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SDF1/CXCR4 signalling regulates two distinct processes of precerebellar neuronal migration and its depletion leads to abnormal pontine nuclei formation

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The development of mossy-fibre projecting precerebellar neurons (PCN) presents a classical example of tangential neuronal migration. PCN migrate tangentially along marginal streams beneath the pial surface from the lower rhombic lip to specific locations in the hindbrain, where they form precerebellar nuclei. Among them, the pontine neurons follow a stereotypic anteroventral-directed pathway to form the pontine nuclei in the pons. The guidance mechanisms that determine the marginal migration of PCN and the anterior migration of pontine neurons are poorly understood. Here, we report that a chemokine SDF1 (also known as CXCL12) derived from the meningeal tissue regulates the migratory pathways of PCN. PCN are chemoattracted by the meningeal tissue, an effect that is mimicked by an SDF1 source. Analysis of knockout mice for the *Sdf1* receptor *Cxcr4* shows that both the marginal migration of PCN and the anterior migration of pontine neurons are disrupted. We provide further evidence that SDF1/CXCR4 signalling regulates these two processes cell-autonomously. As a result of disrupted neuronal migration, pontine nuclei formation was highly abnormal, with the presence of multiple ectopic pontine clusters posteriorly. The ectopic pontine clusters led to ectopic collateral branch formation from the corticospinal tract. Our results together demonstrate crucial roles for SDF1/CXCR4 in multiple aspects of PCN migration and highlight the deleterious consequence of derailed migration on proper nuclei formation. Furthermore, we provide the first in vivo evidence that pontine neurons themselves induce collateral branching from the corticospinal axons.

KEY WORDS: Precerebellar neuronal migration, Chemokine, SDF1/CXCR4, Pontine nuclei, Chemoattraction, Corticospinal tract

INTRODUCTION

To build a functional nervous system depends critically on the migration of newborn neurons from proliferative zones to their final destinations, where they assemble into nuclei, columns and layers, and integrate into neuronal circuits. Tangential migration is essential for the formation of neuronal assemblies that are tangentially displaced from their birthplaces. This is well documented for cortical interneurons (Marín and Rubenstein, 2001) and hindbrain precerebellar neurons (Bloch-Gallego et al., 2005). Theoretically, there are multiple paths neurons can take to move from their birthplace to their final settlement. In reality, however, tangential migration takes place along stereotypic pathways, not only well-defined for their anteroposterior (AP) and dorsoventral (DV) directions, but also their depths within the ventriculopial (VP) span of the neuroepithelium (Marín and Rubenstein, 2001; Sotelo, 2004; Bloch-Gallego et al., 2005). The mechanisms underlying the specification of tangential pathways are only partially understood (Hatten, 2002; Park et al., 2002; Bloch-Gallego et al., 2005). Furthermore, the importance of taking a specific tangential pathway for the subsequent formation of nuclei, layers and columns remains elusive.

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The migration of hindbrain precerebellar neurons presents an excellent model to address these questions, because the tangential migratory pathways and their resultant nuclei have been clearly mapped out and are genetically tractable (Altman and Bayer, 1997; Wingate and Hatten, 1999; Kawauchi et al., 2006; Taniguchi et al., 2006). Precerebellar neurons migrate over long distances, from their germinative zone at the lower rhombic lip (LRL), along distinct tangential pathways to form discrete precerebellar nuclei (see Fig. 1A). The mossy-fibre-projecting precerebellar neurons (PCN) migrate marginally beneath the pial surface of the hindbrain, forming two streams: the anteroventrally directed anterior extramural stream (AES); and the ventrally directed posterior extramural stream (PES). Although the ventral directions of the AES/PES have been attributed to the Netrin and SLIT/ROBO families (Serafini et al., 1996; Yee et al., 1999; Alcantara et al., 2000; Taniguchi et al., 2002; Marillat et al., 2004; Di Meglio et al., 2008), the mechanisms that control the marginal positioning of AES/PES cells, as well as the anterior direction of the AES, are poorly understood.

PCN in the AES and PES migrate abutting the pial meninges (Bourrat and Sotelo, 1990; Altman and Bayer, 1987a; Altman and Bayer, 1987b; Ono and Kawamura, 1990; Kawauchi et al., 2006), which are a source of the potent chemotactic molecule, chemokine stromal cell-derived factor 1 (SDF1; CXCL12 – Mouse Genome Informatics) (McGrath et al., 1999; Tissir et al., 2004). SDF1 and its receptor CXCR4, which were originally identified for their role in leukocyte trafficking (Nagasawa et al., 1996; Tachibana et al., 1998), are employed during the development of the nervous system for regulating the chemotaxis of neurons and axons (Tran and Miller, 2003; Lazarini et al., 2003; Stumm and Hollt, 2007; Li and Ransohoff, 2008). Thus, we hypothesised that the meningeal SDF1 might control PCN migration by confining them to the marginal pathways.

Here, we demonstrate that the meningeal SDF1 and its receptor CXCR4 are critically involved in two distinct aspects of PCN migration: confining the migrating cells to marginal streams, and promoting the anterior migration of AES cells. We also show that disruption of SDF1/CXCR4 signalling results in markedly abnormal formation of the AES-derived pontine nuclei.

MATERIALS AND METHODS

Animals

The generation of *Cxcr4* and *Sdf1* knockout, floxed *Cxcr4*, and *Wnt1-Cre* mice have been described previously (Tachibana et al., 1998; Nagasawa et al., 1996; Tokoyoda et al., 2004; Danielian et al., 1998). Noon of the day on which a vaginal plug was detected was designated as embryonic day (E) 0.5. For expression studies, timed pregnant ICR mice (Nihon SLC, Shizuoka, Japan) were used. All animal maintenance and manipulations were carried out in accordance with the Guidelines for Animal Experiments at Osaka University.

Plasmids and expression vectors

A plasmid with nucleotide 162-1072 of *Cxcr4* mRNA (Accession number D87747) was used to generate riboprobes for in situ hybridisation. The *Mbh2* (*Barhl1* – Mouse Genome Informatics) plasmid was a gift from Dr Tetsuichiro Saito (Chiba University, Japan).

Expression vectors *pCAGGS-Egfp* (Niwa et al., 1991; Hatanaka and Murakami, 2002) and *pCAGGS-Sdf1* (Tanaka et al., 2009), expressing EGFP and SDF1α respectively, were described before. *pCAGGS-Cxcr4-IRES-Egfp* was constructed to co-express *Cxcr4* and *Egfp*.

In vitro electroporation and organotypic culture

In vitro electroporation of DNA into the LRL of E12.5 mouse hindbrains was performed essentially as previously described (Taniguchi et al., 2002). Whole hindbrains were cultured with or without the meninges in open-book configurations on Millicell culture inserts (0.4 μm pore size, Millicell-CM, Millipore). The culture medium contained DMEM-F12 (Sigma-Aldrich), 10% fetal bovine serum (GIBCO, Invitrogen) and 1× N2 supplement (GIBCO, Invitrogen). After 2 days in vitro, the cultured hindbrains were fixed for 2 hours in 4% paraformaldehyde (0.1 M PBS, pH 7.4), and frozen transverse sections (20 μm) were obtained for observation.

In utero electroporation

In utero electroporation was performed as previously described (Hatanaka et al., 2004) with the exception that 2 μl of expression vectors (between 0.5 mg/ml and 2.5 mg/ml) was injected into the fourth ventricle of E12.5 embryos.

