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Embryonic phenotype of *Unc5h3* mutant mice suggests chemorepulsion during the formation of the rostral cerebellar boundary

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SUMMARY

Mutation of the *Unc5h3* (formally known as *rcm*) gene has important consequences on neuronal migration during cerebellar development. *Unc5h3* transcripts are expressed early (embryonic day 8.5) in the hindbrain region and later in the cerebellar primordia. In *Unc5h3* mutant embryos, both the development and initial migration of Purkinje cell progenitors occur as in wild-type controls. The rhombic lip, from which granule cell precursors arise, also appears to form normally in mutants. However, at E13.5, an abnormal subpopulation of granule cell and Purkinje cell precursors becomes detectable in rostral areas of the *Unc5h3* mutant brain stem. These ectopic cerebellar cells increase in number and continue moving in a rostral direction throughout the remainder of embryogenesis and early stages of postnatal development invading the lateral regions of the pontine area and eventually the inferior colliculus. Cell proliferation markers demonstrate the mitotic nature of these subpial ectopic granule neurons indicating the displacement of the rostral external germinal layer in mutant animals. Our data suggest that establishment of the rostral cerebellar boundary may rely on chemorepulsive signaling events that require UNC5H3 expressed by cerebellar neurons and extracellular ligands that are functionally related to the UNC5H3-binding, guidance molecule netrin1. Although the phenotype resulting from the *Unc5h3* mutation is apparently limited to the formation of the cerebellum, additional sites of *Unc5h3* expression are also found during development suggesting the compensatory function of other genes.

Key words: cerebellum, granule cell, Purkinje cell, migration, netrin1, *Unc5h3*, mouse

INTRODUCTION

During the development of the mammalian central nervous system (CNS), the majority of neurons migrate varying distances from their site of origin to their final destination. Although such migrations are critical for the final positioning of cells and the definition of the many different regions of the adult brain, little is known concerning the molecular mechanisms that position neuronal cell bodies. Axonal guidance and invertebrate cell migration studies suggest that cell movements may be controlled by a series of extracellular signals that result in either chemoattraction toward their intermediate/final target or by repulsion by chemorepellants secreted from nontarget cells (reviewed in Tessier-Lavigne and Goodman, 1996).

It has been recently shown that the secretory protein, netrin1, may act as a bifunctional guidance cue simultaneously attracting some axons while repelling others. In the vertebrate neural tube, *netrin1* is expressed in the floor plate region (Kennedy et al., 1994), the normal intermediate target of commissural axons. Explants of floor plate have been shown to attract and redirect commissural axons developing from dorsal parts of the neural tube (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Shirashaki et al., 1995, 1996) and *netrin1*-deficient mice exhibit defects in commissural axon projections (Serafini et al., 1996). Floor plate cells also have a long-range repulsive effect on trochlear motor axons that grow dorsally away from the floor plate in vivo (Colamarino and Tessier-Lavigne, 1995). Genetic analyses in *C. elegans* indicate that, in addition to its role in axonal guidance, the netrin UNC-6 also functions to control cell migrations (Hedgecock et al., 1990; Ishii et al., 1992; Wadsworth et al., 1996).

Evidence suggests that the dichotomous response to netrin may result from the function of different netrin receptors. The *C. elegans unc-5* gene encodes a transmembrane receptor protein that mediates dorsal migrations of pioneer axons and mesodermal cells away from netrin sources (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Hamelin et al., 1993), whereas, netrin-dependent ventral migrations require the expression of another receptor, UNC-40 (Hedgecock et al., 1990; Chan et al., 1996). Furthermore, the vertebrate homologue of UNC-40, Deleted in Colorectal Cancer (DCC), binds netrin1 and is involved in the ventral guidance of commissural axons (Keino-Masu et al., 1996; Fazeli et al., 1997).

Three vertebrate homologues of *unc-5* have been identified: the mouse rostral cerebellar malformation gene, *rcm* (renamed *Unc5h3*), (Ackerman et al., 1997); and two rat genes, *Unc-5h1* and *Unc-5h2* (Leonardo et al., 1997). The proteins encoded by each of these genes bind netrin1 with dissociation constants

consistent with the effective dose for the axon outgrowth-promoting ability of netrin1 (Leonardo et al., 1997). Mice homozygous for either the spontaneous *rcm* mutation (*Unc5h3*^{rcm}) or the transgenic insertion allele (*Unc5h3*$^{rcmTgN(Ucp)1.23Kz}$) display similar abnormalities in the rostral cerebellum and caudal midbrain (Lane et al., 1992; Ackerman et al., 1997). These mice have a dramatic reduction in cerebellar size, abnormal foliation patterns and ectopic cerebellar Purkinje and granule cells that are found in the inferior colliculus of the postnatal midbrain. In addition, mutant cerebella are significantly smaller at birth indicating a possible function for the *Unc5h3* gene during embryogenesis (Ackerman et al., 1997).

To test the hypothesis that *Unc5h3* functions during the early stages of cerebellar development, we examined the *Unc5h3* expression pattern and the ontogeny of Purkinje and granule cells during cerebellar formation in wild-type and *Unc5h3* mutant embryos. Our results strongly implicate a phylogenetically conserved repulsive signaling mechanism for the *C. elegans unc-5* gene and the vertebrate homologue, *Unc5h3*.

