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Contacts between the commissural axons and the floor plate cells are mediated by nectins

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Abstract

During development of the central nervous system (CNS), commissural axons grow toward the ventral midline. After crossing the floor plate, they abruptly change their trajectory from the circumferential to the longitudinal axis. The contacts between the commissural axons and the floor plate cells are involved in this axonal guidance, but their mechanisms or structures have not fully been understood. In this study, we found that nectin-1 and -3, immunoglobulin-like cell–cell adhesion molecules, asymmetrically localized at the contact sites between the commissural axons and the floor plate cells, respectively. In vitro perturbation of the endogenous *trans*-interaction between nectin-1 and -3 caused abnormal fasciculation of the commissural axons and impairment of the contacts, and resulted in failure in longitudinal turns of the commissural axons at the contralateral sites of the rat hindbrain. These results indicate that the contacts between the commissural axons and the floor plate cells are mediated by the hetero-*trans*-interaction between nectin-1 and -3 and involved in regulation of the trajectory of the commissural axons.

Keywords: Nectin; Afadin; The floor plate; Commissural neurons; Heterotypic cell–cell contact; Actin cytoskeleton; Cadherin; Catenin; Central nervous system; Axonal trajectory

Introduction

During early development of the vertebrate central nervous system (CNS), axons grow either longitudinally or circumferentially to reach their targets. Individual growth cones are guided by a variety of attractive and repulsive cues. The floor plate, a source of non-neuronal cells, locates at the ventral midline of the developing vertebrate hindbrain and spinal cord, and connects the right and left halves of the neural tube (Imondi and Kaprielian, 2001). The commissural neurons that originate in the dorsal neural tube elongate their axons, cross the floor plate at the ventral midline, and turn sharply at contralateral sites to project longitudinally within the ventral funiculus (Colamarino and Tessier-Lavigne, 1995; Shirasaki et al., 1995).

Elongation of the commissural axons across the floor plate is mediated by their transmembrane receptors and diffusible chemoattractants released from the floor plate cells (Stoeckli and Landmesser, 1998). The Eph receptor for B-class ephrin is expressed on the surface of the commissural axons. In vitro perturbation of these receptors results in a failure of the commissural axons to reach their final destinations (Imondi and Kaprielian, 2001). A different type of the contacts

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between the commissural axons and the floor plate cells is mediated by the heterophilic interaction between NrCAM on the surface of the floor plate cells and axonin-1/TAG-1 expressed in growth cones of the commissural neurons (Shiga and Oppenheim, 1991; Stoeckli and Landmesser, 1995). In vitro perturbation of axonin-1/TAG-1 with a neutralizing antibody (Ab) results in a failure of the commissural axons to cross the ventral midline of the chick (Stoeckli and Landmesser, 1995).

Electron microscopic analysis indicates that the floor plate cells elongate their basal processes toward the basement membrane (Yaginuma et al., 1991; Yoshioka and Tanaka, 1989). Between the floor plate cells, the junctional complexes are observed at apical surfaces in the ventricular zone, and gap junctions are also observed in the lateral region of the floor plate cells themselves (del Brio et al., 2001; Rodriguez et al., 1996). It has been shown that growth cones are associated with the basal processes of the floor plate cells (Yaginuma et al., 1991). However, any specialized junctional contact between the commissural axons and the floor plate cells or their basal processes has not been reported.

In vertebrates, cell junctions occur at cell–cell and cell–matrix contacts. Cell–cell junctions are categorized into three functional groups: communicating junctions such as gap junctions, anchoring junctions such as adherens junctions (AJs) and desmosomes, and occluding junctions such as tight junctions (TJs). Of these cell–cell junctions, actin-based cell–cell AJs are crucial for organization of other cell–cell junctions (Gumbiner, 1996; Takeichi, 1991). The classic cadherin–catenin system is a key adhesion unit at AJs. At AJs in epithelial cells, E-cadherin directly binds β-catenin that directly binds α-catenin (Nagafuchi et al., 1991; Ozawa et al., 1989; Rimm et al., 1995). α-Catenin directly binds α-actinin and vinculin (Watabe-Uchida et al., 1998; Weiss et al., 1998). Of these proteins, α-catenin, α-actinin, and vinculin are actin filament (F-actin)-binding proteins. The association of E-cadherin with the actin cytoskeleton strengthens its cell–cell adhesion activity (Nagafuchi, 2001). The nectin–afadin unit also localizes at AJs in epithelial cells (Takai and Nakanishi, 2003). Nectin is a Ca^{2+}-independent immunoglobulin-like cell–cell adhesion molecule and constitutes a family of four members: nectin-1, -2, -3, and -4. All nectins first form homo-*cis*-dimers and then homo-*trans*-dimers (*trans*-interactions), causing cell–cell adhesion. Furthermore, nectin-3 forms hetero-*trans*-dimers with nectin-1 and -2 (Satoh-Horikawa et al., 2000). Nectin-4 also forms hetero-*trans*-dimers with nectin-1 (Fabre et al., 2002). All nectins except for nectin-4 have a COOH-terminal conserved motif of four amino acid residues (E/A-X-Y-V) that interacts with the PDZ domain of afadin, an F-actin-binding protein. Nectin-4 does not have the typical consensus motif but binds afadin (Fabre et al., 2002). All nectins are associated with the actin cytoskeleton through afadin (Takai and Nakanishi, 2003).

The nectin–afadin unit is ubiquitously expressed not only in epithelial cells but also in non-epithelial cells (Takai and Nakanishi, 2003; Takai et al., 2003a,b). The nectin–afadin unit is involved in the formation of cell–cell AJs in cooperation with cadherins in epithelial cells and fibroblasts (Honda et al., 2003; Ikeda et al., 1999; Mandai et al., 1997; Tachibana et al., 2000; Takahashi et al., 1999), in the organization of TJs at the apical site of AJs in epithelial cells (Fukuhara et al., 2002), in the formation of synapses in cooperation with N-cadherin in neurons (Mizoguchi et al., 2002), and in the formation of Sertoli cell–spermatid junctions in a cadherin-independent manner in the testis (Ozaki-Kuroda et al., 2002). In fibroblasts, nectin-3 and afadin colocalize with N-cadherin at cell–cell AJs (Honda et al., 2003). In the absorptive epithelial AJs of small intestine, nectins and afadin are highly concentrated at AJs under which F-actin bundles lie (Mandai et al., 1997; Takahashi et al., 1999). This localization pattern is different from that of E-cadherin, which is concentrated at AJs but widely distributed from the apical to the basal sides of the lateral membrane. At the synapses between the mossy fiber terminals and the pyramidal cell dendrites in the CA3 area of the hippocampus, both synaptic junctions and puncta adherentia junctions are highly developed (Amaral and Dent, 1981). Nectin-1 and -3 asymmetrically localize at the pre- and post-synaptic sides, respectively, of puncta adherentia junctions, form hetero-*trans*-dimers, and are involved in the formation of synapses (Mizoguchi et al., 2002). Mutations in the *nectin-1* gene are responsible for cleft lip/palate ectodermal dysplasia (CLPED1)—Margarita island ectodermal dysplasia and Zlotogora-Ogür syndrome—which is characterized clinically by developmental defects of the skin and its appendages, the hands and toes, craniofacial anomaly, and mental retardation (Sozen et al., 2001; Suzuki et al., 2000). At Sertoli cell–spermatid junctions, nectin-2 and -3 asymmetrically localize in Sertoli cells and spermatids, respectively, and form hetero-*trans*-dimers (Ozaki-Kuroda et al., 2002). Nectin-2 is associated with F-actin bundles through afadin on the Sertoli cell side, whereas nectin-3 is not associated with F-actin bundles or afadin on the spermatid side. Nectin-2-deficient mice exhibit male-specific infertility and have defects in the later steps of sperm morphogenesis, including distorted nuclei, an abnormal distribution of mitochondria, and impairment of Sertoli cell–spermatid junctions (Bouchard et al., 2000; Ozaki-Kuroda et al., 2002). Afadin-deficient mice show developmental defects at stages around gastrulation, including disorganization of the ectoderm, impaired migration of the mesoderm, and loss of somites and other structures that are derived from both the ectoderm and the mesoderm (Ikeda et al., 1999; Zhadanov et al., 1999). Cell–cell AJs and TJs in neuroepithelium are improperly organized in the ectoderm of afadin-deficient mice and embryoid bodies (Ikeda et al., 1999). Thus, the nectin–afadin unit plays roles in a wide variety of cell–cell junctions in cooperation with or independently of the cadherin–catenin unit.