Explant co-culture in a three-dimensional matrigel matrix

LRL explants (approximately 200-300 μm) were microdissected from the E12.5 LRL. The meninges of either wild-type or *Sdf1*$^{-/-}$ hindbrains were divided into pieces of about 400 μm in size. Either recombinant murine SDF1 (0.2 μM, PeproTech EC, London, UK) or PBS as a control was embedded into a collagen block (Zhu et al., 2002) by mixing with 10 μl rat tail collagen. The LRL explant was placed next to a meningeal explant or a collagen block 300-500 μm away and covered with 40 μl matrigel (Matrigel Basement Membrane Matrix, BD Biosciences, Massachusetts, USA). The culture medium was the same as for hindbrain organotypic culture. After 2 days, the explants were stained with 0.03% 4,6-diamidino-2-phenylindole (DAPI; Nacalai Tesque) to reveal the migrating cells.

In situ hybridisation on whole-mount embryos and sections

In situ hybridisation on whole-mount mouse hindbrains was performed as previously described (Zhu et al., 2002). In situ hybridisation on 20 μm frozen sections was based on that of Cheng et al. (Cheng et al., 1995) with some minor modifications. The sections were fixed with methanol and the H_2O_2 treatment was omitted.

Immunohistochemistry

Immunohistochemistry was performed on 20 μm frozen sections, as previously described (Zhu et al., 2006). Primary antibodies used were: goat anti-CXCR4 polyclonal (1:300, Abcam), goat anti-SDF1 (c-19) polyclonal (1:50, Santa Cruz Biotechnology), rabbit anti-PAX6 polyclonal (1:400, Chemicon; or a gift from Dr Noriko Osumi), rabbit anti-laminin polyclonal (1:300, Sigma) and rat anti-GFP monoclonal (1:800, Nacalai Tesque). Secondary antibodies used were: Cy3-conjugated donkey anti-goat IgG (1:500, Jackson ImmunoResearch), Cy3-conjugated goat anti-rabbit IgG (1:250, Jackson ImmunoResearch), Alexa Fluor 488-conjugated donkey anti rabbit IgG (1:400, Invitrogen) and Alexa Fluor 488-conjugated goat anti-rat IgG (1:200, Invitrogen).

Anterograde labelling of motor corticospinal axons

Anterograde DiI tracing of motor corticospinal axons was performed essentially as previously described (O'Leary and Terashima, 1988) in postnatal day (P) 3 pups. The injected pups were returned to their mothers until P5 before perfusion with 4% paraformaldehyde. Parasagittal vibratome sections (50 μm) of these hindbrains were obtained and immunostained with a PAX6 antibody under detergent-free conditions to preserve DiI signals.

Image acquisition

Fluorescence and brightfield images were captured with a CCD camera (Axiocam, Zeiss) linked to an upright microscope (BX-60, Olympus). Whole hindbrains after in situ hybridisation were imaged with a Multi-Viewer System (Keyence, Osaka, Japan). In some cases, fluorescence images were obtained by a laser-scanning confocal microscope (TCS SP2 AOBS; Leica Microsystems).

Data quantification

MetaMorph (Version 6.1, Universal Imaging Corporation) was used to quantify cell migration from images of DAPI-stained LRL explants. Each LRL explant was divided into four quadrants (see Fig. 3B) intersecting at the centroid of the explant. The explant itself was masked, and cell migration in the quadrant facing towards (proximal) and away from (distal) the co-culture was quantified as the total fluorescence intensity within each quadrant. The ratio of cell migration in the proximal over distal quadrant represented the bias of cell migration towards the co-culture. A ratio above 1 indicates attraction, whereas close to 1 indicates a neutral effect. Statistical significance was evaluated by Mann-Whitney U-test.

RESULTS

The precerebellar neurons destined to form the mossy-fibre-projecting nuclei begin migrating marginally along the PES and AES from E12.5 and E13.5, respectively (Fig. 1A) (Altman and Bayer, 1997; Taber-Pierce, 1966), whereas those destined to form the climbing-fibre-projecting inferior olivary (IO) nucleus migrate submarginally from E10 (Taber-Pierce, 1973; De Diego et al., 2002). PES cells migrate circumferentially across the midline to form the contralateral external cuneate nucleus (ECN) and lateral reticular nucleus (LRN) in the medulla oblongata. The AES is a more tortuous route, with an initial ventrally directed migration, then an anteriorly directed path and a final ventral turn to the midline to form the predominantly ipsilateral pontine nuclei (PN) in the pons (Kawauchi et al., 2006; Okada et al., 2007).

The meninges confine the migrating PCN to the pial surface

PCN both in the PES and AES migrate beneath the pial surface of the hindbrain neuroepithelium, close to the overlying pial meninges (Altman and Bayer 1987a; Altman and Bayer 1987b; Bourrat and Sotelo, 1990; Ono and Kawamura, 1990). This tempted us to hypothesise that the meninges might serve to confine the migrating PCN to the pial surface. To test this hypothesis, we labelled PCN by electroporating EGFP into the LRL at E12.5 and organotypically cultured these hindbrains either with or without their meninges (Fig. 1B). After culturing for 2 days, many GFP-labelled cells emigrated from the LRL, reaching

the floor plate both in the presence (Fig. 1C) and absence (data not shown) of the meninges. Inspection of transverse sections of these samples showed that with the meninges, migrating cells formed a highly confined stream beneath the pial surface of the hindbrain, like those in vivo (Fig. 1D, *n*=8). By contrast, in the absence of the meninges, many PCN migrated submarginally so that the migratory stream appeared dispersed (Fig. 1E, *n*=5). These results indicate the importance of the meninges in confining the migrating PCN to the marginal route.

Expression of CXCR4 in migrating PCN and SDF1 in the meninges

The meninges may control the migrating PCN by secreting a chemoattractant that recruits and maintains these neurons close to the pial surface. One candidate is the chemokine SDF1, a chemoattractant expressed mainly in the meninges overlying the entire neural tube during development (McGrath et al., 1999; Tissir et al., 2004). Accumulating evidence indicates the importance of SDF1 in regulating the chemotaxis of migrating neurons and growth cones via its cognate receptor CXCR4 (Tran and Miller, 2003; Lazarini et al., 2003; Stumm and Hollt, 2007; Li and Ransohoff, 2008). We therefore investigated the expression of CXCR4 and SDF1 in the developing precerebellar system.

