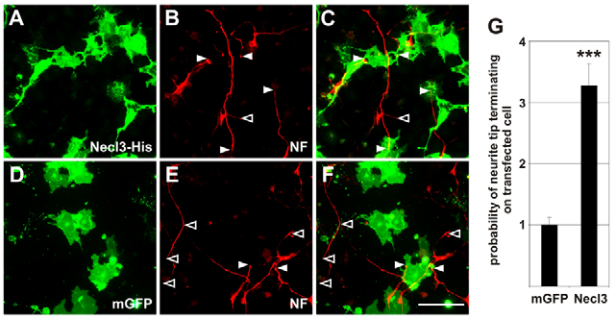

Fig. 7. Necl3/SynCAM2 is an attractive cue for commissural axons. (**A-F**) Commissural neurons dissected from HH25 embryos were cultured on COS7 cells transfected with His-tagged *Necl3* (A-C) or with *MARCKS-GFP* (D-F) for 42 hours. Axons visualized by anti-neurofilament staining were found to preferentially stop on Necl3-positive cells (B,C, arrowheads). The ratio of growth cones on a transfected cell (arrowheads), either a Necl3-positive cell (C, green) or a GFP-expressing cell (F, green) versus a non-transfected COS7 cell (open arrowheads), was calculated (see Materials and methods). (**G**) Growth cones were 3.3-fold more likely to stop on a Necl3-expressing cell compared with a GFP-expressing cell (GFP 1±0.13; Necl3 3.28±0.35; mean±s.e.m., ***, $P \leq 0.001$). Scale bar: 100 μm.

DEVELOPMENT

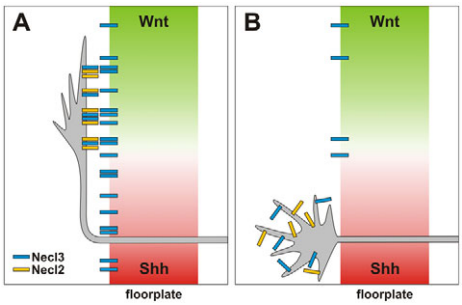

Fig. 8. Necl2/SynCAM1 and Necl3/SynCAM2 are required for commissural axon pathfinding at the contralateral floorplate border. (**A**) Post-crossing commissural axons require Necl3 from the floorplate to cluster Necl2 and Necl3 in their growth cone membrane. The cis-interaction between Necl2 and Necl3 forms a receptor complex that mediates the activity of floorplate-derived Necl3. (**B**) After downregulation of Necl3 in the floorplate, growth cones fail to turn rostrally after midline crossing. The directionality of growth along the floorplate border is also determined by gradients of Shh (Bourikas et al., 2005) and Wnt proteins (Lyuksyutova et al., 2003). It remains to be shown whether these two guidance systems are linked, or whether they act in parallel in a redundant manner.

DISCUSSION

Necls/SynCAMs were shown to be involved in late stages of neural circuit formation, in particular synaptogenesis (Biederer et al., 2002) and myelination in the peripheral (Maurel et al., 2007; Spiegel et al., 2007) and the central nervous system (Park et al., 2008). Here, we demonstrate that Necls/SynCAMs are also involved in early steps of neural circuit formation in the chick spinal cord in vivo. We found expression of all chicken Necl/SynCAM genes in the spinal cord before axons have reached their targets and long before myelination starts (Fig. 3). In fact, we identified Necl3/SynCAM2 in a screen for axon guidance cues expressed in the floorplate. This is in contrast to reports from rat brain, where no expression of Necl3/SynCAM2, but low levels of Necl2/SynCAM1 and Necl4/SynCAM4 were found during embryonic development (Fogel et al., 2007).

Based on the identification of Necl3/SynCAM2 as a candidate axon guidance cue, we used in ovo RNAi to downregulate Necl3 in the developing spinal cord during commissural axon pathfinding. In the absence of Necl3/SynCAM2 post-crossing commissural axons failed to turn rostrally into the longitudinal axis (Fig. 1; Fig. 6). Necls were shown to bind both homo- and heterophilically with other Necls and also with nectins (Takai et al., 2003; Takai et al., 2008b). Nectins are organizers of adherens and tight junctions (Takai et al., 2008a). In analogy, we assumed that Necls could interfere with cell-cell adhesion as well. Therefore, we analyzed the morphology of the floorplate to rule out that the observed phenotype was caused indirectly owing to changes in adhesion between floorplate cells. We found that the floorplate morphology and the contact between commissural axons and the floorplate was unchanged after downregulation of Necl3/SynCAM2, suggesting that the observed phenotype was caused by a direct effect on post-crossing commissural axons (Fig. 2).

