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Netrin-1 is crucial for the establishment of the dorsal column-medial lemniscal system

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Abstract

The dorsal column-medial lemniscal system is a significant sensory pathway that mediates touch and limb position sense. In this system, axons from the second-order neurons in the dorsal column nuclei form the internal arcuate fibers, cross the ventral midline (floor plate) within the medulla oblongata, and then project to the thalamus as the medial lemniscus. Here we demonstrate that Netrin-1, which is secreted from the floor plate in the medulla oblongata, is indispensable to the formation of the dorsal column-medial lemniscal system. Axons from the dorsal column nuclei cross the midline at around embryonic day 11 in mice. Concurrently, *Netrin-1* mRNA and its receptor DCC (deleted in colorectal cancer) were expressed in the floor plate and commissural axons there, respectively. In our explant culture experiments, the floor plates of the embryonic 11-day-old mutant *Netrin-1* homozygous mice did not attract axons from the dorsal column nuclei of ICR mice, while those from the wild type littermates did. Moreover, we observed that although the dorsal column nuclei developed *in situ* in mutant mice, their axons were not attracted toward the floor plate: they did not cross midline and remained ipsilaterally, without forming the internal arcuate fibers, in embryonic 17-day-old mutant *Netrin-1* homozygous mice.

Keywords: axon guidance, chemotropic factor, lipophilic tracer, medial lemniscus, pathfinding, sensory pathway.
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The dorsal column-medial lemniscal system is one of the major sensory pathways. It conveys information from the spinal cord to the thalamus about the mechanical senses of discriminative touch, vibratory sense, and conscious muscle sense, all of which are essential for normal animal activity (Snell 2001). Ascending fibers carrying these signals from the spinal cord make synapses within the dorsal column nuclei (DCN) in the lower medulla oblongata. The axons of the second-order neurons in the DCN then project as internal arcuate fibers, crossing the midline, forming the lemniscal decussation. They finally project to the ventral posterior lateral nucleus of the thalamus on the contralateral side (Biedenbach 1972; Hand and Van Winkle 1977; Martin 2003). The definitive forms of DCN axonal trajectories to the thalamus have been studied in many species, while how they are formed or their underlying molecular mechanisms have not been well explored, despite the importance of the tract (Bovie 1978; Berkley 1980; Massopust *et al*. 1985).

It has been reported that Netrin-1 is a long-range diffusible factor that attracts certain classes of growing axons, such as the spinal commissural axons, during development (Kennedy *et al*. 1994; Mètin *et al*. 1997; Richards *et al*. 1997; Braisted *et al*. 2000; Barallobre *et al*. 2000). In *Netrin-1* mutant mice, commissural axons in the spinal cord, hippocampal commissure, and corpus callosum in the cortex are absent (Serafini *et al*. 1996). Recently, Finger *et al*. (2002) have suggested that interactions between Netrin-1 and its receptors are necessary at multiple choice points for the guidance of the corticospinal tract axons. Unlike these established circuits, the medial lemniscal system is the only one for which the molecular bases

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Abbreviations used: BSA, bovine serum albumin; DCC, deleted in colorectal cancer; DCN, dorsal column nuclei; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiA, 4-(4-(dihexadecylamino)styryl)-*N*-methyl-pyridinium iodide; E, embryonic day; PBS, phosphate-buffered saline.

of its development remain undisclosed. It is of interest whether Netrin-1 is also involved in the formation of the dorsal column-medial lemniscal system, as this system occurs late in evolution compared with another sensory system that mediates the sensation of pain and temperature, the anterolateral system, in the formation of which Netrin-1 plays a crucial role. In the study presented here, we investigated the involvement of Netrin-1 in the formation of the dorsal column-medial lemniscal system using *Netrin-1* mutant mice.

Materials and methods

Animals

Timed-pregnant female ICR mice were obtained from SLC (Hamamatsu, Japan). *Netrin-1* mutant mice were kindly provided by Dr Marc Tessier-Lavigne (Stanford University, Palo Alto, CA, USA). The day that the presence of a vaginal plug was confirmed was defined as embryonic day (E) 0. Embryos between E10 and E17 were used. The mice were housed at a constant temperature and humidity, and were provided with food and water *ad libitum*. Prior to experimental procedures, all pregnant females were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). All experiments were conducted in accordance with the guidelines for the use of laboratory animals of Fukui Medical University (Faculty of Medical Sciences, University of Fukui). Adult male Wistar rats, which were required for extracting collagen, were purchased from Keari (Osaka, Japan), and were housed and treated under the same condition as the mice.