PCN were identified by their expression of a transcription factor *Mbh2*, also known as mammalian *Barhl1* (Bulfone et al., 2000; Li et al., 2004). On E13.5 transverse sections, *Cxcr4* in situ hybridisation labelled a marginal stream of cells extending from the LRL to the floor plate (Fig. 2B). The profile of this stream of cells closely resembles the PES labelled by *Mbh2* in situ hybridisation on an adjacent section (Fig. 2A,C), suggesting the expression of *Cxcr4* in the PES (Fig. 2B,D). Likewise, *Cxcr4* mRNA was also found in the AES at E13.5 (compare Fig. 2F with 2E). At E15.5, *Cxcr4* expression became undetectable in PES neurons undergoing LRN (Fig. 2G,H) and ECN formation (data not shown). By contrast, *Cxcr4* was still expressed in migrating AES neurons at E15.5 (Fig. 2I,J), but was undetectable in PN at E17.5 (Fig. 2K,L). Double immunohistochemistry with a CXCR4 antibody and an antibody against PAX6, a marker for PCN (Engelkamp et al., 1999), demonstrated that CXCR4 protein was expressed in the PES and AES at E14.5 (Fig. 2M-P and insets). SDF1 immunohistochemistry showed that SDF1 protein was predominantly present in the meninges and the pial surface of the hindbrain, adjacent to the PAX6-positive migratory streams (Fig. 2Q,R). Together these data demonstrate the expression of CXCR4

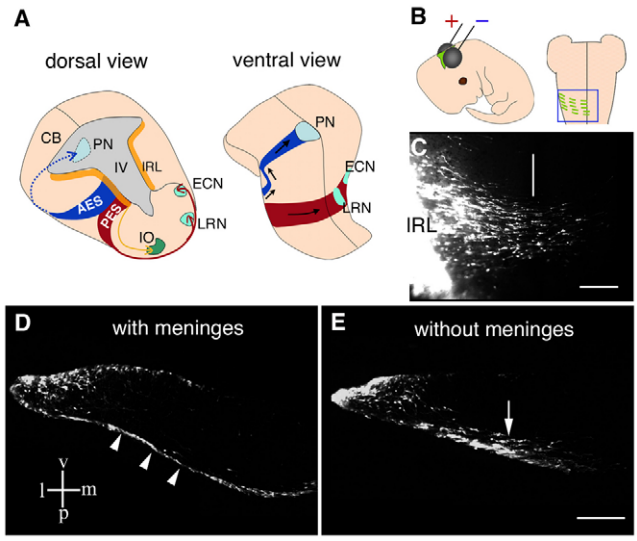

Fig. 1. The meninges are required for the marginal migration of PCN. (**A**) Schematics of the developing mouse precerebellar system. (**B**) Schematics of in vitro electroporation into the lower rhombic lip (LRL) and an open-book organotypic culture. Electroporation was performed at E12.5, a stage when inferior olivary (IO) neurons have already left the LRL. (**C**) After 2 days, many GFP-positive cells emigrated from the LRL towards the floor plate (white line) on an organotypically cultured hindbrain. The image corresponds to the box in B. (**D**,**E**) The migratory stream was confined to the pial surface when the hindbrain was cultured with the meninges as shown on a transverse section (arrowheads in D), whereas removal of the meninges caused many cells to migrate submarginally (arrow in E). IV, fourth ventricle; CB, cerebellum; l, lateral; m, medial; p, pial; v, ventricular. Scale bars: 400 μm in C; 200 μm in E.

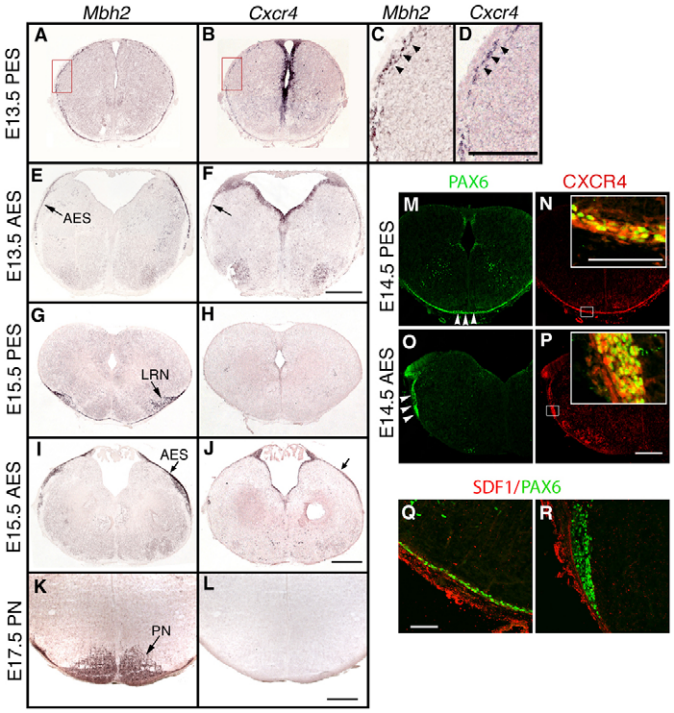

Fig. 2. CXCR4 expression in migrating PCN and SDF1 expression in the meninges. Adjacent sections at various axial levels of mouse hindbrains were subjected to in situ hybridisation for *Mbh2* (A,C,E,G,I,K) and *Cxcr4* (B,D,F,H,J,L). (**A-D**) At E13.5, *Cxcr4* was expressed in a stream of cells (B, arrowheads in D) resembling the posterior extramural stream (PES) labelled by *Mbh2* (A, arrowheads in C). (C,D) Higher-magnification of the boxed areas in A and B, respectively. (**E-L**) *Cxcr4* was also expressed in the anterior extramural stream (AES) at E13.5 (E,F, arrows). Note that *Cxcr4* was expressed in the ventricular zone (B,F). At E15.5, *Cxcr4* expression diminished in the PES, which started to aggregate into LRN (compare H with G), and continued in the migrating AES cells (arrows in I,J). At E17.5, *Cxcr4* was not expressed in pontine nuclei (PN) (compare L with K). (**M-P**) PAX6 and CXCR4 double immunohistochemistry showing co-localization of CXC4 protein and PAX6, a PCN marker, in the PES (M,N) and AES (O,P) at E14.5. The insets are merged higher-magnification images of the boxed areas in N and P. (**Q**,**R**) Double immunohistochemistry for SDF1 and PAX6 on sections neighbouring M and O, respectively, showing the spatial relation between SDF1 protein and migrating PCN. Scale bars: 400 μm in F,J,L; 200 μm in D; 300 μm in P; 75 μm in the insets and Q.

in the migrating PCN and SDF1 in the overlying meninges, suggesting this receptor/ligand pair might mediate the function of the meninges in controlling PCN migration.

The meningeal SDF1 can chemoattract PCN in vitro

To demonstrate directly that SDF1 expressed in the meninges chemoattracts migrating PCN, we performed co-culture experiments in matrigel (Fig. 3A,B). PCN from LRL explants migrated preferentially towards co-cultured meninges or a collagen block embedded with recombinant SDF1 (Fig. 3C,E,I), whereas they showed no preference towards a control collagen block (Fig. 3F,I). The chemoattraction elicited by the meninges was abolished by 20 μM AMD3100, a selective antagonist for CXCR4 (Donzella et al., 1998; Lazarini et al., 2000) (Fig. 3D,I), or when *Sdf1*$^{-/-}$ meninges were used for co-culture (compare Fig. 3H with 3G; Fig. 3I), suggesting that the meningeal attraction is mediated by SDF1/CXCR4 signalling. Thus, the co-culture experiments demonstrate that the meningeal SDF1 can chemoattract migrating PCN in vitro.