Because of its expression in both floorplate and post-crossing commissural axons, we specifically targeted *Necl3/SynCAM2* in the floorplate or in commissural neurons, respectively (Fig. 6). The failure of post-crossing axons to turn rostrally and extend along the contralateral floorplate border was found after downregulation of Necl3 in the floorplate (Fig. 6D,I) and in commissural neurons (Fig. 6G,J). However, our binding assay indicated no homophilic trans-interaction of Necl3 (Fig. 4) in contrast to published reports with mouse Necl3/SynCAM2 (Fogel et al., 2007; Pellissier et al., 2007). In mouse, no homophilic interaction of SynCAM4 was found, indicating that Necls/SynCAMs do not necessarily interact homophilically (Fogel et al., 2007). No ortholog of SynCAM4 has been found in the chicken genome. Thus, chicken Necl3 could be the family member that does not interact homophilically. Alternatively, our assay might have been less sensitive compared with the cell-cell adhesion assays used by Fogel and colleagues (Fogel et al., 2007) and Pellissier and colleagues (Pellissier et al., 2007). In these studies, homophilic interactions of Necl3 was inferred from its accumulation at cell-cell contact sites, whereas we used recombinant proteins fused to alkaline phosphatase and a myc-tag to show binding of Necls to family members expressed in HEK293T cells.

In agreement with published reports (Fogel et al., 2007; Pellissier et al., 2007), we found a strong interaction of Necl2 with Necl3 and of nectin 1 with nectin 3 (Fig. 4) (Kakunaga et al., 2005). In our assay, we found weak heterophilic interactions between Necl1 and Necl2 and between Necl1 and Necl3, again in agreement with published reports (Kakunaga et al., 2005; Shingai et al., 2003). We also confirmed a weak homophilic interaction of Necl2 (Biederer et al., 2002; Fogel et al., 2007; Shingai et al., 2003) and nectin 1 (Shingai et al., 2003).

Based on our binding studies, we concluded that aberrant pathfinding of post-crossing commissural axons was unlikely to be due to the perturbation of a homophilic Necl3 interaction but rather due to the perturbation of the heterophilic interaction between Necl2 and Necl3. Downregulation of Necl2 and Necl3 on commissural axons resulted in aberrant axon guidance at 53% and 58%, respectively, of the injection sites. Similar results were obtained after silencing *Necl3* in the floorplate. In this case, aberrant pathfinding was also observed at 53% of the injection sites. Owing to the strong interaction of Necl2 and Necl3 in vitro (Fig. 4), a heterophilic interaction of floorplate Necl3 with Necl2 on commissural growth cones in vivo is a likely explanation for our findings. The results seen after downregulation of Necl3 in commissural neurons was unexpected, as our binding studies with Necl3-AP-myc did not indicate a homophilic interaction. No other known binding partner for growth cone Necl3 in the floorplate could explain the observed phenotype. Necl1 was shown to bind to Necl3 in our assay, and Necl1 is expressed in the floorplate, but the downregulation of Necl1 in the floorplate did not affect commissural axon guidance. Furthermore, no interaction between Necl3 and nectin 3 was found (this study) (Takai et al., 2008b) also making nectin 3 an unlikely floorplate binding partner for growth cone Necl3. Downregulation of nectin 3 in the floorplate did, however, result in similar guidance defects as seen in the absence of Necl3. This is in line with published results (Okabe et al., 2004). Okabe and colleagues demonstrated a role for nectin 1 and nectin 3 in commissural axon guidance in rat hindbrain explants. The addition of inhibitors of the trans-interaction between nectin 1 and nectin 3 in these cultures resulted in comparable axon guidance defects to those shown in our in vivo experiments using in ovo RNAi to interfere with the heterophilic interaction between growth cone nectin 1 and floorplate nectin 3.