MATERIALS AND METHODS

Mice

We previously described two mutant alleles of the *rcm* (*Unc5h3*) locus; a spontaneous mutation that results from the duplication of an exon in the cytoplasmic region of the UNC5H3 protein and a transgene insertion that results in complete loss of transcript (Ackerman et al., 1997). The spontaneous allele of the *rcm* mutation (*Unc5h3*^{rcm}) is maintained on an F_1 hybrid B6C3HFe-*a/a* background (Lane et al., 1992) and the transgene insertion allele (*Unc5h3*$^{rcmTgN(Ucp)1.23Kz}$) is maintained on a segregating C57BL/6JxSJL/J background (Boyer and Kozak, 1991). Embryos were collected from matings of homozygous *Unc5h3*^{rcm} and control B6C3HFe-*a/a* mice. Results were confirmed in genotyped wild-type and homozygous *Unc5h3*$^{rcmTgN(Ucp)1.23Kz}$ littermates from heterozygous matings. An embryonic age of E0.5 was determined by the appearance of a vaginal plug the morning after conception. Three to eight different animals per time point were examined.

In situ hybridization

All tissues collected for in situ hybridization studies were fixed by immersion in 4% paraformaldehyde/PBS overnight at 4˚C, then dehydrated, cleared in xylene and embedded in paraffin wax prior to sectioning (6 μm) and mounting on Plus slides (Fisher). ^{33}P-labeled sense and antisense riboprobes corresponding to *Unc5h3* (Ackerman et al., 1997), *netrin1* (Serafini et al., 1996), *Sg* (*staggerer)* (Hamilton et al., 1996), *Atoh1* (*math1*) (Akazawa et al., 1995; Ben-Arie et al., 1996) and *RU49* (Yang et al., 1996) were prepared by in vitro transcription according to the manufacturer's protocol (Stratagene). Tissue pretreatment, hybridization and post-hybridization washes were as previously reported (Hui and Joyner, 1993) except that hybridizations were done at 65˚C and β-mercaptoethanol was excluded from post-hybridization washes. Slides were dipped in Kodak NTB2 emulsion, exposed for 1-7 days at 4˚C and developed in Kodak D19. Sections were stained with hematoxylin and photographed as described below. For each of the transcripts tested, in situ hybridization with sense strand riboprobes did not detect any significant signal (data not shown).

Immunohistochemistry

To assess the location of mitotic neuroprogenitors, bromodeoxyuridine (BrdU, Sigma) incorporation studies were performed. Postnatal littermates (P3 to P15; 3 to 15 days after birth) from *Unc5h3*$^{rcmTgN(Ucp)1.23Kz}$/+ matings were injected intraperitoneally with BrdU (50 μg/g) diluted in 7 mM NaOH and killed 60 minutes later. Tissues were removed and fixed at room temperature by immersion in methanol/acetic acid (3:1) overnight, dehydrated, embedded in paraffin and sectioned (6 μm). Sections were prepared for BrdU immunohistochemistry following standard procedures (Hamre and Goldowitz, 1996). Briefly, tissues were deparaffinized and rehydrated prior to treatment with 4 N HCl (20 minutes) to denature DNA. After 3× 3 minute washes in PBT (phosphate-buffered saline, pH 7.4, 0.3% Triton X-100), sections were incubated overnight at 4˚C with mouse anti-BrdU monoclonal antibody (1:50, Dako) in blocking solution (normal sheep serum (1:50) in PBT). Following 3× 3 minute washes in PBT, the sections were exposed to biotin conjugated anti-mouse IgG (Sigma, 1:100 in blocking solution, 30 min), washed 3× with PBT and incubated in avidin-conjugated peroxidase (Sigma, 1:20 in blocking solution). For colorimetric detection, sections were treated with 3,3′ diaminobenzidine hydrochloride (DAB, Sigma) and then counter-stained with cresyl violet. Cells that incorporated BrdU displayed a dense brown precipitate in the nucleus.

Microscopy and image collection

All sections were examined using a Leica DMRXE microscope equipped for bright-/dark-field illumination. High resolution digital images were captured by a Kodak Megaplus 1.4 camera directly linked to the Quantimet Q600HR system (Leica).

RESULTS

Expression of *Unc5h3* during development of the CNS

The temporal and spatial expression patterns of *Unc5h3* during embryogenesis were studied to elucidate its role during CNS formation. Development of the mouse CNS commences at approximately E7.5 (7.5 d.p.c.) with the appearance of the neural plate and is followed by the gradual closure of the neural folds to form the neural tube beginning at E8.5. Within the developing CNS, *Unc5h3* mRNA is first detected at E8.5 in the neuroectoderm associated with the dorsal aspect of the neural tube. Expression is seen in prospective hindbrain and fused cephalic neural folds but not in more caudal regions of the neural tube (Fig. 1A). By E9.5, *Unc5h3* expression domains within the neuroepithelium are well established. High levels of expression are detected in the roof of the mesencephalon, diencephalon, cerebellar plate, and lateral regions of the fourth ventricle with lower levels found in the telencephalon (Fig. 1B). Similar expression patterns are observed at E10.5 (data not shown). By E11.5, high levels of expression are restricted to the cerebellar plate, dorsal mesencephalon and dorsal diencephalon (Fig. 1C). Localization of *Unc5h3* to the dorsal part of the alar laminar in the metencephalon (i.e. the rhombic lip) was identified in transverse sections at E11.5 (data not shown). During this time, the cerebellar primordium begins to enlarge due to cell proliferation and at E12.5, high levels of *Unc5h3* mRNA are detected in this expanding structure (Fig. 1D).