PCN are derailed from marginal streams in *Cxcr4* knockout mice

To address the in vivo role of SDF1/CXCR4, we analysed the PCN migratory paths in E14.5 *Cxcr4*$^{-/-}$ mice. Transverse sections across different axial levels of both wild-type (Fig. 4A, schematic) and *Cxcr4*$^{-/-}$ hindbrains were subjected to *Mbh2* in situ hybridisation. Anteriorly, where the AES transits from anteriorly to ventrally directed migration in the wild type (Fig. 4Aa, arrow), no *Mbh2*-positive cells were detected at the corresponding position in *Cxcr4*$^{-/-}$ (Fig. 4Ab, asterisk). In posterior AES, newborn PCN emigrated marginally from the LRL in the wild type (Fig. 4Ac, arrow). In *Cxcr4*$^{-/-}$, most *Mbh2*-positive cells departed from the marginal streams and headed directly towards the ventral midline deep within the hindbrain parenchyma (Fig. 4Ad, arrow). PCN in the PES also derailed from marginal positions in *Cxcr4*$^{-/-}$ (compare Fig. 4Af with Ae), although to a lesser extent with derailed neurons migrating at a short distance from the pial surface following the general curvature of the marginal zone (Fig. 4Af, arrow). These defects observed in *Cxcr4*$^{-/-}$ (*n*=2) were replicated in *Sdf1*$^{-/-}$ (*n*=2, data not shown), suggesting that SDF1/CXCR4 is the main ligand/receptor pair that mediates the marginal migration of PCN. The phenotypes at E14.5 are consistent with our in vitro data and demonstrate a crucial in vivo role of SDF1/CXCR4 signalling in guiding PCN to migrate marginally.

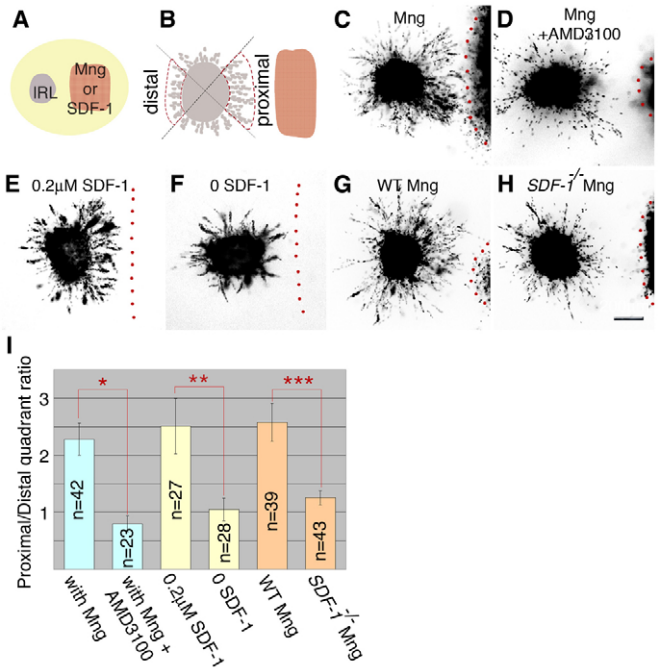

Fig. 3. PCN from LRL migrate preferentially towards co-cultured meninges or recombinant SDF1 protein. (**A**) Schematic of the explant co-culture in matrigel. (**B**) Schematic illustrating the quantification method. (**C-H**) DAPI staining of explant co-cultures to reveal the nuclei of cells. Dotted red lines delineate the borders of the co-cultured meninges (Mng) or collagen blocks to the right. LRL-derived cells migrated preferentially towards the co-cultured meninges (C,G), or towards an SDF1-embedded collagen block (E), but showed no preference towards a control collagen block (F). The attraction elicited by the meninges was abrogated by 20 μM AMD3100 (D), a specific inhibitor of CXCR4, or in co-cultures with *Sdf1*$^{-/-}$ meninges (H). (**I**) Quantification of each condition. *P<0.0001, **P<0.005, ***P<0.0005; Mann-Whitney U-test. Scale bar: 200 μm.

Formation of PN but not LRN/ECN is disrupted in *Cxcr4* knockout mice

We then asked whether precerebellar nuclei could form properly in the absence of SDF1/CXCR4 signalling by *Mbh2* in situ hybridisation on transverse sections across different axial levels (Fig. 4B, schematic) at E16.5. At the pontine flexure, we observed a prominent and symmetrical PN in the wild type (Fig. 4Ba, arrow). By contrast, much smaller and bilaterally asymmetrical PN were present on corresponding sections from *Cxcr4*$^{-/-}$ mice (Fig. 4Bb, arrow, *n*=2), consistent with a previous study (Vilz et al., 2005). Interestingly, posterior to PN, where no *Mbh2*-positive cells were present around the midline in the wild type (Fig. 4Bc), we found a large cluster of *Mbh2*-positive cells spanning the midline in *Cxcr4*$^{-/-}$ (Fig. 4Bd, arrow, *n*=2). *Mbh2*-positive cells could be traced from the LRL to this ectopic cluster (Fig. 4Bd, arrowheads) raising the possibility that the cluster derived from the derailed PCN. At posterior levels, the PES-derived LRN and ECN appeared comparable in size and location between the wild type and *Cxcr4*$^{-/-}$ (compare Fig. 4Be,g with Bf,h).

The marked reduction of PN at their normal position concurrent with the emergence of posterior ectopic *Mbh2*-positive clusters raised the possibility that many pontine neurons are posteriorised in the absence of SDF1/CXCR4 signalling. The migratory pattern of AES at E14.5 in *Cxcr4*$^{-/-}$ also supported this possibility (Fig. 4A). We sought direct evidence for pontine identity of the posterior ectopic *Mbh2*-positive cells. We found that Nuclear factor I (NFI) family members *Nfix* and *Nfib* are expressed at a high level in the PN but weakly in the LRN and ECN from E16.5 (data not shown) (see Fig. S1C,D in the supplementary material). In situ hybridisation on adjacent sections of an E16.5 *Cxcr4*$^{-/-}$ hindbrain with *Mbh2*, *Nfix* and *Nfib* showed that the ectopic *Mbh2*-positive cells were strongly positive for *Nfix* and *Nfib* signals (see Fig. S1A,B in the supplementary material), suggesting that these cells were indeed pontine neurons.

Therefore, whereas formation of the LRN and ECN appears normal, PN formation is largely posteriorised in animals depleted of SDF1/CXCR4 signalling.

Multiple posteriorised pontine clusters are present in *Cxcr4*$^{-/-}$

To better appreciate the spatial distribution of derailed pontine neurons in *Cxcr4*$^{-/-}$, we performed *Mbh2* in situ hybridisation on whole-mount E16.5 hindbrains from wild type, *Cxcr4*$^{+/-}$ and *Cxcr4*$^{-/-}$ embryos. In wild type (*n*=2) and heterozygotes (*n*=6),