Owing to the absence of a candidate trans-binding partner for axonal Necl3 in the floorplate, the most parsimonious explanation for the phenotype obtained after targeting *Necl3* in commissural neurons is therefore a cis-interaction between Necl2 and Necl3

on commissural axons (Fig. 8). It is widely accepted that Necls/SynCAMs form cis-interactions before they form trans-interactions (Takai et al., 2008b). Alternatively, growth cone Necl3 could interact with a so far unidentified binding partner in the floorplate.

Because the phenotypes seen after perturbation of the heterophilic interaction between nectin 1 and nectin 3 are virtually indistinguishable from those observed after downregulation of Necl2 and Necl3 in commissural neurons, it could be possible that cis-interactions would also include Necl2/nectin 1 and Necl3/nectin 1, or even higher order complexes between Necls and nectin 1. Although we cannot formally rule out such a possibility, we consider this an unlikely scenario as nectin 1 has not been shown to bind to any Necl in cis so far (Takai et al., 2008b). By contrast, Necl2 was shown to be excluded from areas of Afadin clusters, indicating that cis-complexes between nectins, which bind to Afadin, and Necls, which do not bind to Afadin, are unlikely (Shingai et al., 2003). Necls were shown to bind to MAGUKs, membrane-associated guanylate kinases, and to the Band4.1 family of scaffolding proteins (Murakami, 2005). Thus, it appears that nectins and Necls act in parallel, rather than in a cooperative manner, in post-crossing commissural axon guidance.

Finally, the question remained whether Necls were acting as guidance cues, or whether commissural axons were just slowed down in the absence of Necl2 and Necl3 interactions. The distinction between guidance and growth is often not possible because guidance of axons that fail to grow cannot be studied. Many IgCAMs were shown to have a dual function as neurite growth promoters and as axon guidance molecules; whether they exert one or the other function, or in fact both, depends on the context. An example where this was shown best, is axonin 1/TAG1. Axonin 1 guides commissural axons without affecting their growth (Fitzli et al., 2000; Stoeckli and Landmesser, 1995). Similarly, downregulation of axonin 1/TAG1 in the developing cerebellum interferes with guidance, but not growth, of parallel fibers (Baeriswyl and Stoeckli, 2008). By contrast, axonin 1/TAG1 affects the guidance of sensory afferents (Perrin et al., 2001) and promotes sensory axon growth (Stoeckli et al., 1991; Stoeckli et al., 1996).

Post-crossing commissural axons were shown to extend along the floorplate border in an NgCAM- and NrCAM-dependent manner (Fitzli et al., 2000). Detailed in vitro and in vivo analyses demonstrated that the presence of either NgCAM or NrCAM was sufficient for axonal extension along the contralateral floorplate border. Axons only failed to grow and lingered at the floorplate exit site in the absence of both NgCAM and NrCAM. Importantly, the absence of both NgCAM and NrCAM only interfered with axonal extension and not with the rostral orientation of post-crossing axons, as growth cones were still biased toward rostral turns. By contrast, here we found no rostral bias of growth cones (Fig. 6, insets). In fact, many growth cones even showed a caudal orientation, indicating that Necls/SynCAMs were not interfering with the growth of post-crossing commissural axons, but rather with their guidance. This was supported by our in vitro experiment where growth cones preferred COS7 cells expressing Necl3 compared with mock-transfected cells (Fig. 7). Additional evidence came from our analysis of HH28 spinal cords, where post-crossing commissural axons were still lingering at the floorplate exit site arguing against a decreased growth rate. Similarly, the absence of axons stalling at or within the floorplate rather than at the exit site argues against the idea that commissural axons fail to grow in the absence of interactions between floorplate Necl3 and Necl2/Necl3 expressed on commissural axons.

Conclusion

Based on our in vitro and in vivo experiments, we postulate a model for Necls/SynCAMs in post-crossing commissural axon guidance that involves both cis- and trans-interactions (Fig. 8). Necl3/SynCAM2 expressed by floorplate cells is recognized by a cis-complex formed by Necl2/SynCAM1 and Necl3/SynCAM2 on commissural axons. This model is supported by our in vivo studies that revealed a failure of post-crossing axons to extend rostrally along the floorplate border when Necl3/SynCAM2 was downregulated in the floorplate or when either Necl2/SynCAM1 or Necl3/SynCAM2 was downregulated in commissural neurons. Our findings of a role for Necls/SynCAMs in post-crossing axon guidance extend the known functions of Necls/SynCAMs to early aspects of neural circuit formation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.042515/-/DC1

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