We have recently demonstrated that nectin-1 and -3 proteins are highly expressed in the floor plate in the middle stages of mouse embryonic development (Okabe et al.,

2004). In this study, we have examined whether nectins play roles in the formation and/or maintenance of the contacts between the commissural axons and the floor plate cells in the ventral midline of the neural tube. We have found here that the contacts are mediated by the hetero-*trans*-interaction between nectin-1 and -3 and involved in regulation of the trajectory of the commissural axons.

Materials and methods

Antibodies, recombinant proteins, and embryos

A rabbit anti-l-afadin polyclonal Ab (pAb) and a mouse anti-l-afadin monoclonal Ab (mAb) were obtained as described (Mandai et al., 1997; Sakisaka et al., 1999). A mouse anti-β-catenin mAb was purchased from Transduction Laboratories. A rabbit anti-nectin-1α pAb and rat anti-nectin-1, anti-nectin-2, and anti-nectin-3 mAbs were obtained as described (Satoh-Horikawa et al., 2000; Takahashi et al., 1999). A mouse anti-ZO-1 mAb was kindly supplied by Dr. Sh. Tsukita (Kyoto University, Kyoto, Japan). A mouse anti-F84.1 mAb was kindly supplied by Dr. Stallcup (La Jolla Cancer Research Center, California) (Prince et al., 1992). gD and Nef-3 were prepared as described (Sakisaka et al., 2001; Satoh-Horikawa et al., 2000). Embryonic mice were obtained from ICR strains (Nihon SLC, Hamamatsu, Japan). The noon, when the vaginal plug was found, was embryonic day 0.5 (E0.5). Embryonic rats were obtained from pregnant rats (Wistar strain). The day of the vaginal plug was E0.

Flat whole-mount in vitro preparations of the hindbrain

Tissue dissection and explant culture were performed as described (Shirasaki et al., 1995) with some modifications. Briefly, the hindbrains of mouse embryos at E11.5–12.5 or rat embryos at E13 were dissected. The hindbrain was then cut open along the dorsal midline and flat-mounted with ventricular side down, followed by each procedure. In brief, E13 Wistar rats were dissected in cold DMEM/F12 (Sigma). The whole-mount hindbrain explants were cut along the dorsal midline, put on the transwell membrane (Corning) with the ventricular side down, and cultured in the same medium in the presence of either 60 μg/ml of human IgG (Sigma) as a control or the mixture of 60 μg/ml of gD and 60 μg/ml of Nef-3 at 37°C for 48 h. For DiI labeling, these explants were washed with PBS and fixed with 4% paraformaldehyde. Small crystals of DiI were implanted into the ventral midline in the rostral hindbrain (Godement et al., 1987). After the DiI implantation, the whole-mount preparations were rinsed to remove unattached DiI particles and again placed in 4% paraformaldehyde. They were stored in the dark at room temperature for 6–7 days and then observed by epifluorescence microscopy and confocal laser scanning microscopy (Radians 2000, BioRad).

In situ hybridization

In situ hybridization was performed as described (Takemoto et al., 2002) with some modifications. In brief, mRNA probes were labeled with digoxigenin-UTP by RNA in vitro transcription according to the manufacture's protocol (Roche). Cryosections were prepared as described above. The sections were air-dried and postfixed in 4% paraformaldehyde/PBS at room temperature for 5 min. They were washed with water for 3 min and then in 0.1 M triethanolamine (pH 8.0) at room temperature for 3 min. They were vigorously washed in 0.25% dehydrated acetic acid/0.1 M triethanolamine at room temperature for 10 min, followed by washing with PBS at room temperature for 3 min twice. The sections were incubated with a prehybridization solution (50% deionized formamide [GIBCO], 5× SSPE, 5% SDS, and 1 mg/ml of yeast tRNA) at 60°C. Each mRNA probe was mixed with the prehybridization solution and added to the samples. After incubation at 60°C overnight, the samples were washed with 5× SSC and then with 2× SSC containing 50% formamide at 60°C for 30 min three times. After being blocked with a blocking buffer (2× BMB [Roche], 1× TN [pH 7.5], and 0.1% Tween-20), the samples were incubated with the anti-digoxigenin Ab conjugated to alkaline phosphatase at room temperature for 2 h. The samples were washed with PBS and with the washing buffer (100 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) at room temperature for 30 min five times. The samples were incubated with the TNM-T buffer (100 mM Tris–HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$, and 0.1% Tween-20) at room temperature for 3 min. The color was developed in BM-purple solution until intense staining appeared. The preparation of the mouse afadin mRNA probe was previously described (Okabe et al., 2004). We used two mouse afadin mRNA probes (1450–1470 bp and 2211–2235 bp, antisense) and the mouse nectin-1, -2, and -3 mRNA probes (84–1035 bp, antisense, 1–1014 bp, antisense, and 162–1200 bp, antisense, respectively).

Microscopy

Immunofluorescence microscopy

Immunohistostaining was performed as described (Ozaki-Kuroda et al., 2002). The samples were observed with a confocal laser scanning microscope (Radians 2000, BioRad).

Immunoelectron microscopy

Immunoelectron microscopy, using the silver-enhanced immunogold method, was performed as described (Mizoguchi et al., 1994) with some modifications. Briefly, the whole-mount brains dissected from E12.5 embryonic mice were perfused with 0.05% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) at pH 7.4. After the immersion fixation, the samples were soaked in 25% sucrose/PBS and placed into a 1:3 mixture of 30% sucrose in PBS and OCT compound. The samples were

frozen in liquid nitrogen and stored at −80°C. The 3- to 5-μm-thick sections were incubated with a primary Ab, followed by the incubation with a secondary Ab. The secondary Abs for the anti-nectin-3 mAb and anti-nectin-1α pAb were coupled with 1.4-nm gold particles (Nanoprobes Inc.), and that for β-tubulin III was biotinylated and fixed with 1% glutaraldehyde. The sample-bound gold particles were silver-enhanced by the HQ-silver kit (Nanoprobes Inc.) at 25°C for 8 min. The ABC Kit was used for the β-tubulin III staining with DAB. The samples were washed again and postfixed with 1% osmium oxide in 0.1 M PB at 4°C for 1 h. They were stained with uranyl acetate in a moist chamber at 4°C for 30 min, dehydrated through ethanol and propylene oxide, and embedded in epoxy resin. The 50- to 70-nm ultrathin sections were cut and then observed with an electron microscope (H-7500, Hitachi).

Transmission electron microscopy

Electron microscopy was performed as described (Ozaki-Kuroda et al., 2002). In brief, flat whole-mount brains were perfused with 4% paraformaldehyde, 2.5% glutaraldehyde/0.1 M PB at 4°C overnight, cut into the longitudinally long pieces of 1 × 1 × 3 mm, and immersed in the same fixative at 4°C overnight. They were refixed with 2.5% glutaraldehyde and 1% tannic acid (Merck) at 4°C for 4 h and washed with PB overnight and with 1% osmium oxide/0.1 M PB at 4°C for 1 h. They were then stained with uranyl acetate at 4°C for 30 min. They were dehydrated by passage through a graded series of ethanol and propylene oxide, and embedded in epoxy resin. From this sample, 50- to 70-nm ultrathin sections were cut and then observed with the electron microscope (H-7500, Hitachi).