Unc5h3 mutant mice have ectopically positioned Purkinje and granule neurons in midbrain regions (Ackerman et al., 1997) therefore, we studied *Unc5h3* expression in the wild-type precursors of these cell types. Purkinje cells arise from

the ventricular zone between E11 and E13 and subsequently migrate along the radial glial fiber system to the middle of the cerebellar anlage where they establish the differentiating zone composed of immature neurons (Inouye and Murakami, 1980; Hatten and Heintz, 1995; Altman and Bayer, 1997). During this period, we detect *Unc5h3* transcripts in both the ventricular and differentiating zones (Fig. 1D). Hybridizations with probes to *stagger* (*Sg)*, a nuclear hormone receptor localized in Purkinje cells (Hamilton et al., 1996), confirmed the identity of these *Unc5h3*-expressing cells. Around E13 in the mouse, cell proliferation begins at the rhombic lip. This separate neuroepithelium generates a population of mitotic neuroprogenitors that migrate in a rostromedial direction over the surface of the cerebellar anlage where they establish a displaced germinal epithelium called the external granular layer (EGL) (Hatten and Heinz, 1995; Altman and Bayer, 1997). The EGL primarily gives rise to embryonic precursor cells, which are specified to become cerebellar granule cells (Alder et al., 1996). Cells within the rhombic lip express *Unc5h3* transcripts prior to the formation of the EGL (Fig. 1B and data not shown). The identity of these *Unc5h3*-expressing cells as granule cell precursors was confirmed by hybridization studies using *Atoh1*,