To establish the presence of *Netrin-1* mutant alleles, Southern blot analysis was performed to confirm the presence of the *Lac Z* sequence in the mutant, as described previously (Serafini *et al*. 1996). A probe specific for *Lac Z* was synthesized from a *BamH*I/*Hind*III fragment of pBudCE4/*Lac Z*/CAT (Invitrogen, Carlsbad, CA, USA). As homozygous mutant mice cannot suckle, and die within a few days after birth, mutants were maintained only in the heterozygous form.

In principle, the procedure for genotyping embryos was the same as described previously (Serafini *et al*. 1996). In brief, RT–PCR was performed against RNAs isolated from the caudal portions of embryos containing a neural tube (E11) or from excised caudal spinal cord (E17), using the following primers: forward 5′-TGACTGTAGGCACAACACGG-3′, reverse for wild-type 5′-CTCCATGTTGAATCTGCAGC-3′, and reverse for mutant 5′-GCCTTCCATCTCAACTCTCC-3′. The difference in length between the wild allele (154 bp) and the mutant allele (312 bp) was checked with the aid of gel electrophoresis.

Tracing procedures

E10–E12 embryos were immersed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). In the case of E17 embryos, the brain and spinal cord was dissected out as a whole and immersed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). After being fixed, small crystals of the axonal tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) were injected directly into the DCN with a micropipette.

The trajectories of the axons of the medial lemniscus were visualized by injecting crystals of DiI into that brain area or, in the case of E17 embryos, crystals of 4-(4-(dihexadecylamino)styryl)-*N*-methyl-pyridinium iodide (DiA; Molecular Probes) were injected into the dorsal funiculus. Injected embryos were left in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) at 60°C for over 3 weeks to allow the lipophilic tracers to diffuse the full length of the axons. The embryos or brain/spinal cord specimens were then embedded in 3.5% agar containing 8% sucrose, and 80- or 100-μm transverse sections were cut using a vibratome. Labeled axons were photographed with the aid of a fluorescence microscope.

Photoconversion

The fine morphological structures of axons were examined in some sections containing DiA-labeled axons after subjecting them to photoconversion (Singleton and Casagrade 1996). Sections were preserved in phosphate-buffered saline (PBS, pH 7.2) at 4°C. They were flattened onto a slide glass without a coverslip, and the excess buffer on the flattened section was removed and replaced with one to three drops (or enough to cover the area of interest) of fresh 3,3′-diaminobenzidine (DAB; Dojindo Laboratories, Tokyo, Japan) solution [1.5 mg DAB/mL diluted in Tris–HCl; Tris(hydroxymethyl)aminomethane, pH 7.4]. The area of interest was then located and irradiated using an Olympus Inverted Research Microscope model IX-70 equipped with epifluorescence and a 100 W mercury bulb. The DAB solution was changed after 10 min of irradiation and then replaced with fresh, cold DAB solution every 20–30 min during the remainder of the photoconversion. The progress of the reaction was monitored by switching periodically between fluorescent and white light until a dense brown DAB product was visualized.

In situ hybridization

A conventional protocol was used for *in situ* hybridization (Nagano *et al*. 2000). In brief, 14-μm-thick frozen tissue sections were fixed with formaldehyde, acetylated with acetic anhydride, and then dehydrated. Mouse *Netrin-1* cDNA fragment (GenBank accession number NM_008744, nucleotides 1201–1793) was cloned by RT–PCR from mouse E11 hindbrain RNA extract using the following primers inserted into the pGEM plasmid (Promega, Madison, WI, USA) that was then used as a template plasmid: forward 5′-AAAGCCTGTGATTGCCACCC-3′ (nucleotides 1201–1220), reverse 5′-CCCTTCTTCTCCCGTTGCTG-3′ (nucleotides 1774–1793). [α-^{35}S]UTP-labeled, single-stranded RNA synthesized with the *Nco*I-digested template plasmid containing the *Netrin-1* cDNA fragment and SP6 RNA polymerase, and that synthesized with the *Not*I-digested template and T7 polymerase were used as antisense and sense probes, respectively. Hybridization signals were visualized by macro-autoradiography (film autoradiography) and micro-autoradiography (emulsion autoradiography).