Results

Localization patterns of nectin-1 and -3 in the floor plate

We first examined the localization of nectin-1, -2, and -3, and afadin in the floor plate during the mouse embryogenesis in detail by immunofluorescence confocal microscopy. We focused on the ventral midline region of the transverse planes (Figs. 1A–C) and the coronal planes (Figs. 1D–E) of the neural tube. As early as at E9.5, the floor plate cells were differentiated in the ventral midline of the mouse hindbrain, and the immunofluorescence signal for nectin-3 started to be concentrated at the specific cell–cell contact sites in the ventral midline (Fig. 1A, bracket). The floor plate develops from anterior to posterior in general. The signal for nectin-3 was also observed at the cell–cell contact sites in one or two cell layers of the ventral midline at the thoracic level at E9.5 (data not shown), suggesting that the floor plate cells start to express nectin-3 at the hindbrain level and subsequently at the thoracic level. The signal for nectin-1 was not observed in the region of the floor plate around E9.5. Based on the previous report (Shirasaki et al., 1995), the commissural axons are expected to reach the ventral midline and cross the floor plate around E10.5 during the mouse embryogenesis. The signal for nectin-1 was first observed at E10.5 only at the basal region of the ventral midline, which was the expected ventral commissure (Fig. 1B, arrowheads). The signal for nectin-3 was always observed at the cell–cell contact sites of the midline cells themselves (Fig. 1A, bracket) and at cell–cell contact sites between the midline cells and the ventral commissure (Fig. 1B). The signals for nectin-1 and -3 in the ventral midline were the most significant around E12.5 (Fig. 1C). It is noted that the signal

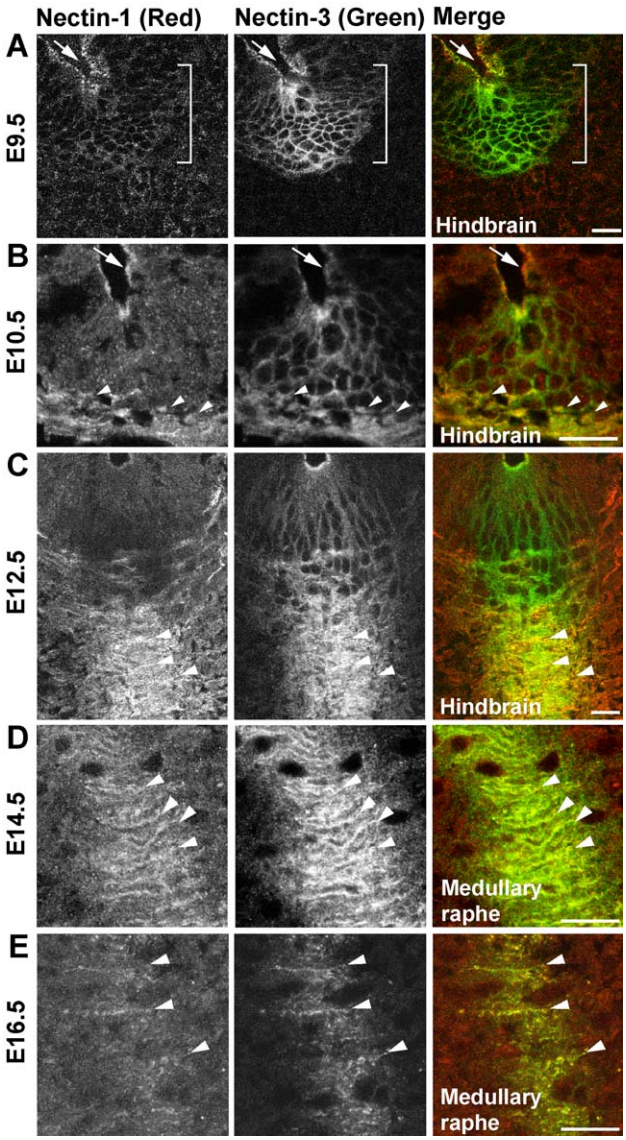

Fig. 1. Localization of nectin-1 and -3 in the floor plate. Frozen sections were obtained from the embryonic mouse hindbrains and observed by immunofluorescence confocal microscopy. (A–C) Transverse sections of the ventral midline and (D–E) coronal sections of the medullary raphe in the mouse hindbrains at (A) E9.5, (B) E10.5, (C) E12.5, (D) E14.5, and (E) E16.5 were doubly immunostained by the anti-nectin-1α pAb (red) and the anti-nectin-3 mAb (green). Brackets: the signals for nectin-3 at the cell–cell contacts. Arrowheads: the colocalization of nectin-1 and -3. Arrows: the ventricular zone. Scale bars = 20 μm.

for nectin-1 was always concentrated only in the midline regions. The concentrated signal for nectin-1 was never visible on pre- or post-floor plate trajectory outside of the floor plate (data not shown). At E14.5, the signals for nectin-1 and -3 in the ventral midline were markedly observed at medullary raphe (Fig. 1D, arrowheads). In most cases, the floor plate disappears around the neonatal stages (Prince et al., 1992). The signals for nectin-1 and -3 decreased at E16.5 (Fig. 1E, arrowheads), indicating that the expression patterns of nectin-1 and -3 in the ventral midline are apparently parallel to the developmental process of the floor plate in embryonic mice. It may be noted that the signals for nectin-1 and -3 were observed at the apical AJs of neuroepithelial cells in the ventricular zone of the neural tube from E8.5 to E14.5 and reduced in the later development (data not shown and also see Figs. 1A and B, arrows).

The signal for nectin-2 was observed in the region of the ventral commissure, but the intensity of the signal for nectin-2 was much less than those of the signals for nectin-1 and -3 (data not shown). The signal for afadin was hardly observed in the region of the ventral commissure (data not shown). On the other hand, signals for both nectin-2 and afadin were markedly observed at the apical AJs of the neuroepithelial cells in the ventricular zone of the neural tube from E11.5 to E18.5 (data not shown).

Expression patterns of the nectin and afadin mRNAs

We next examined expression patterns of the *nectin* and *afadin* mRNAs in the floor plate by in situ hybridization using transverse sections of E11.5 mice. The signal for the *nectin-1* mRNA was hardly observed in the region of the floor plate (Fig. 2A, large arrow) but observed in the neuroepithelial layer of the neural tube as well as of the dorsal root ganglia (Fig. 2A, NE and DRG). The weak signal for the *nectin-1* mRNA was also detected in the dorsal region of the neuroepithelium, in which some of the commissural neurons localize (Fig. 2A, small arrows). The signal for the *nectin-3* mRNA was prominently observed at the cell bodies of the floor plate cells (Fig. 2B, large arrow and inset). The signals for the *nectin-2* and *afadin* mRNAs were observed in the neuroepithelial layer of the neural tube (Figs. 2C and D, NE and DRG), but not prominent in the floor plate (Figs. 2C and D, large arrows). These results indicate that the floor plate cells express the *nectin-3* mRNA but not the *nectin-1*, *-2*, or *afadin* mRNA. Therefore, it is likely that the localization of nectin-1 protein in the ventral midline (Fig. 2, small arrows) is due to nectin-1 protein expressed in the commissural axon of the neurons in the dorsolateral neural tube.

Hetero-trans-interaction between nectin-1 and -3 at the contacts between the commissural axons and the floor plate cells

We examined the detailed localization of nectin-1 and -3 in the floor plate by immunoelectron microscopy. The floor plate cells elongated their basal processes toward the basement membranes and the contact surface membranes of the commissural axons as described (Campbell and Peterson,