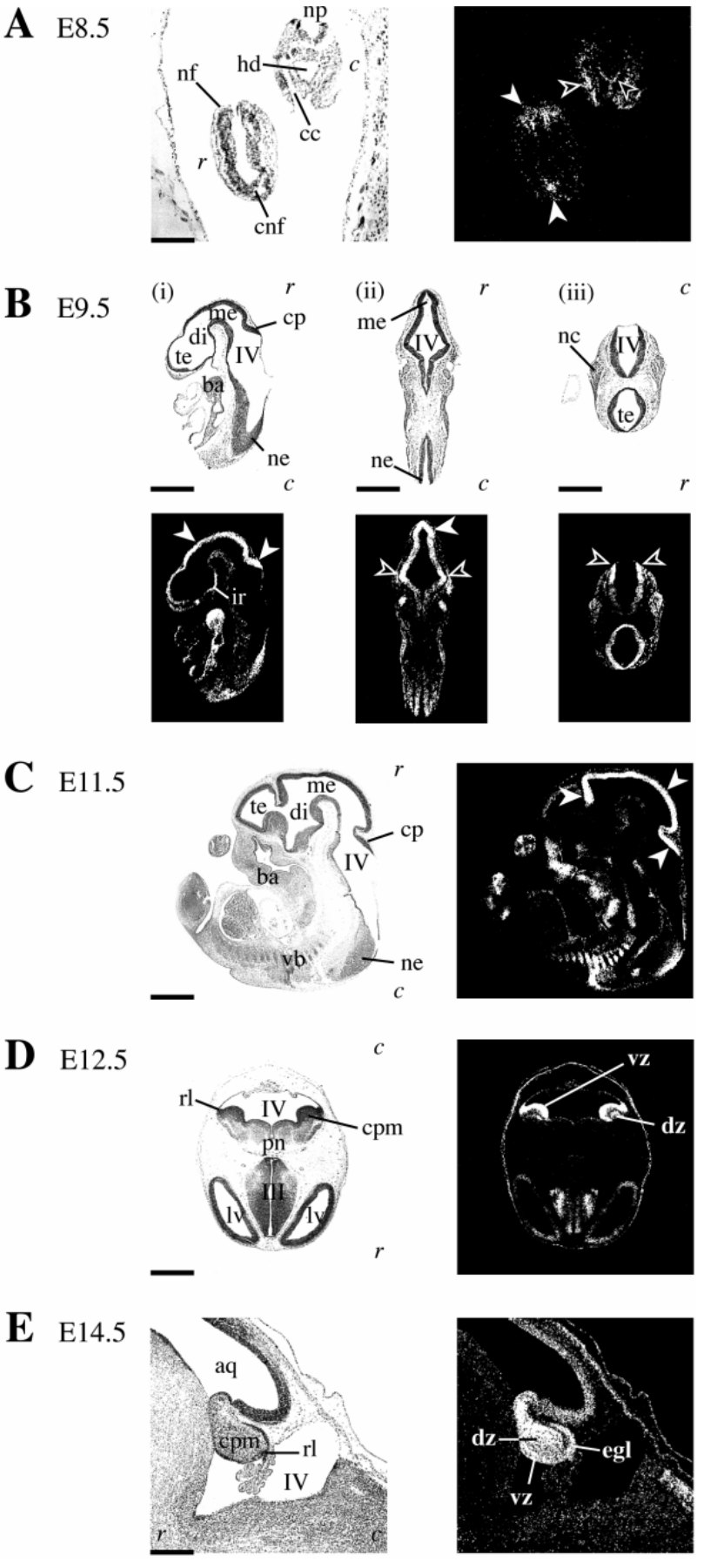


Fig. 1. *Unc5h3* expression during embryonic development of the wild-type cerebellum. Bright-field and corresponding dark-field sections are shown. (A) Section through the rostral (*r*) and caudal (*c*) regions of an E8.5 embryo within the decidua. Note *Unc5h3* expression in the neural folds (white arrowheads) and cells near to the coelomic cavity and hindgut diverticulum (open arrowheads). Scale bar: 200 μm. (B) Midsagittal (i), longitudinal (ii) and transverse (iii) sections of E9.5 embryos. *Unc5h3* expression is detected in the cerebellar plate, dorsal mesencephalon and dorsal diencephalon (white arrowheads) and in the neuroepithelium in lateral regions of the fourth ventricle (open arrowheads). Scale bars: 530 μm (C) Midsagittal section of an E11.5 embryo. *Unc5h3* expression is restricted to the cerebellar plate and the dorsal mesencephalon and diencephalon (arrow heads). Scale bar: 900 μm. (D) An E12.5 transverse section demonstrates *Unc5h3* expression in the differentiating zone, ventricular zone and rhombic lip of the cerebellar primordium. Scale bar: 700 μm. (E) Midsagittal section of an E14.5 embryo shows *Unc5h3* expression in the various regions of the developing cerebellum. Scale bar: 450 μm. (Abbreviations: aq, aqueduct; ba, branchial arch; cc, entrance to the intraembryonic coelomic cavity; cnf, cephalic neural fold; cp, cerebellar plate; cpm, cerebellar primordium; di, diencephalon; dz, differentiating zone; egl, external granular layer; IV, fourth ventricle; hd, hindgut diverticulum; ir, infundibular recess; lv,lateral ventricle; me, mesencephalon; nc, neural crest; nf, neural fold; np, neural plate, caudal part of caudal neuropore; ne,ventral neuroepithelium; pn, pontine area; rl, rhombic lip; te, telencephalon; III, third ventricle; vz, ventricular zone; vb, vertebral bodies.) Orientation: *r*, rostral; *c*, caudal.

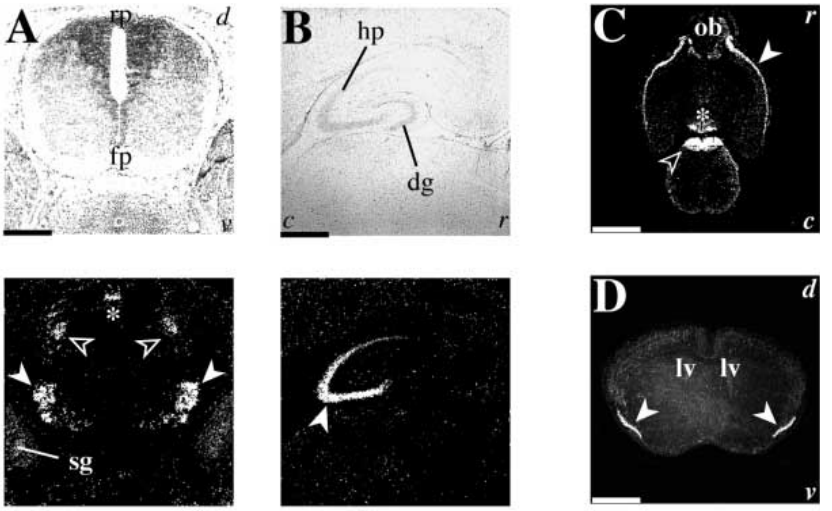


Fig. 2. Additional *Unc5h3* CNS expression domains in wild-type animals. Bright-field and corresponding dark-field sections are shown. (A) Transverse section of E12.5 thoracic spinal cord demonstrating high expression in motor columns (white arrowheads) and lower levels of specfic expression in dorsal regions (open arrowheads) and roof plate (*). Scale bar: 200 μm. (B) Horizontal section of P7 brain showing *Unc5h3* expression in hippocampus (arrowhead) but not dentate gyrus. Scale bar: 600 μm. (C) *Unc5h3* expression in the lateral olfactory tract (white arrowhead), pontine nuclei (open arrowhead) and posterior mammillary nuclei (*) in horizontal section of P7 brain. Scale bar: 2.5 mm. (D) Coronal section through the rostral adult brain displays flanking *Unc5h3* expression domains corresponding to the lateral olfactory tract (white arrowheads). Scale bar: 2.0 mm. (Abbreviations: dg, dentate gyrus; fp, floor plate; hp, hippocampus; lv, lateral ventricle; ob, olfactory bulb; rp, roof plate; sg, spinal ganglion.) Orientation: *r*, rostral; *c*, caudal; *d*, dorsal; *v*, ventral.