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde containing 0.1 M phosphate buffer (pH 7.4) for 3 h, embedded in 3% agarose, then 50-μm transverse sections were cut using a vibratome. Sections were pre-treated for 30 min with 0.3% hydrogen peroxide, 1% Triton X-100 following 10% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA), 1% Triton X-100 in PBS. They were then incubated with an anti-deleted in colorectal cancer (DCC) antibody (BD PharMingen, San Diego, CA, USA) diluted at 1 : 200 overnight at 4°C. The second antibody was a biotinylated goat

anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 200. Sections were then processed with a Vector ABC kit according to the manufacturer's instructions. The horseradish peroxidase reaction was completed with DAB (0.05% in PBS) and 0.03% hydrogen peroxide. Alternate sections were incubated without the primary antibody as a control.

Explant cultures and immunostaining their neurites

The floor plate and the DCN in the lower medulla oblongata were dissected out from E11 embryos. Explants were embedded in rat collagen gel matrix and cultured in Dulbecco's modified Eagle medium/F-12 medium (Invitrogen) containing 10% fetal bovine serum (Cansera, Etobicoke, Ontario, Canada) at 37°C under 5% CO_2. Culture procedures were the same as described previously (Sato *et al*. 1994). The distance between the floor plate and the DCN was 200–500 μm. DCN explants were cultured alone as a control. Rat collagen was purified from the tails of Wistar rats, as described previously (Heffner *et al*. 1990). These explants were fixed with 4% formaldehyde containing 0.1 M phosphate buffer after 48 h of culturing.

To quantify the neurite-inducing and outgrowth-promoting effects of the floor plates, the number of neurites out of the explants and the length of these neurites were measured. Prior to the measurement, the closest point to the floor plate was identified in each DCN explant. Next, the focus of the microscope was adjusted so that the largest number of neurites were observed. Then, neurites of the DCN explant, extending from the side spanning 500 μm with this selected point in the middle in that focal plane, were studied.

For immunostaining, some sets of the explants were cultured on the 3.0-μm pore collagen coated membrane (Transwell; Corning, NY, USA). These explants were washed as a whole with membrane, then fixed with 4% formaldehyde containing 0.1 M phosphate buffer for 1 h. They were pre-treated with 10% BSA (Sigma-Aldrich), 1% Triton X-100 in PBS. They were then incubated with an anti-DCC antibody (BD PharMingen) diluted at 1 : 200 overnight at 4°C. The second antibody was a goat anti-mouse IgG-Cy2 diluted 1 : 200. Alternate membranes were incubated without the primary antibody as a control. They were photograghed with the aid of a fluorescence microscope.

Results

The majority of axons from the DCN crossed the midline at around E11

First of all, we examined the point in time when the axons from the DCN cross the midline. The lipophilic tracer DiI was injected into the region where the DCN will appear at a later stage of development, of E10, E11, and E12 mice ($n = 2$ each). The position of the future DCN was determined based on the data of Altman and Bayer (1995) and Kaufman (1994). At these developmental stages, the dorsal closure of the medulla is not completed, thus DiI was injected into the lower dorsal one-third portion of the medulla oblongata, where the DCN is located longitudinally (Fig. 1a). At E10, no axons were recognized to cross the midline (Fig. 1b). In E11 mice, several axons were observed to cross the midline, forming the lemniscal decussation, then turn rostrally (Fig. 1c). In E12 mice, numerous axons were observed to cross the midline, then turn rostrally (Fig. 1d). Concomitantly, the expression patterns of *Netrin-1* mRNA and its receptor, DCC, were investigated. At E11, *Netrin-1* mRNA expression was abundant in the floor plate of the lower medulla oblongata, and DCC was observed immunohistochemically in the axons that cross the midline (Figs 1e, i and j). Signals of *Netrin-1* mRNA expression were detected, while the intensity of DCC-immunoreactivity in the crossing axons was very weak on E12 (Figs 1f, k and l). *Netrin-1* mRNA expression in the floor plate decreased gradually in later stages (E14; Fig. 1g, E16; Fig. 1h).