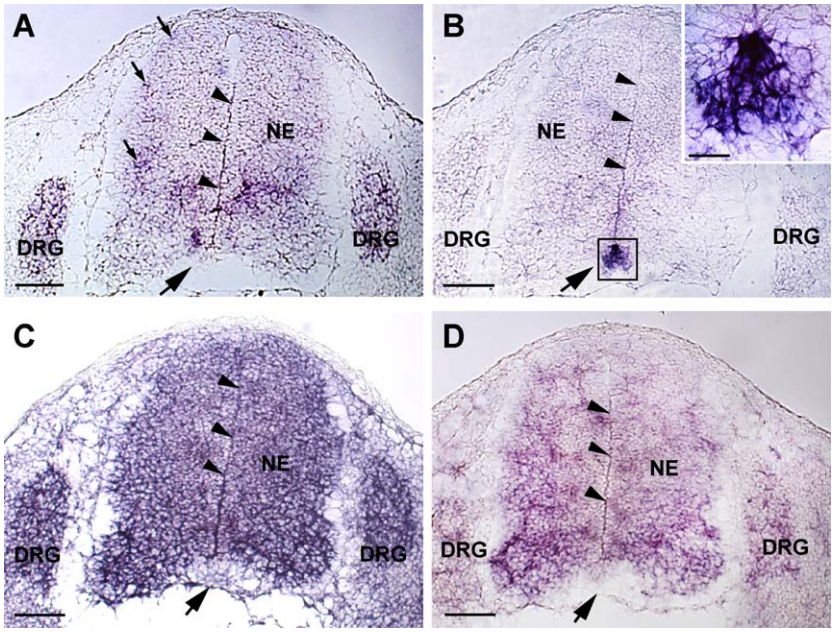

Fig. 2. Expression of the *nectin* and *afadin* mRNAs in the floor plate cells. Transverse sections of the E11.5 mouse hindbrains including the upper cervical region of the spinal cords were prepared from frozen sections, followed by in situ hybridization with the antisense *nectin-1*, *-2*, *-3*, and *afadin* mRNA probes. (A) The signals for the *nectin-1* mRNA, (B) the *nectin-3* mRNA, (C) the *nectin-2* mRNA, and (D) the *afadin* mRNA. Inset: high magnification of the box in B. Arrowhead: the ventricular zone. Large arrows: the ventral midline cells. Small arrows in A: commissural neurons. DRG, dorsal root ganglia; NE, neuroepithelial cells. Scale bars = 100 μm in A–D and 40 μm in inset in B.

1993). The feature of the contact sites between their basal processes and the axons were shown in the midsagittal sections of the E12.5 mouse hindbrain (Fig. 3). All axons were stained in dark with the anti-β-tubulin III mAb labeled with DAB. The surfaces of the DAB labeled axons were observed as circles attached to the edge of the basal process of the floor plate cell, which contained mitochondria (Figs. 3A and B). More than 80% of the immunogold particles detected by the anti-nectin-1α pAb, which recognized the cytoplasmic tail of nectin-1α, were only aligned beneath the plasma membrane of the axons that faced the floor plate cells (Fig. 3B, arrowheads). There was no signal for nectin-1 on the axons detached from the floor plate cell, on the free edge of the basal process (Fig. 3B, double-headed arrows), between the axons themselves (Figs. 3A and B, arrows), or between the basal processes of the floor plate cells (Fig. 3C, bracket). The signal detected with the anti-nectin-3 mAb, which recognized the extracellular region of nectin-3, was observed only at the space between the axon and the basal process of the floor plate cell (Fig. 3D, arrowheads). There was no signal for nectin-3 on the free edge of the basal process of the floor plate cell (Fig. 3D, double-headed arrows) or between the axons themselves (Fig. 3D, arrows). These results indicate that the signals for nectin-1 and -3 are concentrated only at the contact sites between the commissural axons and the floor plate cells in the ventral midline. We could not detect the signal for nectin-3 using our Ab against the cytoplasmic tail of nectin-3 by immunoelectron microscopy (data not shown). Based on the result that the *nectin-3* mRNA, but not the *nectin-1* mRNA, was expressed in the floor plate cells, it is most likely that nectin-3 localizes in the floor plate cells, and that nectin-1 and -3 asymmetrically localize in the commissural axons and in the floor plate cells, respectively, and form hetero-*trans*-dimers there. It may be noted that a small number of the commissural axons showed larger surfaces at the contact sites (Fig. 3C, large arrow). These sizes were bigger than those of the typical axons and they contained electron-dense materials. This structure might be a growth cone and nectin-1 might localize not only in the commissural axons but also in the growth cones.

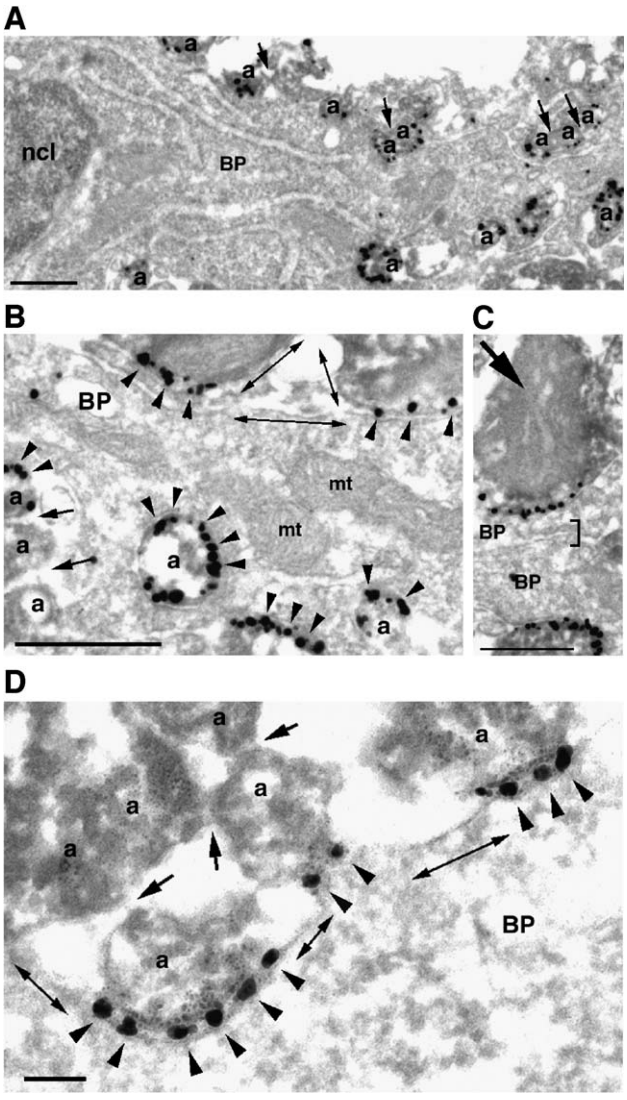

Fig. 3. Asymmetric localization of nectin-1 and -3 at the cell–cell contact sites in the floor plate. Midsagittal sections of the E12.5 mouse floor plate were prepared from the open-book preparations of the mouse hindbrains and analyzed by immunoelectron microscopy using the anti-nectin-1α pAb and the anti-nectin-3 mAb. The commissural axons were labeled with DAB against anti-β-tubulin III mAb. (A–C) Immunogold particles for nectin-1α, and (D) for nectin-3 at the contacts. Arrows: the contact sites between the commissural axons. Arrowheads: the contact sites between the commissural axons and the basal process of the floor plate cells. Bracket: the contact sites between the basal processes of the floor plate cells. Double-headed arrows: the free edges of the axons or the floor plate cells. Large arrow: a growth cone. a, axons; BP, basal processes of the floor plate cells; mt, mitochondria; ncl, nucleus. Scale bars = 500 nm in A–C and 100 nm in D.

Absence of a prominent actin cytoskeletal structure at the contacts between the commissural axons and the floor plate cells

We have recently shown that the expression level of afadin is very low in the floor plate (Okabe et al., 2004). Moreover, the expression level of the *afadin* mRNA was not significant in the floor plate cells (Fig. 2D, arrow). These results suggest that the contacts are different from the classically categorized AJs, which are undercoated with thick F-actin bundles, in epithelial cells and fibroblasts (Mandai et al., 1997; Takahashi et al., 1999). Therefore, we next examined whether the contacts are associated with F-actin-binding proteins and undercoated with thick F-actin bundles. The immunofluorescence signals for F-actin and ZO-1 were observed, but not significantly concentrated in the region of the floor plate, compared with the signals for nectin-1 and -3 that were the most prominent in the floor plate of E12.5 mice (Figs. 4A1 and A2, arrows). The signals for F-actin and ZO-1 were constantly observed at the apical end of the floor plate cells and at AJs of the neuroepithelial cells in the ventricular zone (Figs. 4A1 and A2, arrowheads). The signals for N-cadherin and β-catenin