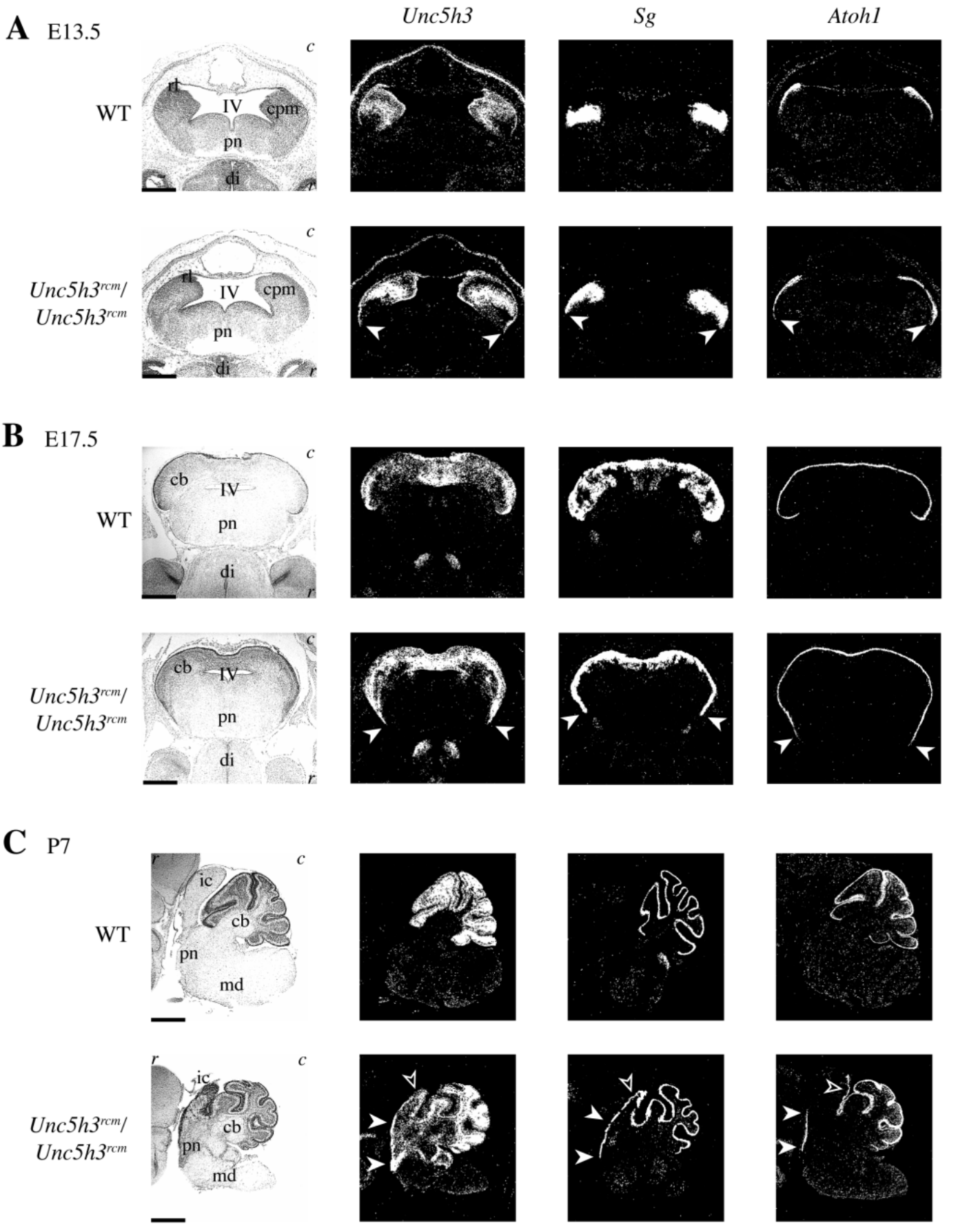
a bHLH gene expressed in granule cell neuroprogenitors (Akazawa et al., 1995; Ben-Arie et al., 1996). Purkinje and granule cell neurons expressed *Unc5h3* throughout the remainder of embryonic (Fig. 1E) and postnatal development although, by P21, *Unc5h3* mRNA levels were significantly downregulated as previously noted (Ackerman et al., 1997).

Distinct areas of *Unc5h3* expression are also found in other regions of the CNS. Sagittal sections of embryos at various ages show *Unc5h3* mRNA in extracranial neural tube of E9.5 and older embryos (Fig. 1B,C and data not shown). Transverse sections of the embryonic spinal cord revealed expression in cells of the alar and basal plates of grey matter in the mantal layer. These cells give rise to the dorsal and ventral grey horns (columns), respectively. Spinal ganglia and cells of the roof plate also express *Unc5h3* (Fig. 2A). During postnatal development and in the adult, *Unc5h3* expression is detected in the pyramidal neurons of the hippocampus but not in the granule cells of the dentate gyrus (Fig. 2B). The expression domain surrounding the region of the infundibular recess of the diencephalon (Fig. 1B) becomes enlarged during embryogenesis and appears to correspond with increasing levels of expression seen in the thalamus at later stages of embyronic/postnatal development (data not shown). In addition, the thalamic posterior mammillary nuclei, the pontine nuclei of the pons and the lateral olfactory tract also express *Unc5h3* (Fig. 2C,D).