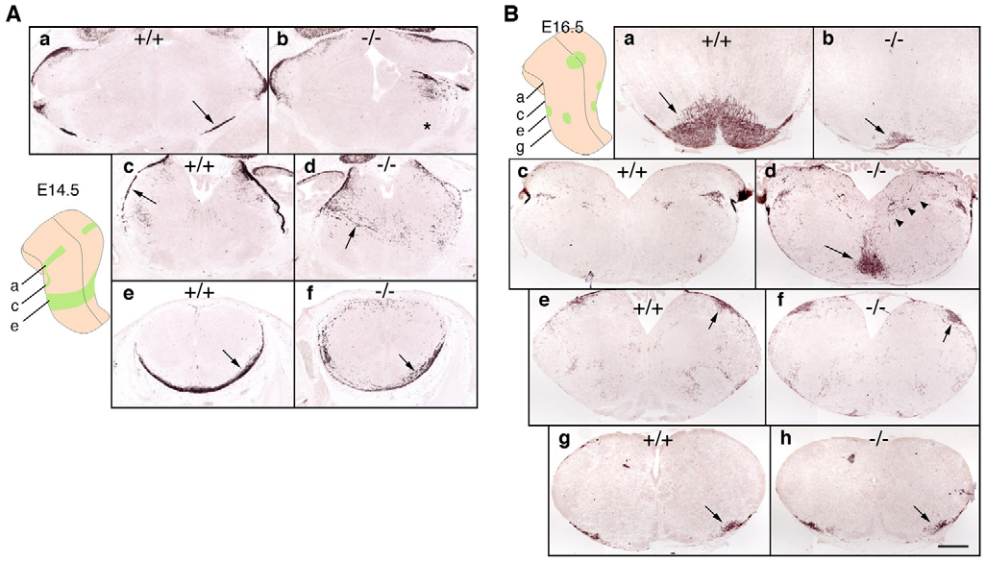

Fig. 4. PCN migration is markedly disrupted in *Cxcr4*$^{-/-}$ mice. The migration of PCN and subsequent nuclei formation were analysed by *Mbh2* in situ hybridisation on transverse sections of E14.5 (A) and E16.5 (B) hindbrains. (**A**) The schematic shows a wild-type E14.5 hindbrain with the AES and PES depicted in green and the approximate axial levels of the illustrated sections indicated. (a,b) The frontier of the AES turning ventrally in the E14.5 wild type (a, arrow) was not observed in a corresponding section from *Cxcr4*$^{-/-}$ (b, asterisk). (c,d) At posterior AES, PCN migrated marginally from the LRL in the wild type (c, arrow), whereas in *Cxcr4*$^{-/-}$ most *Mbh2*-positive cells left the marginal stream heading straight towards the midline (d, arrow). (e,f) Within PES, many PCN migrated at a distance from the pial surface in *Cxcr4*$^{-/-}$ (f, arrow), as opposed to marginally in the wild type (e, arrow). These results suggest that PCN derailed from marginal migration in *Cxcr4*$^{-/-}$. (**B**) The schematic shows a wild-type E16.5 hindbrain with precerebellar nuclei depicted in green and the approximate axial levels of the illustrated sections indicated. (a,b) Prominent and symmetrical PN at the level of pontine flexure seen in the wild type (a, arrow) appeared much smaller and asymmetrical in *Cxcr4*$^{-/-}$ (b, arrow). (c,d) Posterior to PN, where no *Mbh2*-positive cells were found medially in the wild type (c), an ectopic pontine-like cluster emerged in *Cxcr4*$^{-/-}$ (d, arrow). Loose trails of *Mbh2*-positive cells appeared to link between the LRL and the cluster (d, arrowheads). (e-h) By contrast, the ECN (f, arrow) and LRN (h, arrow) in *Cxcr4*$^{-/-}$ were comparable in size and position to those of the wild type (e and g, arrows). Scale bar: 400 μm.

Mbh2 labelled the bilaterally symmetrical PN anteriorly at the level of the trigeminal ganglion (gV) (Fig. 5A,B) and the LRN (Fig. 5A,B, asterisks) and ECN (data not shown) posteriorly. In *Cxcr4*$^{-/-}$, the LRN and ECN appeared normal in both position and size (Fig. 5C,D, asterisks; data not shown), consistent with our data on transverse sections (Fig. 4B). By contrast, the disruption in PN revealed on the whole-mount was dramatic (Fig. 5C,D; n=6). PN in their normal position (at or above the gV) were very small (Fig. 5C,D, notched arrowheads); instead multiple ectopic pontine clusters were found around the midline posterior to the gV. Closer inspections on whole-mount and transverse sections (Fig. 5E) enabled us to discern two types of ectopic pontine clusters: type I, defined by a single large cluster located deeply and spanning the midline (appears out of focus in Fig. 5C,D, arrowheads; Fig. 5E, arrowheads), corresponding to the cluster shown in Fig. 4Bd; and type II, defined by multiple superficial clusters situated adjacent to the midline (Fig. 5C-E, arrows). Most type II clusters were anterior to the type I cluster and appeared to derive from distinct marginal migratory streams, some of which were still visible (Fig. 5C,D). Another feature of type II clusters was their left-right asymmetrical distribution, the pattern of which varied among individual samples (Fig. 5C,D; n=6). In summary, whole-mount in situ hybridisation provided a three-dimensional perspective of the mutant phenotype that could not be easily appreciated on sections. Two types of defects were observed in pontine neurons (Fig. 5F): (1) those migrating deeply forming a deep cluster posteriorly; and (2) those migrating marginally, but departed from the anterior migratory path prematurely, ending up in multiple posterior locations.

SDF1/CXCR4 signalling functions cell-autonomously in developing PCN

We have shown so far that depletion of SDF1/CXCR4 signalling results in derailment of PCN from their marginal migration as well as posteriorised pontine migration. Although their expression pattern and the co-culture data imply that SDF1/CXCR4 signalling may function cell-autonomously, we cannot rule out the possibility that these mutant phenotypes are secondary to other defects in the hindbrain cytoarchitecture or a possible earlier defect in AP patterning. Indeed, CXCR4 is expressed in the ventricular zone and several other hindbrain nuclei (Fig. 2B,F; data not shown) (McGrath et al., 1999; Tissir et al, 2004; Lieberam et al., 2005).

To address this question, we asked if the defects in PCN migration could be rescued by expressing *Cxcr4* in *Cxcr4*$^{-/-}$ PCN progenitors in the LRL. Either *pCAGGS-Cxcr4-IRES-Egfp* expressing full-length *Cxcr4* or *pCAGGS-Egfp* as a control was electroporated into the LRL of wild-type and *Cxcr4*$^{-/-}$ embryos at E12.5 by in utero electroporation. *Egfp* electroporation in the wild type labelled the PES and AES unilaterally (Fig. 6A,E) (Kawauchi et al., 2006). The GFP-positive PES was confined marginally abutting the laminin-positive meninges, as shown at E14.5 on a transverse section (Fig. 6A; n=5). The labelling of AES and PN, better appreciated on E16.5 whole-mount hindbrains (n=12), showed the characteristic anterior migratory path laterally (Fig. 6E, arrow) and its ventral turn just below the rootlet of the gV (Fig. 6E, asterisk). Forced expression of *Cxcr4* in the wild-type LRL did not cause notable changes in migratory profiles of the PES (Fig. 6B) or AES (Fig. 6F). Electroporation of *Egfp* alone into the LRL of *Cxcr4*$^{-/-}$ embryos labelled the aberrant PES and AES similar to those revealed by