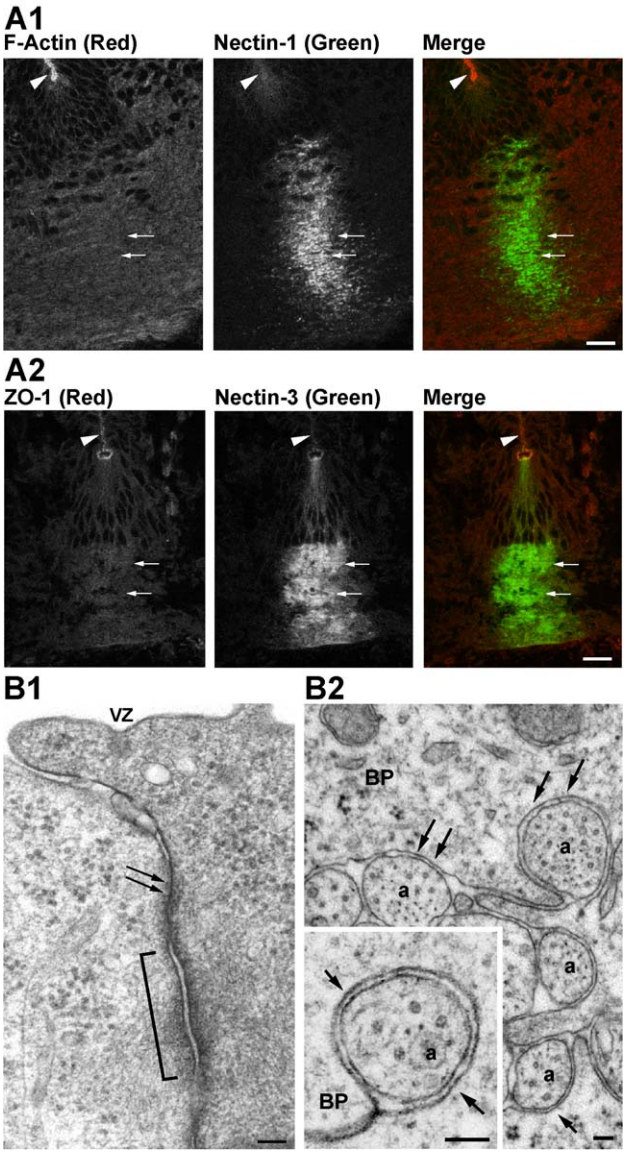

Fig. 4. Absence of prominent actin cytoskeletal structures in the floor plate. (A) Confocal laser scanning microscopy on actin cytoskeletal structures. Transverse sections of the E12.5 mouse floor plate were immunostained by the anti-nectin-1α pAb, the anti-ZO-1 mAb, and the anti-nectin-3 mAb, and F-actin was labeled with phalloidin. (A1) The signals for F-actin (red) and nectin-1 (green). (A2) The signals for ZO-1 (red) and nectin-3 (green). Arrowheads: the AJs in the ventricular zone. Arrows: the contact sites in the ventral midline. (B) Transmission electron microscopy on actin cytoskeletal structures. Sagittal sections of the E12.5 mouse floor plate were observed by transmission electron microscopy. (B1) The cell–cell junctions at the apical sites of the floor plate cells along the ventricular zone. Bracket and arrows: the electron-dense junctional complex. (B2) The contact sites of the floor plate. Inset: high magnification of the contact between the axon and the basal process. Arrows: the contact sites between the basal process of the floor plate cell and surfaces of the commissural axons. a, surfaces of the commissural axons; BP, the basal process of the floor plate cell; vz, ventricular zone. Scale bars = 20 μm in A and 100 nm in B and in inset in B2.

or other F-actin-binding proteins, including α-actinin and vinculin, were not significantly concentrated in the region of the floor plate, compared with the signals for nectin-1 and -3 (data not shown). Furthermore, a typical prominent electron-dense structure observed at general AJs was not observed by transmission electron microscopy at the contacts between the commissural axons and the basal process of the floor plate (Fig. 4B2 and inset, arrows), consistent with the earlier observations (Yaginuma et al., 1991; Yoshioka and Tanaka, 1989). These results indicate that the contacts between the commissural axons and the floor plate cells are different from the classically categorized AJs. In contrast, the supranuclear region of the floor plate cells showed electron-dense structures at the apical poles (Fig. 4B1, bracket and double-arrows). It has been shown that the apical region of the floor plate cells contains TJs (Rodriguez et al., 1996). Therefore, these electron-dense structures might be AJs and/or TJs.

Impairment by nectin inhibitors of the contacts between the commissural axons and the floor plate cells

We next examined whether the contacts between the commissural axons and the floor plate cells are inhibited by nectin inhibitors. We used a mixture of gD and Nef-3 as nectin inhibitors. gD is an extracellular fragment of glycoprotein D, an envelope glycoprotein of herpes simplex virus type 1, fused to the Fc portion of human IgG, which binds to nectin-1 and inhibits its hetero-*trans*-interaction with nectin-3 (Mizoguchi et al., 2002). Nef-3 is an extracellular fragment of nectin-3 fused to the Fc portion of IgG, which binds to nectin-1 and inhibits its hetero-*trans*-interaction with nectin-3 (Honda et al., 2003). A mixture of gD and Nef-3 inhibits the *trans*-interaction between nectin-1 and -3 more effectively than gD or Nef-3 alone (Fukuhara et al., 2002). Cerebellofugal axons begin to grow toward the ventral midline in the rat rostral hindbrain from E12 to E13, cross the ventral midline floor plate around E14, and then make a right-angled turn to elongate along longitudinal paths at a distance from the floor plate from E15 to E16 (Shirasaki et al., 1995). We prepared the flat-mount open-book rat hindbrain at E13.0 (Figs. 5A1–A3) and cultured it in the presence of the nectin inhibitors or human IgG as a control for 48 h. Transverse sections of the flat-mount open-book hindbrain (Fig. 5A3) were doubly immunostained by the anti-nectin-1α pAb and the anti-F84.1 mAb. The nectin inhibitors did not affect the localization of F84.1, a floor plate cell marker (Prince et al., 1992), in the rat floor plate cells (Figs. 5B1 and B2, red), but reduced the signal for nectin-1 in the ventral commissure (Figs. 5B1 and B2, green, arrowheads). These results indicate that the hetero-*trans*-interaction between nectin-1 and -3 in the floor plate is inhibited by the nectin inhibitors. We next prepared sagittal sections of the floor plate (Fig. 5A3) and analyzed the morphological changes by transmission electron microscopy. In the presence of control IgG, most of commissural axons did not contact each other (Figs. 5C1, arrows, and 6A) and made axon–floor plate cell (fpc) contacts (axon–fpc contacts), and a few commissural axons made a fascic-