Abnormal granule and Purkinje cell migration in *Unc5h3* mutant mice

Purkinje and granule cells have previously been identified in the adult inferior colliculus in *Unc5h3* mutants (Ackerman et al., 1997). To determine the ontogeny of these ectopic cells, we followed their development in cerebella of *Unc5h3^{rcm}* mutant animals that express a mutated form of the *Unc5h3* transcript. At E12.5, *Unc5h3* expression domains in mutant animals are identical to those of controls (data not shown). In addition, no differences in the expression of *Atoh1* are detected at this or earlier stages of development (data not shown). However, by E13.5, subtle differences in the spatial localization of *Unc5h3*, *Atoh1* and *Sg* transcripts are observed between control and mutant embryos (Fig. 3A). In *Unc5h3^{rcm}* homozygotes, subpopulations of both Purkinje and granule cell progenitors extending slightly more rostrally towards the pontine area were identified (Fig. 3A, arrowheads). As development progresses, more ectopic *Unc5h3*-positive cells were observed. By E17.5, these ectopic cells extend further rostrally, invading the lateral regions of the pontine area (Fig. 3B). In situ hybridizations with *Atoh1* identified neuroprogenitors immediately beneath the pia mater in ectopic regions and in the EGL within the normal cerebellar territory (Fig. 3B). At E17.5, these ectopic *Atoh1*-positive cells extend more rostrally compared to *Sg*-expressing Purkinje cell progenitors (Fig. 3B, arrowheads). A decrease in the overall size of the mutant cerebellum is also apparent by E17.5 (Fig. 3B). Abnormal subpial cell populations in very lateral regions of the mutant embryonic brain were confirmed by examination of serial sagittal sections of *Unc5h3^{rcm}/Unc5h3^{rcm}* brains. Furthermore, the presence of identical ectopic cell populations in embryos homozygous for the transgene insertion allele, *Unc5h3$^{rcmTgN(Ucp)1.23Kz}$*, which do not express *Unc5h3* mRNA (Ackerman et al., 1997), confirmed these ectopic cell

Fig. 3. Detection of ectopic cerebellar neurons in *Unc5h3^{rcm}/Unc5h3^{rcm}* mice. Bright-field and corresponding dark-field images show the expression domains of *Unc5h3*, *Sg* and *Atoh1* during cerebellar morphogenesis at embryonic stages (A) E13.5, (B) E17.5 and (C) P7. A and B display horizontal sections through the developing cerebellum and brain stem while C shows lateral sagittal sections of the cerebellar region. (A) Subtle differences in the expression domain for each of the transcripts are seen in *Unc5h3^{rcm}* homozygotes compared to their wild-type controls (arrowheads). (B) Ectopic expression of the *Unc5h3*, *Sg* and *Atoh1* transcripts is detected in the lateral regions of the pontine area in mutant mice (arrowheads). (C) Comparison of control and mutant expression patterns clearly shows the presence of ectopic cerebellar neurons in the pontine area (white arrowheads) and inferior colliculus (open arrowheads). Scale bars: A, 580 μm; B, 700 μm; C, 900 μm. (Abbreviations: cb, cerebellum; cpm, cerebellar primordia; di, diencephelon; IV, fourth ventricle; ic, inferior colliculus; rl, rhombic lip; md, medulla; pn, pontine area.) Orientation: *r*, rostral; *c*, caudal.


A E13.5
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populations were not due to misexpression of wild-type or mutant UNC5H3 (data not shown).

Analysis of medial/lateral sagittal sections of postnatal *Unc5h3* brains allowed the identification of ectopic cerebellar cells in the inferior colliculus (Ackerman et al., 1997). The location of ectopic cerebellar cells in the late stages of embryogenesis and throughout postnatal development was studied by examining sections from more lateral regions of the *Unc5h3* mutant brain. Movement of Purkinje and granule neurons into the inferior colliculus in P3 mutant brains appears to be a continuation of the rostral migration of cerebellar cells. In P7 *Unc5h3*^{rcm} homozygotes, lateral sagittal sections clearly demonstrate the presence of ectopic cerebellar cells throughout the lateral parts of the inferior colliculus and pontine area (Fig. 3C). Note the expression domain of *Atoh1* is discontinuous due to the subpial localization of the ectopic granule precursors passing behind the unlabelled region, around the side of the brain (Fig. 3C). *Atoh1* mRNA is down-regulated in postmitotic granule precursors and is not expressed in granule neurons migrating inwards to establish the internal granular layer (IGL) or in cells in the IGL (Akazawa et al., 1995). Therefore, hybridization studies with *RU49*, a zinc finger gene that is expressed in later stages of cerebellar granule neuron differentiation (Yang et al., 1996) were performed. These studies revealed *RU49*-positive cells in internal regions of the pontine area and inferior colliculus, demonstrating that ectopic postmitotic granule cells in these areas undergo inward migrations postnatally (data not shown). As previously noted, ectopic cerebellar cells are not found in the adjoining superior colliculus and thalamus (Ackerman et al., 1997).

Atoh1 has been shown to be expressed in the neurogenic component of the external granular layer. To confirm that ectopic *Atoh1*-positive cells in the inferior colliculus and pontine area are mitotically active during postnatal cerebellar development, we performed BrdU-labelling studies on *Unc5h3*$^{rcmTgN(Ucp)1.23Kz}$ mutant and wild-type brains. After exiting the cell cycle, granule cell precursors begin their inward migrations at P3 to form the internal granule layer (IGL). This process continues until about P15 at which time the EGL is no longer present (Hatten and Heintz, 1995). BrdU immunoreactivity in mutant brains was detected in the proliferative layer of the EGL within the normal cerebellar territory and was continuous with the labelling of ectopic cerebellar granule cells in the inferior colliculus (Fig. 4). Abnormal cohorts of granule cells that extend from the EGL down into the molecular layer have been described in lateral regions of postnatal cerebella from homozygous *Unc5h3*^{rcm} mice (Ackerman et al., 1997). BrdU immunoreactivity was also detected in these clusters of granule cells (data not shown), suggesting either migration of mitotically active granule cells or abnormal invaginations of the EGL. Immunodetection of BrdU injected into P3 and P15 wild-type and mutant littermates, demonstrated that temporal control of cell proliferation and the postmitotic migration of ectopic granule cells is similar to that of progenitors in the normal EGL (data not shown).