DCN neurites were induced and enhanced elongation by the floor plates of the wild-type but not the *Netrin-1* homozygous mutant mice

Because Netrin-1 in the floor plate plays an essential role in attracting commissural axons in the spinal cord, it is assumed that Netrin-1 is also pivotal for the formation of the internal arcuate fibers-medial lemniscal decussation by acting on the DCN axons. To explore this possibility, floor plates in the lower medulla oblongata of E11 *Netrin-1* mutant mice and the DCN from E11 ICR mice were cut into small pieces and then co-cultured in collagen gel. After culturing for 48 h, neurites from the DCN explants were observed. Based on their morphological features, almost all neurites out of explants were axons. In the case of the floor plates of wild-type littermates (fp+/+), the majority of neurites from the DCN explants projected to the floor plates ($n = 4$, Fig. 2a). The DCN neurites also extended toward the floor plates obtained from *Netrin-1* heterozygous mutant mice (fp+/–), even in the presence of fp+/+ (Fig. 2b). By contrast, floor plates taken from the *Netrin-1* homozygous mutant mice (fp–/–) did not attract DCN neurites. As shown in Fig. 2(c), despite the fact that fp–/– was placed closer to the DCN explant than fp+/–, most DCN neurites projected toward the fp+/−. Furthermore, expression of DCC in the DCN neurites were studied, then they exhibited DCC immunoreactivity (Fig. 2d). Then, neurite inducement and neurite outgrowth-promoting effects of the floor plate were quantified by measuring the number and the length of the DCN neurites with or without the floor plate explant (Figs 2a and e). When DCN were cultured with floor plates, the mean number (± SEM) of extended neurites from the DCN explant was 44.3 ± 5.8 ($n = 4$), while 14.8 ± 1.3 when the DCN was cultured alone ($n = 4$). The mean neurite length of DCN axons in the presence of floor plates was significantly longer (318.9 ± 38.4 μm) than that in the absence of floor plates (117.2 ± 20.8 μm, Figs 2f and g). These *in vitro* findings clearly show that Netrin-1 plays an important role for the initiation and outgrowth-promotion of the DCN neurites.