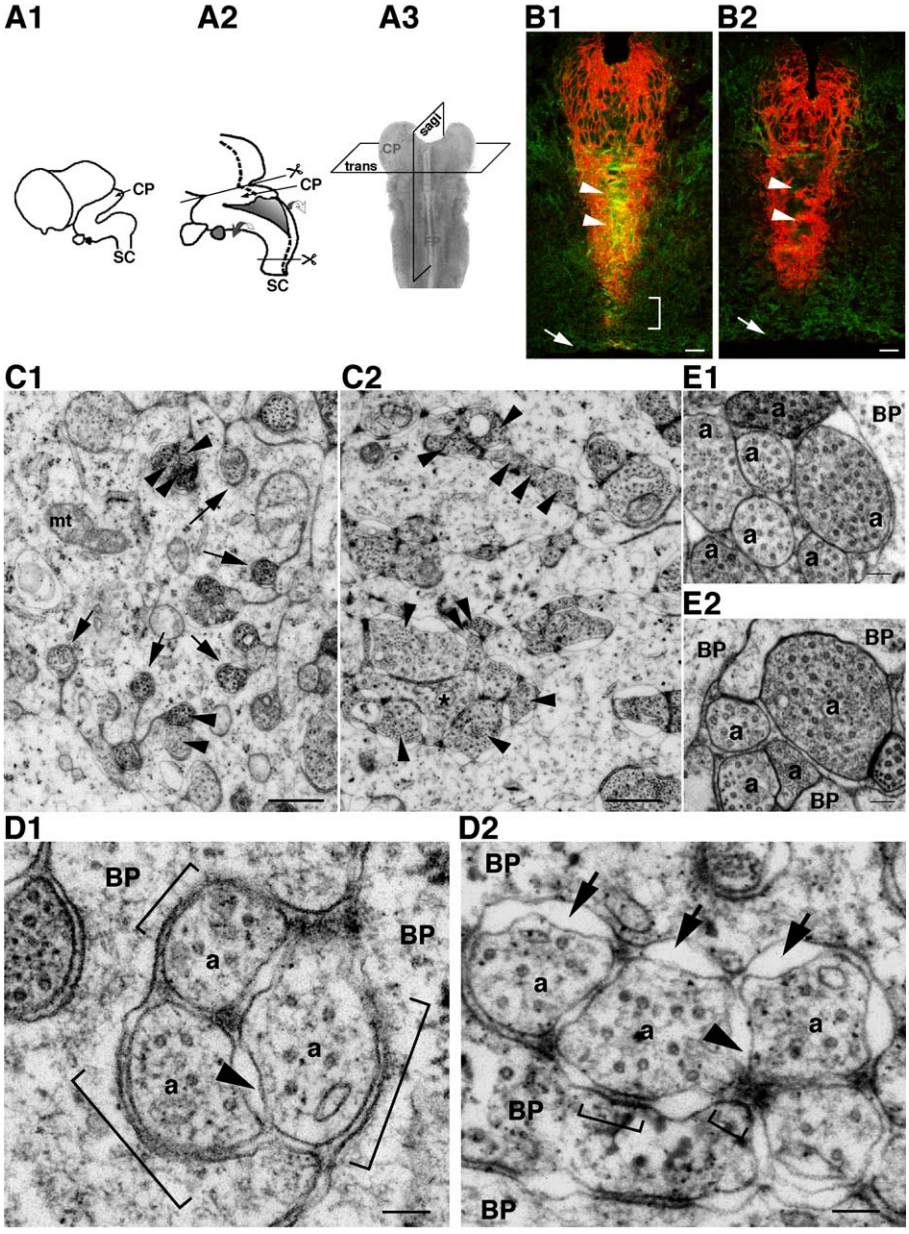

Fig. 5. Impairment of the contacts between the commissural axons and the floor plate cells by the nectin inhibitors. (A) Schematic diagram of the preparation for the flat-mounted open-book hindbrain, modified from Tashiro et al. (2000). (A1) The flat-mounted open-book hindbrains were dissected from rat embryos at E13.0. (A2) The hindbrains cut open along the dorsal midline. (A3) The flat-mounted open-book hindbrains were cultured for 48 h in the presence of 60 μg/ml of the nectin inhibitors or 60 μg/ml of human IgG as a control. After the culture, either transverse (trans) or sagittal (sagi) sections of explants were prepared. (B) Transverse sections were immunostained by the anti-nectin-1α pAb (green) and anti-F84.1 mAb (red) in the presence of IgG as a control (B1), or in the presence of the nectin inhibitors (B2). Bracket: the ventral commissure along the basement membrane. White arrows: the ventral commissure attached to the basal membrane. White arrowheads: the signals for nectin-1 and F84.1 shown in yellow in merge. Scale bars = 20 μm. (C–E) Sagittal sections were observed by transmission electron microscopy in the presence of IgG as a control (C1, D1, and E1) and in the presence of the nectin inhibitors (C2, D2, and E2). The contact sites near the floor plate cell bodies in low magnifications (C) and high magnifications (D). (C) Arrows: single axons. Arrowheads: the fasciculated axons. Asterisk: the axon surface locating in the center of the clustering. (D) Arrowheads: detachments of the axon–axon contacts. Arrows: detachments of the axon–fpc contacts. Brackets: the axon–fpc contacts. (E1, E2) The contacts near the basement membrane indicated by a white arrow in (B1). a, surfaces of the commissural axons. BP, the basal processes of the floor plate cells; CP, cerebellar plate; mt, mitochondoria. Scale bars = 100 nm.

ulation (axon–axon contacts) (Figs. 5C1, arrowheads, and 6A). In contrast, in the presence of the nectin inhibitors, most of axons made fasciculations (Figs. 5C2, arrowheads, and 6A), and the axons in the center of the cluster axons lost axon–fpc contact (Fig. 5C2, asterisk). In the presence of IgG, commissural axons were tightly attached to the basal processes of the floor plate cells with the round shapes of their surfaces (Fig. 5D1, brackets), although small spaces were observed between the commissural axons and the floor plate cells in some cases (data not shown). In contrast, in the

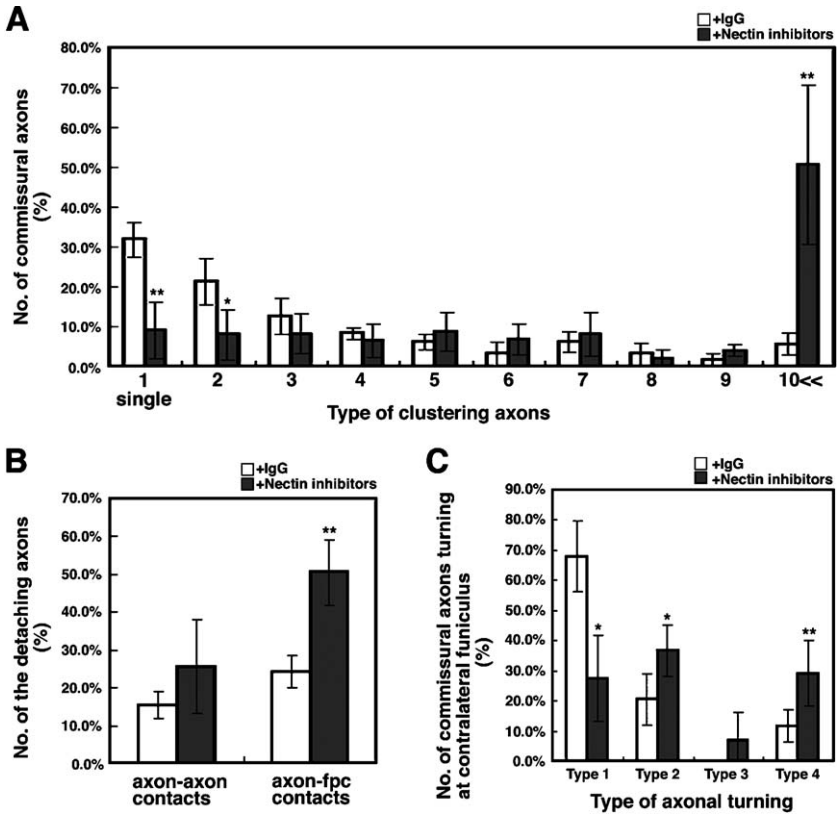

Fig. 6. Quantitative analysis of the effects of the nectin inhibitors on the contacts between the commissural axons and the floor plate cells. The flat-mounted open-book hindbrains were dissected from rat embryos at E13.0 and were cultured for 48 h in the presence of 60 μg/ml of the nectin inhibitors or 60 μg/ml of human IgG as a control. About 10 ultrastructural features (7000×) with 300–500 nm intervals were randomly picked up for each count of axons or spaces. (A) Effects on the fasciculation of the commissural axons. About 700–1000 axons were counted from each individual explant ($n = 5$ in controls and $n = 4$ in the presence of the nectin inhibitors, three independent experiments). (B) Effects on the axon–axon or axon–fpc contacts. About 30–50 of detachments were counted from each individual explant and calculated versus the total number of contacts. ($n = 5$ in controls and $n = 4$ in the presence of the nectin inhibitors, three independent experiments). (C) Effects on axonal trajectory errors. Any visible axons labeled with DiI (totally about 30 axons) were counted from each individual explant. ($n = 5$ in controls and $n = 6$ in the presence of nectin inhibitors, three independent experiments). Type 1, sharply turning axons; type 2, roundly turning axons finally reaching the longitudinal ends, type 3, returning axons to the floor plate, and type 4, randomly turning axons losing the longitudinal directions. $^*P < 0.05$, $^{**}P < 0.01$.

presence of the nectin inhibitors, the axon–fpc contacts were markedly loosened and the size and shape of the axons were variable (Figs. 5C2 and D2, arrows, and 6B), although this inhibitory effect of the nectin inhibitors was incomplete and the partial axon–fpc contacts remained (Fig. 5D2, brackets). Moreover, axon–axon contacts were loosened in the presence of the nectin inhibitors (Figs. 5D1 and D2, arrowheads, and 6B). However, the axon–fpc contacts near the basement membrane were not significantly inhibited by the nectin inhibitors (Figs. 5E1 and E2). Immunofluorescence signals for nectin-1 and -3 at the axon–fpc contacts near the floor plate cell body (Fig. 5B1, arrowheads) were more intense than that near the region along the basement membrane (Fig. 5B1, bracket), suggesting that the inhibitory effect of the nectin inhibitors is specific for the hetero-*trans*-interaction between nectin-1 and -3. Taken together, these results indicate that the hetero-*trans*-interaction between nectin-1 and -3 prevents commissural axons from clustering and induces formation of heterotypic contacts between the commissural axons and the floor plate cells.