Expression of *Unc5h3* and *netrin1* during cerebellar embryogenesis

We have previously demonstrated that cells expressing the UNC5H3 protein can bind netrin1 in vitro (Leonardo et al., 1997), suggesting a potential receptor/ligand interaction. To determine whether such a relationship may occur in vivo, we examined the expression domains of netrin1 in contrast to those of *Unc5h3* during cerebellar formation. The *Unc5h3*-expressing cerebellar primordia are located to the caudal/dorsal and lateral aspects of the fourth ventricle. In contrast, *netrin1* is expressed in a rostral/ventral and medial fashion within this region (Fig. 5). At E12.5, *netrin1* mRNA is detected in the median sulcus and basal plate of the fourth ventricle, juxtaposed to the expression domain of *Unc5h3* (Fig. 5A). Examination of midsagittal sections also show an enlarged *netrin1* expression domain associated with the median sulcus, located directly opposite the developing cerebellum (Fig. 5B). The expression pattern of *netrin1* is similar during the remainder of embryogenesis and early postnatal development and did not vary between wild-type and *Unc5h3* mutant mice (Fig. 5C and data not shown).

Unc5h3 expression exclusive of CNS development

Unc5h3 transcripts are first detected at E7.5 in the allantois and parts of the amniotic membrane (data not shown). At E8.5,

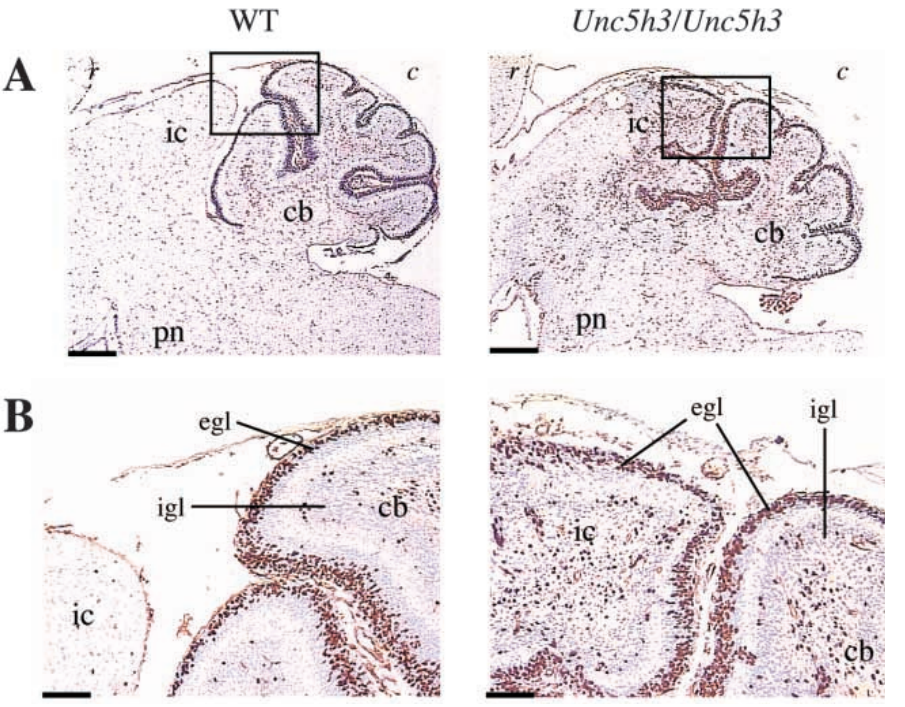

Fig. 4. Detection of mitotically active granule neurons in P7 wild-type and *Unc5h3*$^{rcmTgN(Ucp)1.23Kz}$ mutant mice. (A) BrdU-labeled granule cell progenitors within the normal cerebellar territory and in the inferior colliculus of mutant animals. Scale bars: 400 μm. (B) Higher magnification images of the boxed region depicted in A. Scale bars: 110 μm. (Abbreviations: cb, cerebellum; egl, external granular layer; ic, inferior colliculus; igl, internal granular layer; md, medulla; pn, pontine area.) Orientation: *r*, rostral; *c*, caudal.

Unc5h3 is expressed in the mesodermal cells surrounding the coelomic cavity and in cells ventral to the hindgut diverticulum (Fig. 1A). By E10.5, *Unc5h3* expression is found in cells of the dorsal gut mesentery and coelomic angles which adjoin the urogenital ridge (Fig. 6A), locations corresponding to those of migrating germ cells (Rugh, 1990). In addition, we detected *Unc5h3*-positive cells at later stages of gonadogenesis (E14.5). *Unc5h3* expression is also detected in subectodermal migrating neural crest cells (Fig. 6B). Expression in the developing lung is identified at E10.5 (Fig. 6B). At E14.5, the level of *Unc5h3* message is highest in the interstitial cells of the immature lung while much lower levels are detected in the cells lining the developing bronchioles (Fig. 6C). Also at this time, *Unc5h3* mRNA is found throughout the embryonic renal system with the cells surrounding the collecting ducts and those of the ureter showing the strongest signal (Fig. 6C). Components of the E9.5 branchial arch express *Unc5h3* (Fig. 1B) and structures derived from the arch, including the jaw and cartilages of the palate, remain *Unc5h3*-positive during the later stages of embryogenesis (Fig. 6C). *Unc5h3* transcription increases from E10.5 in the cartilagenous primordia of many bones and cartilages (Figs 1C and 6C) including the somitic sclerotome, which gives rise to the vertebrae and vertebral discs (Fig. 1C and data not shown). The *Unc5h3* transcript is not detectable in several other tissues including heart, liver and gastrointestinal tract (Fig. 6C).

