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

Vera Niederkofler, Thomas Baeriswyl, Regula Ott and Esther T. Stoeckli*

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 Slits 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$^{\text{high}}$ to caudal$^{\text{low}}$ Wnt4 gradient in the floorplate (Lyuksyutova et al., 2003). By contrast, in chick, Shh expressed in a rostral$^{\text{low}}$ to caudal$^{\text{high}}$ 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 (Biederer et al., 2002). In contrast to nectins, Necls are not involved in junction formation. Necl1/SynCAM3 and Necl4/SynCAM4 were shown to be required for myelination in the peripheral and the central nervous system (Maurer 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-
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 Necl/SynCAMs affect late aspects of neural development. Here, we describe a function for Necl/SynCAMs in axonal pathfinding in the developing spinal cord in vivo providing compelling evidence that Necl/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 promoter was always co-electroporated to verify the specificity of targeting. Necl2 (3 µg/µl dsRNA; ChEST1028m10), Necl2 (3 µg/µl dsRNA; ChEST764m1), 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-SCI-BEN (16.9H2), 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; RM0270, 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 (Zuelig 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 pDNA3-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 mM 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 (RM0270) 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
express three Necl family members, pathfinding. Database searches revealed that chicken embryos Necl and nectin genes during the time of commissural axon navigation towards the midline at HH19, cross the floorplate at HH22-23, and then turn rostrally along the contralateral floorplate border at HH24. At HH26, when visualized by injections of the lipophilic dye Dil 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). 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.

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-L), 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 coding sequences to test for homophilic and heterophilic interactions of Necl3/SynCAM2 with floorplate and extend along the contralateral border of the floorplate in untreated (data not shown) and control-injected embryos (arrow). 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.

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-L), 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 coding sequences to test for homophilic and heterophilic interactions of Necl3/SynCAM2 with floorplate cells. Instead, they stalled or made random turns once they had reached the contralateral side. This phenotype was seen at approximately two thirds of all Dil 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.
Fig. 3. Three Necl/SynCAMs and two nectins are expressed in the embryonic chicken spinal cord. (A–R) Expression patterns of Necl1 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,L,O). Necl1 was expressed in the floorplate throughout this time window (A–C, arrows). Commisural 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 commisural 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). Inserts in L and J show sections processed with the sense probes of Necl3 and nectin 1, respectively. Scale bar: 100 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. We again 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.

**Necl3/SynCAM2 guides post-crossing axons**

![Image](image-url)
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 has an attractive effect on commissural axons was unexpected. The most probable explanation is that a cis-complex formed between Necl3 and either Necl2 or nectin 1 on commissural axons.

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. 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 withnectins (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 of 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,J) 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 Necl3, 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 Necl3 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 here we found no rostral bias of growth cones (Fig. 6, insets). In fact, many growth cones even showed a caudal orientation, indicating 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 Necl3, 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 Necl3 act in parallel, rather than in a cooperative manner, in post-crossing commissural axon guidance.

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.

Acknowledgements

We thank Silvia Arber (Biozentrum, University of Basel) for constructs, Matthias Gesemann for help and suggestions on the cloning of Necl3, and all members of the laboratory for comments and discussions. The project was supported by the Swiss National Science Foundation and by the Olga-Mayenfisch Stiftung.

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

References


Necl3/SynCAM2 guides post-crossing axons