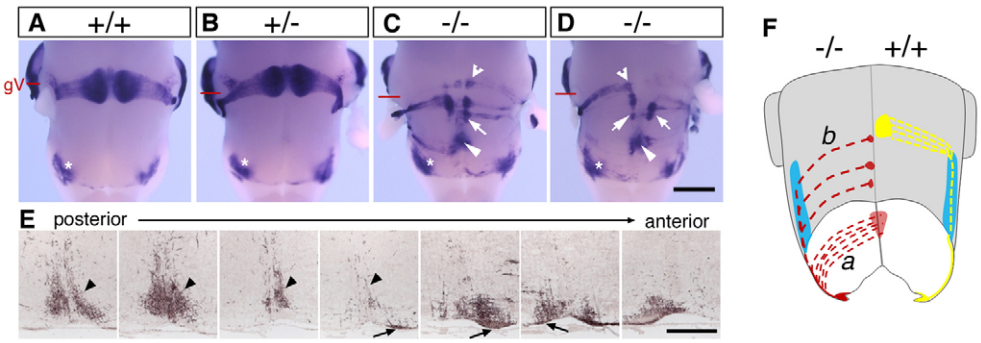

Fig. 5. Multiple posterior pontine clusters with bilaterally asymmetrical distribution are present in *Cxcr4*$^{-/-}$ mice. *Mbh2* in situ hybridisation was performed on whole-mount E16.5 wild-type, *Cxcr4*$^{+/-}$ and *Cxcr4*$^{-/-}$ hindbrains. (**A**,**B**) A wild-type (A) and a heterozygotic (B) hindbrain showed bilaterally symmetrical PN anteriorly and LRN posteriorly (asterisks). (**C**,**D**) Two *Cxcr4*$^{-/-}$ hindbrains presented here had reduced PN at their presumptive positions (notched arrowheads), accompanied by an emergence of multiple ectopic clusters. One large ectopic cluster across the midline was located deeply (arrowheads). The other ectopic clusters adjacent to the midline, as well as their migratory streams, were located superficially (arrows) and displayed left-right asymmetry. (**E**) The nature of these ectopic clusters was confirmed by *Mbh2* signals on sections. Seven sections approximately 200 μm apart spanning posteriorly from the deep ectopic cluster (arrowheads) to the more anterior superficial clusters (arrows) are shown. (**F**) Schematic summarizing the results from sections and whole mounts to show the two abnormal behaviours in pontine neuron migration in *Cxcr4*$^{-/-}$: (a) pontine neurons departed from the marginal stream and headed straight to the ventral midline to form the deep ectopic cluster; (b) pontine neurons migrated marginally but departed from the anterior path prematurely to form the superficial clusters. Scale bars: 800 μm in D; 400 μm in E.

Mbh2 in situ hybridisation: broadened PES (Fig. 6C; *n*=2), many AES cells migrating deeply anterior to the PES (see Fig. S2 in the supplementary material), and the posteriorised type I and II clusters (Fig. 6G, arrowhead and open arrowhead, respectively; *n*=5). Of particular note, the characteristic anterior path of the AES was largely missing in the mutant. Inspection on transverse sections from these samples confirmed that many GFP-positive cells were located within the type I (Fig. 6I; *n*=2) and type II ectopic clusters (data not shown), which were double positive for PAX6 (Fig. 6I, inset).

We then tested if these phenotypes could be rescued by expressing *Cxcr4* in the *Cxcr4*$^{-/-}$ LRL. *Cxcr4* expression restored a PES confined to the marginal position at E14.5 (*n*=2; compare Fig. 6D with 6C), and resulted in few labelled cells migrating deeply anterior to the PES (data not shown, *n*=2). The anterior migration of pontine neurons was also restored (*n*=6), as evidenced by the re-emergence of the characteristic anterior migratory path (Fig. 6H, arrow) and prominent PN in the normal anterior position. This occurred concomitantly with few labelled cells in type I and II ectopic pontine clusters (compare Fig. 6H with 6G; a transverse section is shown in Fig. 6J), suggesting that expression of CXCR4 directed the strayed cells to their normal anterior-migratory pathway. Taken together, these results suggest that CXCR4 functions cell-autonomously in controlling the marginal migration of PCN and anterior migration of pontine neurons.

Ectopic pontine clusters induce ectopic collateral branching from corticospinal tract

What are the consequences of ectopic PN on the later event of axon innervations? PN are the main hindbrain targets of the corticospinal tract (CST). Corticopontine innervation is formed by interstitial collateral branching from the trunk of CST axons (O'Leary and Terashima, 1988). Although collagen co-culture experiments implied that cues from PN control the formation of collateral branching (Heffner et al., 1990), direct in vivo evidence supporting this possibility has been missing. The ectopic pontine clusters resulted from depleted *Sdf1/Cxcr4* signalling provided us with an opportunity to seek the missing evidence.

The mature pattern of motor corticopontine collaterals emerges at around P2 in mice (Bastmeyer and O'Leary, 1996), but neonates of *Cxcr4*$^{-/-}$ and *Sdf1*$^{-/-}$ die at birth (Tachibana et al., 1998; Nagasawa et al., 1996). To circumvent this obstacle, we conditionally knocked out *Cxcr4* in *Wnt1*-expressing LRL using a *Wnt1-Cre* driver and a *Cxcr4*$^{fl/fl}$ responder line. The *Wnt1-Cre* driver was used because PCN precursors were shown to locate within the *Wnt1*-expressing domain of LRL (Rodriguez and Dymecki, 2000). *Wnt1-Cre*;*Cxcr4*$^{fl/fl}$ mice survived into adulthood. First we confirmed that PN formation was indeed disrupted in *Wnt1-Cre*;*Cxcr4*$^{fl/fl}$ mice similarly to *Cxcr4*$^{-/-}$ (data not shown) and ectopic pontine clusters persisted until at least P5 (see Fig. S3 in the supplementary material). The CST was then traced anterogradely by DiI from the motor cortex between P3 and P5 (Fig. 7A). In control *Cxcr4*$^{fl/fl}$ hindbrains, labelled motor CST showed a stereotypic pattern of collateral branches directed exclusively into PN (Fig. 7B-D; *n*=3 brains). Interestingly, the motor CST in *Wnt1-Cre*;*Cxcr4*$^{fl/fl}$ hindbrains showed extensive collateral branching at multiple ectopic sites between the pontine flexure and the pyramidal decussation (Fig. 7E; *n*=4 brains). Notably, each ectopic cluster of collateral branches extended towards a PAX6-positive ectopic pontine cluster (Fig. 7F-H). The formation of ectopic branching could not be due to changes in corticospinal neurons in the motor cortex because *Wnt1* expression was excluded from the developing forebrain (Parr et al., 1993). Although we could not formally exclude the possibility that the ectopic branching could be caused by disruption of hindbrain nuclei other than PN, the high correlation between the sites of ectopic collateral formation and ectopic pontine clusters suggest that pontine neurons themselves control the collateral branch formation from the corticospinal tract.

DISCUSSION

In the developing brain, a directed tangential migratory pathway is specified for AP and DV directions, as well as depth within the VP span of the neuroepithelium. By focusing on the migrating precerebellar neurons, we have demonstrated that the chemokine