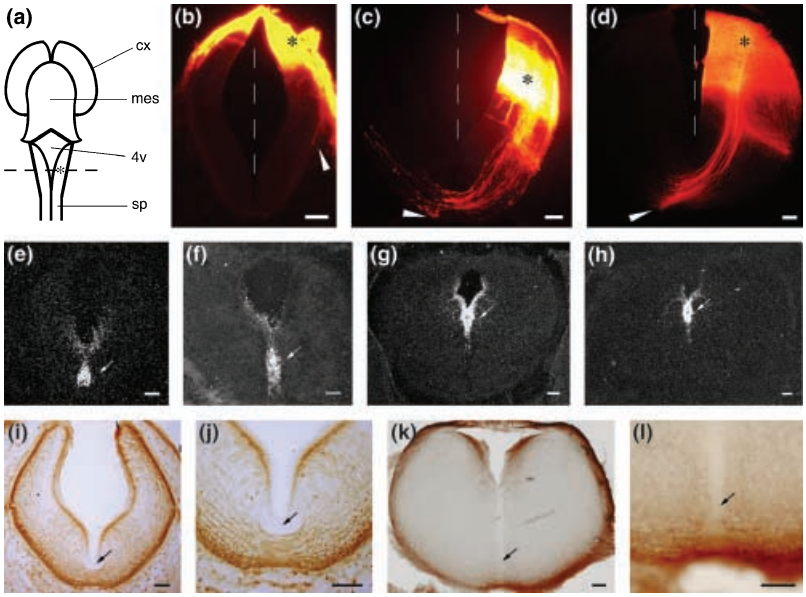

Fig. 1 Axons from the dorsal column nuclei (DCN) cross the midline on E11 in a mouse. Simultaneously, *Netrin-1* mRNA and its receptor DCC (deleted in colorectal cancer) are expressed in the floor plate and commissural axons, respectively. (a) A schematic dorsal view of the brain and the upper cervical spinal cord of E10–12 mice; because of the pontine flexure, the cortex (cx) is seen behind the mesencephalon (mes) in this view. The dotted line indicates the level of the lower medulla oblongata at which transverse sections were made (b–l); 4v, the fourth ventricle; sp, spinal cord. (b–d) Transverse sections taken at the level of the lower medulla oblongata show that the labeled axons from the DCN (arrowhead) cross the midline (dotted line) then turn rostrally as the development advances. Asterisks (a–d) indicate the site of the DCN at which DiI was injected. At E10, axons were barely labeled and no axons were observed to cross the midline (b). The labeled axons from the DCN crossed the midline and then turn rostrally at E11 (c). Numerous crossing axons were observed at E12 (d). (e) Strong expression of *Netrin-1* mRNA was observed in the floor plate (white arrow) at E11. In later stages, *Netrin-1* mRNA expression is still observed, but the signal intensity is gradually weakened and the positive region is getting smaller (f: E12; g: E14; h: E16). (i) DCC-immunoreactivity was detected on the commissural axons at E11. A higher magnification view of the site at which commissural axons cross the midline is shown in (j). At E12, DCC-immunoreactivity decreased much (k; l is a higher magnification view of k). Black arrows indicate the floor plates (i–l). Scale bars = 100 μm.

DCN was located in the lower medulla oblongata in both wild-type and *Netrin-1* homozygous mutant mice

Axonal tracers were injected into the dorsal funiculus and the medial lemniscus of E17 *Netrin-1* mutant mice in order to determine the DCN locale and to visualize the axonal trajectories of the medial lemniscal pathway in the absence of Netrin-1 (Fig. 3a). The dorsal funiculus terminates within the DCN. As *Netrin-1* mutant mice die neonatally, experiments were carried out using mice in the late embryonic stage. Moreover, E17 was chosen in order to observe the formation of DCN axonal pathways as close to the mature form as possible. DiA was injected into the dorsal funiculus, while DiI was injected into the medial lemniscus of the contralateral side. In the wild-type littermates (control), DiI-labeled medial lemniscal axons were traced retrogradely back to the contralateral side, and neurons located at the dorsal medulla were filled with DiI (fibers are shown in red, Fig. 3b). The labeled neurons were localized in the DCN. In the *Netrin-1* homozygous mutant mice, no DiI-labeled axons crossed the midline (also shown in red, Fig. 3c). DiA-labeled axons of the dorsal funiculus were observed to terminate in the dorsal side of the lower medulla oblongata in both the wild-type littermates and the homozygous mutant mice (fibers are shown in green, Figs 3b and c). In the homozygous mutant mice, some ectopic fibers were labeled (Fig. 3c, small arrowheads) and DiA also labeled some of the adjacent blood vessel systems. Photoconversion was performed to allow visualization of the fine morphology of the DiA-labeled dorsal funiculus axons. The dorsal funiculus fibers projected to the dorsal lower medulla oblongata, there being some spread in both the wild-type and the *Netrin-1* homozygous mutant mice (Figs 3d and e). In the wild-type mice, axons fanned out to end with fine fibers, while in the *Netrin-1* mutant mice, axons appeared to be slightly deformed, having a frizzled appearance (Figs 3d and e). These findings suggest that *Netrin-1* homozygous mutant mice have no contralaterally projecting medial lemniscal axons of the DCN, as seen in the controls, although the dorsal funiculus and the DCN were in evidence.