In vitro perturbation of the trajectory of the commissural axons by the nectin inhibitors

In the last set of experiment, we examined whether the nectin inhibitors affect the trajectory of the commissural axons. We labeled the commissural axons with DiI anterogradely to observe the trajectory of the axons using the open-book preparations of the rat hindbrain (Shirasaki et al., 1995). There were mainly two types of trajectories observed in the controls. One type of trajectory (type A) is generated by the commissural axon that leaves the cerebellar plate (Fig. 7A, CP), crosses the floor plate (Fig. 7A, FP), and makes longitudinal turns to grow either rostrally or caudally (Fig. 7A, line a). The other type of trajectory (type B) is generated by the commissural axon that crosses the floor plate and straightly projects toward the dorsal region (Fig. 7A, dotted line b). This type B trajectory, without any longitudinal turn, was observed both in the presence of IgG and the nectin inhibitors (Figs. 7B1 and B2, arrows). The frequency of the type B trajectory in the presence of IgG (50.10% ± 6.29) was

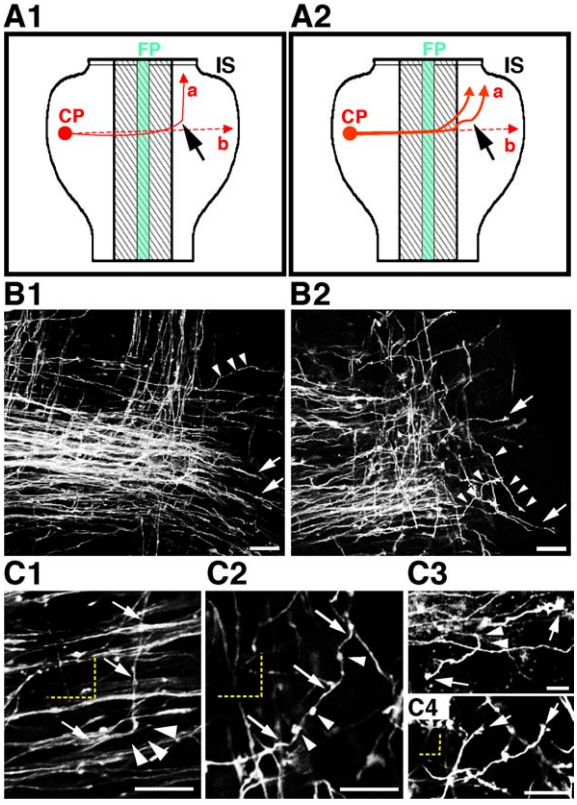

Fig. 7. Perturbation of the trajectory of the commissural axons by the nectin inhibitors. The flat-mounted open-book hindbrains were obtained from rat embryos at E13.0. They were cultured for 48 h in the presence of 60 μg/ml of the nectin inhibitors or 60 μg/ml of human IgG as a control. After the culture, DiI crystal was placed into the cerebellar plate of the fixed explant. (A) Schematic diagrams for the pial side view of the explants with trajectories of axons. Black arrows: longitudinal turns at contralateral sites. Line a: the trajectory of the turning axons. Dotted line b: the trajectory of axons without turning. A1, in the presence of IgG; A2, in the presence of the nectin inhibitors. (B) Longitudinal turns of DiI-labeled commissural axons at contralateral sites indicated by black arrows in A. B1, in the presence of IgG; B2, in the presence of the nectin inhibitors. (C1) Type 1, (C2) type 2, (C3) type 3, and (C4) type 4 axons labeled with DiI. Arrows in B: axons without turning, in C1: trajectory of type 1 axon, in C2: that of type 2 axon, and in C3 and C4: the growth cones. Arrowheads in B: type 4 axons, in C1, C3: turning points, and in C2: varicoses. CP, cerebellar plate; FP, floor plate; IS, isthmus. Scale bars = 60 μm in B and 30 μm in C.

not significantly different from that in the presence of the nectin inhibitors (50.71% ± 6.68). Therefore, the type B trajectory was considered as an artifact by in vitro culture. Then, we carefully observed the type A trajectory (Fig. 7A, line a), which was affected at the contralateral sites after crossing the floor plate by the nectin inhibitors (Figs. 7A1 and A2, arrows, B1 and B2). We therefore characterized the type A trajectory in view of the turning axons. The type A included four stereotyped trajectories: type 1 axons vertically turned at the contralateral sites (Fig. 7C1, arrows), sharply forming 90° angles (Fig. 7C1, arrowheads). Type 2 axons roundly turned at the contralateral sites and their growth cones finally reached on the longitudinal line toward the rostral or caudal end (Fig. 7C2, arrows). Type 3 axons returned back to the ventral midline even after crossing the floor plate (Fig. 7C3, arrowheads). Two different growth cones projected toward the opposite directions (Fig. 7C3, arrows). Type 4 axons moderately turned at the contralateral sites but lost the longitudinal directions (Figs. 7B1 and B2, arrowheads, and C4, arrows). We judged the trajectories by the observation using a 20× objective. Quantitative analysis (Fig. 6C) was shown by the type 1–4 axons based on the total number of axons in the type A trajectory, and axons in the type B trajectory were not included. As results, the number of type 1 axons significantly decreased in the presence of the nectin inhibitors, whereas those of types 2 and 4 axons significantly increased (Fig. 6C). Type 3 axons were observed in the presence of the nectin inhibitors, but not in the presence of IgG (Fig. 6C). The commissural axons often had varicoses in the presence of the nectin inhibitors (Fig. 7C2, arrowheads). These results indicate that the inhibition of the hetero-*trans*-interaction between nectin-1 and -3 at the contacts between the commissural axons and the floor plate cells causes failure in the longitudinal turns of the commissural axons. For additional information, we observed the pre-floor plate trajectories in the earlier development (data not shown). When the explants were cultured for 24 h, most growth cones just reached the contralateral sites after crossing the midline. None of axons made longitudinal turns yet, and the pathfindings in the presence of IgG were not significantly different from those in the presence of the nectin inhibitors (data not shown). As described above, misprojecting axons were observed after the explants were cultured for 48 h, in which the longitudinal turns of axons were observed.

Discussion

Here we have shown that nectin-1 and -3 asymmetrically localize at the contact sites between the commissural axons and the floor plate cells, respectively. We have clearly shown the evidence that nectin-1 localizes only on the commissural axons by immunoelectron microscopy with the anti-nectin-1α pAb, which recognizes the cytoplasmic tail of nectin-1α. Although we could not detect the signal for nectin-3 by the Ab against the cytoplasmic tail of nectin-3, we have shown here two lines of evidence that nectin-3 localizes on the floor plate cells: the signal for the *nectin-3* mRNA is detected in the floor plate cells, and the immunogold particles for nectin-3 was detected by the anti-nectin-3 mAb, which recognizes the extracellular region of nectin-3, localized at the space between the axon and the basal process of the floor plate cell. The localization of nectin-1 on the commissural axons is consistent with our earlier observation that nectin-1 localizes at the presynaptic side of the synapses (Mizoguchi et al., 2002). We have furthermore demonstrated here that the mixture of gD and Nef-3, the inhibitors for the hetero-*trans*-interaction between nectin-1 and -3, impairs the contacts between the commissural axons and the floor plate cells and alters the

trajectories of the commissural axons. Taken together, it is likely that nectin-1 and -3 asymmetrically localize at the contacts between the commissural axons and the floor plate cells, respectively, form hetero-*trans*-dimers, and lead to correct axonal trajectory.