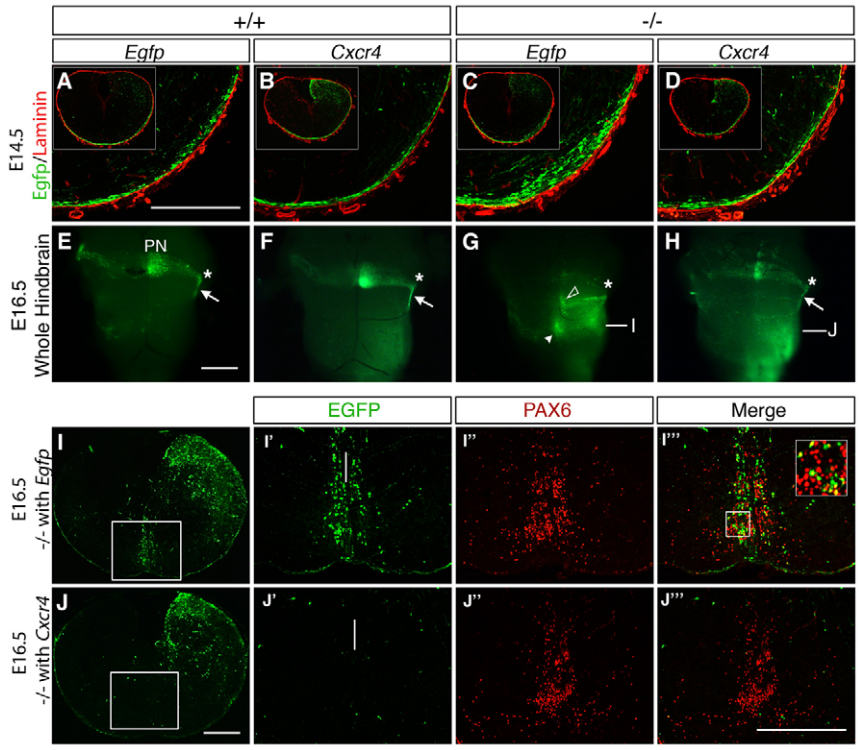

Fig. 6. CXCR4 functions cell-autonomously in the migrating PCN. *Egfp* or *Cxcr4* (with co-expressed *Egfp*) was introduced into the LRL of wild-type (A,B,E,F) or *Cxcr4*$^{-/-}$ (C,D,G,H) mouse embryos by in utero electroporation at E12.5. (**A-D**) Transverse sections of E14.5 samples after EGFP and laminin (labels the meninges) double immunohistochemistry. Insets show views of whole sections. *Egfp* electroporation labelled a marginal PES directly abutting the laminin-positive meninges in the wild type (A). Expression of *Cxcr4* in the wild type did not affect the appearance of the PES (B). In *Cxcr4*$^{-/-}$, PES appeared broadened, with many cells migrating at a distance from the pial surface (C). This defect was rescued by expressing *Cxcr4* in *Cxcr4*$^{-/-}$ LRL (D). (**E-H**) Whole-mount E16.5 hindbrains after electroporation at E12.5. Asterisks indicate the gV rootlets. In the wild type, expressing either *Egfp* (E) or *Cxcr4* (F) labelled similar profiles: a largely ipsilateral PN and the stereotypic anterior path of the AES (arrows in E and F). *Egfp* electroporation in *Cxcr4*$^{-/-}$ labelled the posterior type I and type II clusters (G, arrowhead and open arrowhead, respectively). Note that the characteristic anterior migratory path was missing. *Cxcr4* expression in *Cxcr4*$^{-/-}$ LRL restored PN in their normal position as well as the anterior migratory path (H, arrow). (**I-J'''**) Transverse sections at indicated axial levels in G and H, respectively. Higher-magnification of the boxed areas in I and J are shown in I′-I‴ and J′-J‴. (I) In *Cxcr4*$^{-/-}$ expressing only EGFP, many GFP-labelled cells were located in the type I ectopic cluster, many of which were PAX6-positive (inset). But in *Cxcr4*$^{-/-}$ expressing CXCR4, few labelled cells were found in the type I ectopic cluster. Scale bars: 400 μm in A-D,I-I‴,J-J‴; 800 μm in E-H.

SDF1 in the pial meninges and its receptor CXCR4 in PCN are crucial for controlling two distinct processes of PCN migration: (1) positioning the migratory streams to the appropriate VP level; and (2) regulating the anterior migration of pontine neurons.

Role of SDF1/CXCR4 signalling in the tangential migration of PCN

Evidence suggests that SDF1/CXCR4 signalling controls the VP position of migrating PCN in both the PES and AES, most likely via chemoattraction by the meningeal SDF1: (1) PCN emigrating from the LRL were attracted by the meningeal SDF1 in vitro; (2) the highest SDF1 protein concentration was found in the pial meninges and the pial surface of the hindbrain (Fig. 2), suitably located to attract PCN to migrate along the marginal path. Thus, the scenario presented here resonates with recent findings in the developing cortex, where the meningeal SDF1 confines the tangential migration of hem-derived Cajal-Retzius cells and a subset of cortical interneurons to the marginal zone via chemoattraction (Borrel and Marín, 2006; Li et al., 2008; Lopez-Bendito et al., 2008; Tanaka et al., 2009). As SDF1 is expressed in the pial meninges surrounding the entire central nervous system (CNS) (McGrath et al., 1999; Tissir et al., 2004), these findings imply that chemoattraction by the meningeal SDF1 could be a general mechanism for controlling pathfinding events taking place in the CNS marginal zone.

The second role of SDF1/CXCR4 signalling appears to be regulating the anterior migration of pontine neurons. Two types of defective anterior migration were observed in *Cxcr4* knockout mice. The first comprises pontine neurons that derail from the marginal stream and migrate towards the midline deep within the neuroepithelium. Two lines of evidence indicate that these neurons may have completely failed to turn anteriorly. First, their leading processes appear to orient circumferentially towards the midline in *Egfp*-electroporated *Cxcr4*$^{-/-}$ samples (see Fig. S2 in the supplementary material). Second, the deep ectopic cluster they formed was located in rhombomeres (r) 6 and 7 (Y.Z. and F.M., unpublished), the axial levels where the pontine progenitors were shown to originate by genetic fate mapping (Farago et al., 2006). The second type of defect comprises pontine neurons that appear to migrate marginally. Many of them are likely to have migrated for some distances anteriorly, as most superficial type II clusters they formed were located more anterior than the deep type I cluster. Nevertheless, they failed to complete the full course of the anterior pathway, turning towards the ventral midline prematurely in a rather