DCN axons did not cross the midline in *Netrin-1* homozygous mutant mice

To confirm further that DCN axons do not cross the midline in the absence of Netrin-1, anterograde tracing of DCN axons

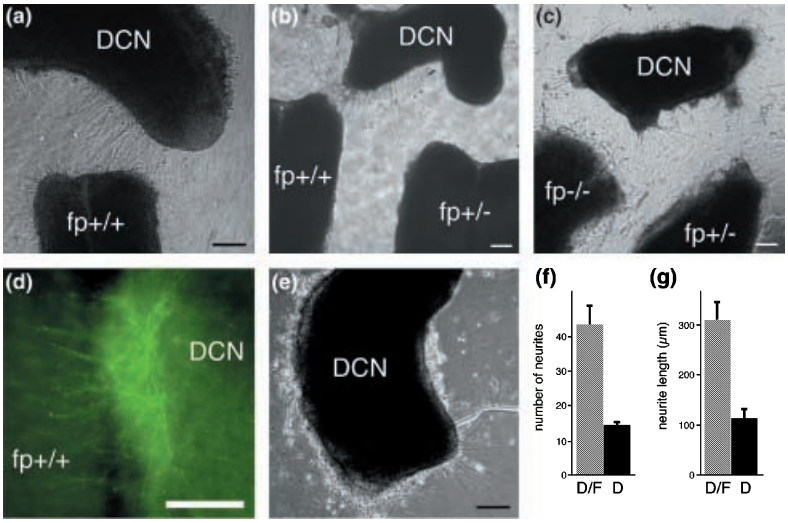

Fig. 2 Netrin-1 from the floor plate plays an important role for the initiation and outgrowth-promotion of the DCN neurites *in vitro*. (a) Many DCN neurites extended toward the floor plate of wild-type (fp+/+). (b) DCN neurites extended toward the floor plate of wild-type (fp+/+) and the floor plate of *Netrin-1* heterozygous mutant mice (fp+/–). (c) DCN neurites were not attracted toward the floor plate of *Netrin-1* homozygous mutant mice (fp–/–), although they were attracted toward the floor plate of the *Netrin-1* heterozygous mutant mouse (fp+/–), despite fp+/– being located further away than fp–/–. (d) DCC-immunopositive neurites out of the DCN explant extended toward the floor plate of wild-type. Please note that the floor plate is DCC-negative. (e) DCN were cultured alone as a control. (f, g) The quantitative data of the number of neurites (f) and the neurite length (g) out of the DCN explant with or without a floor plate explant ($n = 4$ each). Mean number and length of extended neurites from the DCN explants were calculated in each culture, then results of four independent experiment were averaged. Representative photographs are shown in (a) and (e); D/F, DCN with a floor plate; D, DCN only for control. Values are means with SEM. Floor plates and DCN explants were taken from E11 embryos (a–e). Scale bars = 200 μm.

using DiI was performed in E17 mice. The injection site of DiI is shown in Fig. 4(b), which is adjacent to the area postrema. The appropriate injection site was determined based on the termination sites of DiA-labeled dorsal funiculus (Fig. 3). In the *Netrin-1* heterozygous mice, the internal arcuate fibers were clearly labeled. Fibers were highly fasciculated, forming the lemniscal decussation (Fig. 4a; a schematic view Fig. 4b). In the *Netrin-1* homozygous mutant mice, labeled axons were much less fasciculated and did not cross the midline, although a few apparent randomly extending axons that were close to the midline were recognized, but they failed to cross it (Fig. 4c). These data indicate that DCN axons fail to cross the midline in the absence of Netrin-1.

Discussion

Using lipophilic tracers, the morphology of the medial lemniscal pathway was clearly visualized, and it was demonstrated that Netrin-1 is involved in the formation of the dorsal column-medial lemniscal system, especially in the formation of the internal arcuate fibers and lemniscal decussation. It has been demonstrated in the rat that Netrin-1 attracts commissural axons in the lower brain stem and that, once they cross the midline, they crucially trun rostrally (Shirasaki *et al*. 1995, 1998; Shirasaki and Murakami 2001). These data are consistent well with our findings of mice, although it is open whether DCN axons are included in such commissural axons or not. Although there are currently no specific markers for the DCN, our use of lipophilic tracers proved advantageous for confirming the position of the DCN. Moreover, with the aid of explant culture experiments, we first confirmed the universal neurite-promoting, or apparently axon-promoting, effect of Netrin-1 on DCN neurites (Kennedy *et al*. 1994; Richards *et al*. 1997; Braisted *et al*. 2000).