Each cadherin family member mainly forms homo-*trans*-dimers (Takeichi, 1991), but each nectin family member forms both homo- and hetero-*trans*-dimers (Takai and Nakanishi, 2003; Takai et al., 2003a). Among various combinations, the hetero-*trans*-dimers of nectin-1 and -3 is the strongest and that of nectin-2 and -3 is the next strongest as estimated by the aggregation assay using an L-cell line stably expressing each nectin family member (Honda et al., 2003). We have first shown the hetero-*trans*-dimers of nectin-1 and -3 at the synapses between the mossy fiber terminals and the pyramidal cell dendrites in the CA3 area of the hippocampus (Mizoguchi et al., 2002), and then shown the hetero-*trans*-dimers of nectin-2 and -3 at the Sertoli cell–spermatid junctions in the testis (Ozaki-Kuroda et al., 2002). We have shown here the third example of the hetero-*trans*-dimers of nectin-1 and -3 at the contacts between the commissural axons and the floor plate cells.

AJs and TJs in epithelial cells are known to be associated with the actin cytoskeleton (Gumbiner, 1996). Particularly, AJs are undercoated with thick F-actin bundles. Many F-actin-binding proteins directly or indirectly bind to E-cadherin and nectin: they include afadin, β-catenin, vinculin, and α-actinin (Nagafuchi, 2001; Takai and Nakanishi, 2003). Puncta adherentia junctions are also associated with the actin cytoskeleton (Spacek and Harris, 1998), which may be mediated by afadin, α-catenin, and other F-actin-binding proteins (Mizoguchi et al., 2002; Uchida et al., 1994). At Sertoli cell–spermatid junctions, nectin-2 binds afadin and is associated with F-actin bundles surrounding the head of spermatids in Sertoli cells, whereas nectin-3 is not associated with F-actin bundles on the spermatid side (Ozaki-Kuroda et al., 2002). In contrast to these junctions, the contacts between the commissural axons and the floor plate cells may not be associated with at least F-actin-binding proteins, including afadin, ZO-1, vinculin, or α-actinin. The contacts are not associated with the prominent actin cytoskeleton visualized by transmission electron microscopy, consistent with the earlier observations (Yaginuma et al., 1991). It has been shown that the association of the cell–cell adhesion molecule with the actin cytoskeleton strengthens cell–cell adhesion (Nagafuchi, 2001). Taken together, the contacts between the commissural axons and the floor plate cells mediated by nectin-1 and -3 might be different from and not as strong as other junctions associated with the actin cytoskeleton and involved in the dynamic regulation of the developing CNS.

Both the nectin–afadin and cadherin–catenin units comprise AJs in epithelial cells and puncta adherentia junctions in neurons (Takai and Nakanishi, 2003; Takai et al., 2003b), whereas the nectin–afadin unit is a major adhesion system at Sertoli cell–spermatid junctions in the testis, and the cadherin–catenin system is unlikely to be involved in this type of junctions (Ozaki-Kuroda et al., 2002). Like Sertoli cell–spermatid junctions, we could not detect the concentrated signal for N-cadherin or β-catenin in the floor plate (data not shown). Therefore, it is likely that nectins play more important roles than N-cadherin and β-catenin at the heterotypic contacts between the commissural axons and the floor plate cells, although we cannot completely exclude the possible roles of N-cadherin and β-catenin there. It has previously been reported that αN-catenin is expressed in the commissural axons of embryonic mice (Uchida et al., 1994). The commissural neurons express N-cadherin as well as β1-class integrin and neurofascin in embryonic chicken to form homotypic axon–axon contacts and regulate the growth and fasciculation of the commissural axons (Boisseau et al., 1991; Dodd et al., 1988; Krushel et al., 1993; Shiga and Oppenheim, 1991). Therefore, N-cadherin might be involved in the growth and fasciculation of the commissural axons rather than in the contacts between the commissural axons and the floor plate cells.

We have shown that in vitro perturbation of the nectin-1- and -3-based contacts by the nectin inhibitors causes the abnormal trajectories of the commissural axons and that the defects occur after the axons have left the midline. We cannot rule out the possibility that the abnormal trajectories of the axons may be secondary to the disruption of axon–axon contacts rather than axon–floor plate cell contacts. We have found, however, that perturbation of the endogenous hetero-*trans*-interaction between nectin-1 and -3 causes abnormal fasciculation of the commissural axons. Therefore, nectins prevent abnormal fasciculation of the commissural axons by forming the axon–floor plate cell contacts.

The commissural axons and the floor plate cells communicate or transfer signals through their contact sites (Stoeckli and Landmesser, 1998). Therefore, the impairment of the nectin-1- and -3-based contacts by the nectin inhibitors may cause the axons unable to transfer signals inside the cells and result in abnormal turns and loss of the proper direction. It may be noted that the immunofluorescence signals for nectins in the floor plate are restricted to the ventral midline. Compared with the ordinary floor plate labeled with the floor plate cell marker such as F84.1, the localization of nectins is restricted in the horizontally narrow and vertically long region from the top of the hindbrain to the end of the spinal cord. Even within the floor plate region, the signals for nectin-1 and -3 are concentrated only at the cell–cell contacts in the midline. This expression pattern is very unique and different from those of soluble guidance molecules such as netrin and TAG-1 (Colamarino and Tessier-Lavigne, 1995; Kennedy et al., 1994). Nectins may be required for structurally fixing the commissural axons to the floor plate cells until the floor plate disappears. In spite of in vitro perturbation of the nectin-1- and -3-based contacts by the nectin inhibitors, the commissural axons still cross the floor plate and some can still go toward rostrally and caudally. This observation is consistent

with the previous findings that the commissural axons are navigated by multiple guidance molecules including netrin (Kennedy et al., 1994; Shirasaki et al., 1996). The inhibition of the hetero-*trans*-interaction of nectins loosens the adhesion sites and the trajectory of the axons does not become sharp. The hetero-*trans*-interaction of nectins may play a role in mechanical fixation of the commissural axons to the floor plate cells and in maintenance of the contact sites for axonal pathfindings.

Immunoelectron microscopy shows the signals for nectin-1 not only on the axons but also on growth cones of the commissural neurons at the ventral midline, indicating that nectin-1 and -3 form hetero-*trans*-dimers at the contacts even between growth cones and the floor plate cells. The motile growth cones at the tip of the axons are sensors triggered by attractive and repulsive cues in the environment, and they extend and retract filopodia and lamellipodia. We have recently shown that the *trans*-interactions of any combinations of nectins induce the formation of filopodia and lamellipodia through the respective activation of Cdc42 and Rac through c-Src in epithelial cells and fibroblasts (Shimizu and Takai, 2003). Signals are transduced bidirectionally from nectin-1 to -3 and from nectin-3 to -1. Eventually, the extension or retraction of filopodia and lamellipodia occurs at this leading edge to make growth cones to determine the direction of migration. The hetero-*trans*-interaction between nectin-1 and -3 may function in the axonal pathfindings in the floor plate. One directional signaling from nectin-3 to -1 may regulate growth cones to determine their directions. Such signaling from nectin-3 to -1 may regulate the up- or down-regulation of receptors for the guidance cues.

Beyond the focus of our current studies, it is worth knowing whether nectins are involved in the pathfindings either of the pioneer (the earlier) or the follower (the later) axons (Colamarino and Tessier-Lavigne, 1995). However, there is not any molecular marker that is specifically expressed in either axon. It might be interesting to speculate that nectins are more important for the earlier projections, which require more heterotypic manner of the cell–axon contacts, than other adhesion molecules such as N-cadherin, which are required for axonal fasciculations caused by homotypic axon–axon contacts.

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