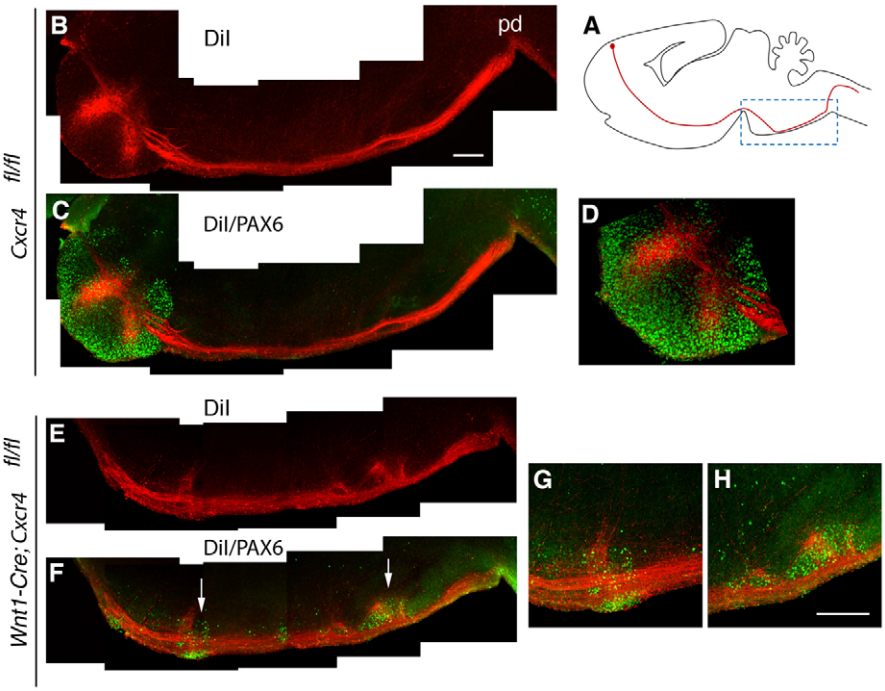

Fig. 7. Corticospinal tract axons extend ectopic collateral branches towards the ectopic pontine clusters. (**A**) Schematic depicting motor corticospinal tract (CST) in a parasagittal plane of an early postnatal brain (see O'Leary and Terashima, 1988). The box outlines the approximate area shown in B,C,E,F. (**B**,**C**) The DiI-labelled CST in a P5 *Cxcr4*$^{fl/fl}$ hindbrain shows stereotypic collateral branching initiated from the CST segment overlying PN. PN were labelled by PAX6 immunoreactivity (C). No notable site-specific collateral branching occurred from the trunk of CST posterior to PN. (**D**) Higher-magnification of the PN region. (**E-H**) In a *Wnt1-Cre;Cxcr4*$^{fl/fl}$ hindbrain, ectopic collateral branching occurred at multiple locations along the trunk of CST in the hindbrain, each one of which was correlated with a PAX6-positive ectopic pontine cluster. Two such sites (arrows in F) are shown at higher magnification in G and H. pd, pyramidal decussation. Scale bars: 200 μm.

unpredictable manner. As both defects were rescued by expressing *Cxcr4* in the *Cxcr4*$^{-/-}$ LRL, it is unlikely that they were secondary to disruptions in other hindbrain architectures.

Mechanisms of SDF1/CXCR4 signalling in the anterior migration of pontine neurons

What is the mechanism(s) for the anterior migration of pontine neurons? We think the two anterior migration defects reflect the requirement of SDF1 in two distinct aspects.

The first type of defect in which cells went straight to the ventral midline without apparent anterior turning may be secondary to their failure in migrating marginally. This is because most pontine neurons that migrated marginally travelled anteriorly for some distances, as discussed above. If the guidance cue(s) instructing the anterior migration is spatially confined to the pial surface, the pontine neurons derailed from the marginal stream would miss the cue(s) and escape its influence.

The second type of defect may reflect a direct involvement of SDF1/CXCR4 signalling in the anterior migration of pontine neurons. A most straightforward explanation would be that an anterior-high posterior-low SDF1 gradient instructs the anterior migration of pontine neurons by way of chemoattraction. Indeed, we detected such a graded distribution of SDF1 protein underlying the anterior migrating pontine neurons (see Fig. S4 in the supplementary material). An instructive role of SDF1 is further supported by the gain-of-function experiment that showed that ectopic expression of SDF1 in the LRL prevented pontine neuron precursors from leaving the LRL (see Fig. S5 in the supplementary material). However, the fact that some marginally migrating pontine neurons can move anteriorly for some distances suggests the existence of other instructive cues. Alternative to, or in addition to, the instructive role of SDF1, SDF1/CXCR4 signalling may serve to modulate the responsiveness of pontine neurons to other anterior guidance cue(s), as has been demonstrated in developing retinal ganglion cell axons and sensory axons (Chalasani et al., 2003; Chalasani et al., 2007).

SLIT2 expressed by the facial motor nucleus appears to regulate the anterior migration of pontine neurons by preventing the ROBO1/ROBO2-expressing pontine neurons from leaving their anterior pathways prematurely (Geisen et al., 2008). It is unlikely that SDF1/CXCR4 signalling promotes the anterior migration of pontine neurons chiefly via modulating the SLIT-ROBO signalling pathway, because the anterior migration defects reported here in *Cxcr4* or *Sdf1* knockout mice were more severe than those in mice depleted of SLIT-ROBO signalling: in the former, a majority of pontine neurons failed to reach their normal anterior positions, whereas in the latter only a minority failed.

Consequences of disrupted migration on precerebellar nuclei formation

Precerebellar nuclei formation takes place as a consequence of proper migration of PCN. The derailment from marginal migratory streams in *Cxcr4* knockout mice appears to be manifested differently in the PES- and AES-derived nuclei. Whereas the derailed cells in the PES can form the LRN and ECN in their normal positions, those in the AES fail to migrate anteriorly and form a deep ectopic pontine cluster at a posterior position. This difference could be due to two reasons. First, the displacement from the marginal position in the PES is smaller than that in the AES, meaning that the derailed PES cells may be able to encounter instructive cues for nuclei formation. The smaller derailment in PES might be due to the presence of additional guidance cues for PES neurons in the marginal/submarginal region in the caudal hindbrain. Second, whereas the PES is a straightforward ventral migration, the AES is a tortuous trajectory comprising ventral-anterior-ventral switches. Therefore, the consequence of failing to migrate marginally earlier on was amplified by their failure to encounter the anterior migratory cues.

Those pontine neurons that migrated marginally but fell short of anterior migration formed multiple ectopic clusters that exhibited left-right asymmetry. The fact that the pattern of this asymmetry varies among individuals raises the possibility that it might arise as a result of intrinsic and environmental noises that the developing

pontine neurons experience during their production and migration. The fluctuations in phenotypes owing to noises might be normally buffered and equalised between the left and right by an intact and robust anterior guidance mechanism, but become unmasked phenotypically when the robustness of that mechanism is weakened, as might be the case in the *Cxcr4* knockout. Interestingly, similar left-right variations were reported in a recent study in which anterior migration of pontine neurons was affected in *Hoxa2*-, *Hoxb2*-, *Phox2b*-, *Robo1-Robo2*- and *Slit1-Slit2*-deficient mice (Geisen et al., 2008). These observations taken together suggest that several molecular mechanisms may contribute additively to the optimal robustness of the anterior migration of pontine neurons.

Pontine neurons control the formation of collateral branching from the CST

The presence of multiple ectopic pontine clusters in *Cxcr4* knockout mice tempted us to test in vivo whether the pontine neurons themselves trigger the formation of collateral branches from the CST, a possibility that was only inferred from in vitro evidence (Heffner et al., 1990). By using a conditional knockout strategy to circumvent lethality at birth, we could obtain postnatal pups that had multiple ectopic pontine clusters. The motor CST in these samples extended few collateral branches at the pontine flexure, as PN were largely missing at their normal positions. Instead, we observed multiple ectopic clusters of collateral branches posteriorly along the CST, each of them correlated with an ectopic pontine cluster, suggesting a causal relationship between these two events. As the ectopic pontine clusters exist before the growth of motor CST, it is reasonable to conclude that the ectopic pontine neurons trigger the ectopic collateral formation.

It should be noted that not every ectopic pontine cluster was accompanied by a collateral cluster. This could be because our focal DiI injection labelled only a small fraction of motor CST axons. However, an alternative is that only subpopulations of pontine neurons can induce collaterals from the motor CST. In support of this alternative is the mature pattern of motor corticopontine projection in wild-type mice, in which collaterals arise at a specific rostral and a caudal site from CST segments overlying PN. The motor CST axons, however, seem to be capable of extending collateral branches from diverse rostrocaudal positions within the hindbrain in response to the induction of ectopic pontine clusters, suggesting that these axons themselves do not have an intrinsic mechanism that governs the sites of collateral extension. These observations, taken together, provide the first in vivo evidence that the formation of collateral branches from CST is controlled by cues derived from pontine neurons.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/11/1919/DC1

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