Neurogenesis in the DCN has been studied extensively in rats; neurons in the DCN are generated in the most caudal part of the medulla oblongata on E11–E13 in the rat (Altman and Bayer 1980). In mice, we observed that most DCN axons begin to cross the midline at around E11. Therefore, considering that the developmental course of mice generally occurs approximately 1–2 days earlier than that of rat during middle and late embryogenesis, it is likely that axons from the DCN begin to extend immediately after birth, and then cross the midline soon after. It has been reported that commissural axons in the spinal cord also begin to project just after birth, and numerous axons cross the midline on E13 in rats and on E11 in mice (Keino-Masu *et al*. 1996; Serafini *et al*. 1996).

In the lower rhombic lip, several kinds of progenitor neurons are generated, such as neurons in the pontine nuclei, external cuneate nuclei, lateral reticular nuclei, and olivary nuclei, which are grouped together into the pre-cerebellar

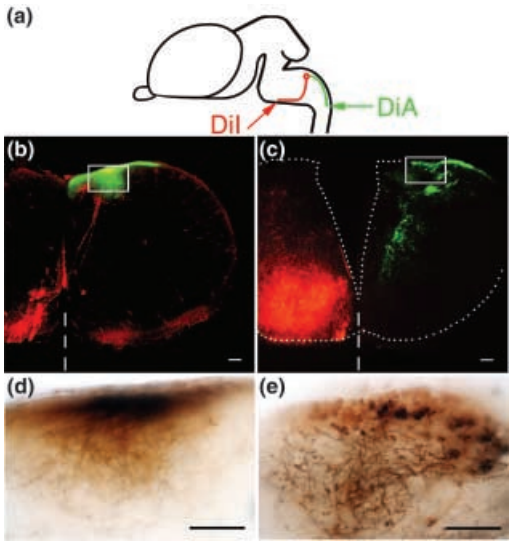

Fig. 3 The DCN was located at the dorsal lower medulla in both the normal and the *Netrin-1* mutant mice. (a) Schematic lateral view of the E17 mouse CNS. Arrows indicate where lipophilic tracers were injected. DiI was injected into one side of the medial lemniscus (red arrow), while DiA (green arrow) was injected into the opposite side of the dorsal funiculus. The red circle indicates the site of the DCN. (b) Transverse section of the lower medulla of the wild-type mouse. The medial lemniscus was traced back to cell bodies in the DCN in the lower medulla by labeling with DiI. In addition, DiA labeled dorsal funiculus axons that terminated in the DCN. The midline is shown by a dotted line. The boxed area in which fibers of both the dorsal funiculus (green) and the medial lemniscus (red) terminate is the DCN. (c) Transverse section of the lower medulla of the *Netrin-1* homozygous mouse. Fibers were labeled as in (b). In comparison with the wild-type, no DiI-labeled fibers (red) were observed to cross the midline (dotted line). (d, e) DiA-labeled fibers, shown in the boxed areas in (b) and (c), were photoconverted (d and e, respectively). Fibers of the dorsal funiculus spread and terminated in both the wild-type and mutant mice. Some blood vessels were stained randomly by the diffusion of DiA (arrow). Scale bars = 200 μm.

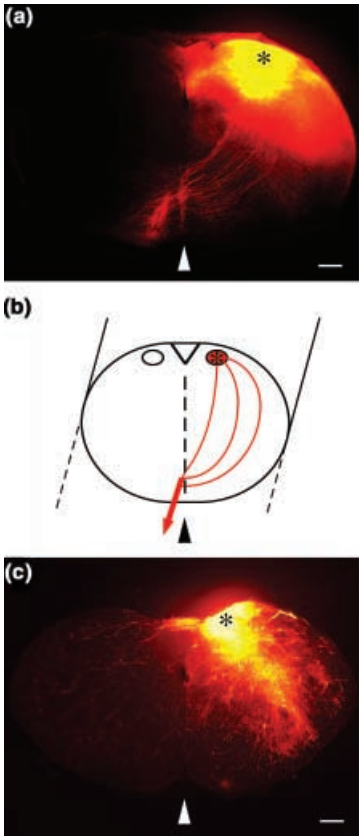

Fig. 4 DCN axons of the *Netrin-1* homozygous mutant mouse did not cross the midline. (a) Transverse section from an E17 *Netrin-1* heterozygous mutant mouse at the lower medulla. DiI was injected into the DCN, and labeled axons crossed the midline. (b) Schematic view of (a). DiI-labeled DCN axons form internal arcuate fibers, cross the midline and then project rostrally, forming the medial lemniscus (red arrow). (c) Transverse section from an E17 *Netrin-1* homozygous mutant mouse. DiI-labeled DCN fibers failed to cross the midline. Labeled axons were less fasciculated in the mutant compared with the control (a). Asterisks indicate the site of the DCN (where DiI was injected). Arrowheads (a–c) indicate the midline. Scale bars = 200 μm.

system (Rodriguez and Dymecki 2000). In rats, these nuclei in the brainstem are generated sequentially over the E12–E18 period, and neurons in the external cuneate nuclei are generated on E14 (Altman and Bayer 1987a,b). By contrast, the DCN is generated in the most caudal part of the medulla oblongata on E11–E13 in rats (Altman and Bayer 1980). As explants of DCN were taken from the most caudal part of the medulla oblongata of E11 mice, the involvement of pre-cerebellar neurons, especially the external cuneate nuclei neurons, was minimized in our explant culture experiments using E11 mouse explants. The region from which the explants were taken was not the where most pre-cerebellar neurons are generated, and the external cuneate nuclei, which are the closest to the DCN, are unlikely to be generated on E11 in mice, considering the 1- to 2-day difference in embryogenesis that exists between mice and rats, as mentioned above. If any neurons of the pre-cerebellar system were included in the explants, it is highly likely that they migrated out into the collagen gel by being attracted by the Netrin-1 from the floor plates (Yee *et al*. 1999; Alcántara *et al*. 2000). Because we did not observe such migrating cells, it is unlikely that the pre-cerebellar neurons, the axons of which may be attracted by Netrin-1, were included in the DCN explants in our experiments.

It has been demonstrated that the pre-cerebellar system is established by migration; the pontine nuclei migrate on the extramural migratory stream and the inferior olivary neurons migrate in the submarginal stream from the rhombic lip (Altman and Bayer 1987a,b). It has been suggested that Netrin-1 is involved in these migrations of the pre-cerebellar system (Bloch-Gallego *et al*. 1999; Yee *et al*. 1999; Alcántara *et al*. 2000; de Diego *et al*. 2002). These nuclei are absent or

disorganized in *Netrin-1* homozygous mutant mice. For instance, the pontine nuclei are completely absent and the inferior olivary nuclei are disorganized (Serafini *et al*. 1996; Bloch-Gallego *et al*. 1999). However, as the DCN do not belong to the pre-cerebellar system, they are not involved in these migrations. We certainly observed that the DiA-labeled fibers of the dorsal funiculus terminate in the dorsal part of the caudal medulla oblongata in the *Netrin-1* homozygous mutant mice, as they do in the DCN in the wild-type littermates (Fig. 3). It has been demonstrated that the dorsal funiculus ascends in the dorsal part of the spinal cord in *Netrin-1* homozygous mutant mice, although the location in the spinal cord is slightly different from wild-type mice because of the defect of the corticospinal pathway in the mutant mice (Finger *et al*. 2002). Furthermore, in these homozygous mutant mice, the axonal extension was clearly visualized by the injected DiI (Fig. 4). It is of interest that some DiI-labeled axons extending from the dorsal medulla oblongata, apparently including the DCN, projected ventrally, although the trajectories were very tortuous in the *Netrin-1* homozygous mutant mice (Fig. 4), suggesting that there exist other molecular machineries that repel or attract axons in the dorsoventral direction. It appears therefore that the DCN are generated *in situ* in *Netrin-1* homozygous mutant mice. However, the morphology of the axons of the dorsal funiculus, which reached the dorsal part of the caudal medulla oblongata, were not exactly the same between the wild type and *Netrin-1* homozygous mutant mice. Although small number of labeled axons were seen to project in different directions, and a small disturbance of the pathfinding of the dorsal funiculus might have occurred, most axons terminated in the dorsal part of the caudal medulla oblongata in the homozygous mutant mice.

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