DISCUSSION

UNC5H3/netrin1 signaling is critical for the establishment of the rostral cerebellar boundary

To further define the mechanisms underlying abnormal cell migrations in UNC5H3-deficient mice, we examined the embryonic movements of *Unc5h3*-expressing cerebellar neurons in mutant animals. Our results indicate that both Purkinje and granule cells migrate rostrally into ectopic areas during the time of normal embryonic granule cell migrations. Further, they suggest that these cells fail to respond to a chemorepulsive cue expressed in areas adjoining the rostral cerebellum that would normally function to prevent such movements.

The secreted protein netrin1 is a ligand that may be involved in the guidance of *Unc5h3*-receptor-mediated migration of neuronal precursors during cerebellar development. *netrin1* encodes a secretory protein and has the capacity to act at some distance from its source (Serafini et al., 1994, 1996; Colamarino and Tessier-Lavigne, 1995). It has been shown that UNC5H3 can bind netrin1(Leonardo et al., 1997) and, in *C. elegans*, cells expressing the homolog of UNC5H3, UNC-5, migrate away from sources of netrin (Hedgecock et al., 1990; Leung-

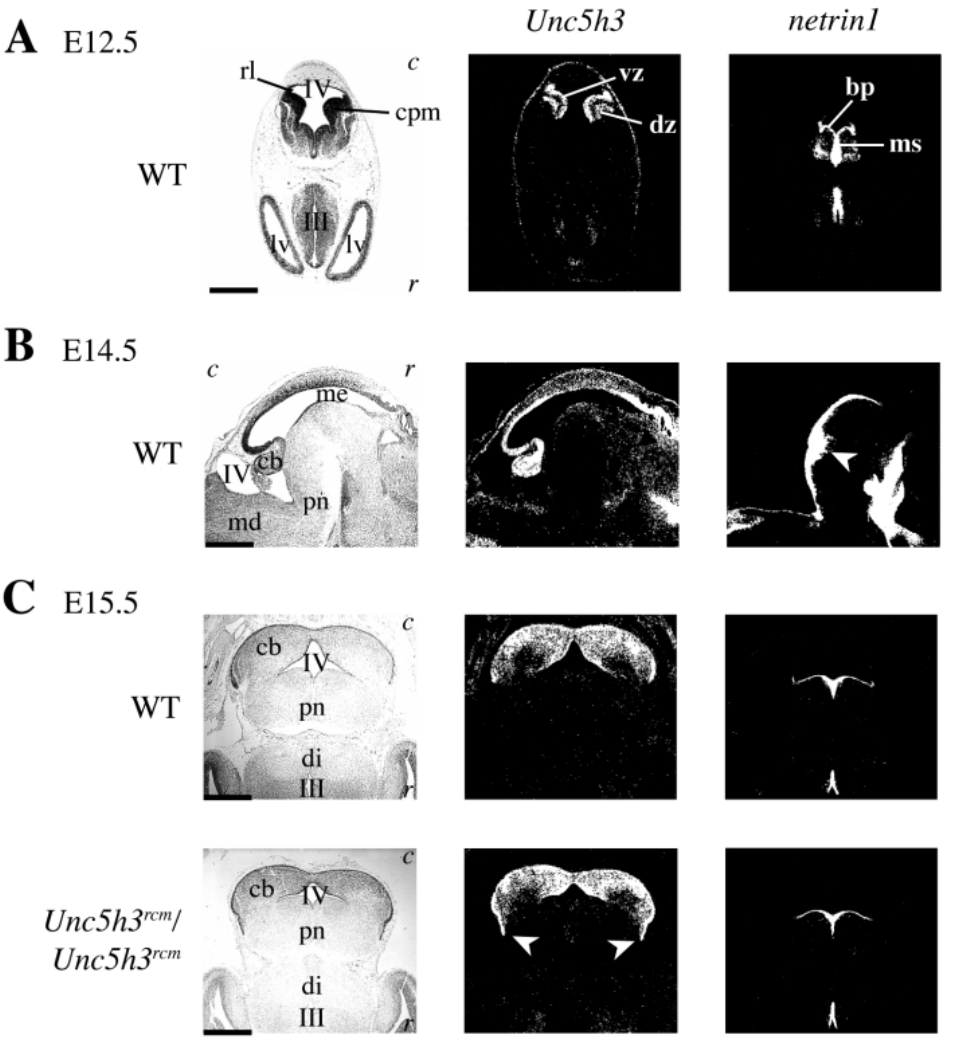

Fig. 5. *Unc5h3* and *netrin1* expression domains during cerebellar development. Bright-field and corresponding dark-field images of wild-type (A-C) and *Unc5h3*^{rcm}/*Unc5h3*^{rcm} (C) tissues. (A) Transverse sections of E12.5 embryo. Note *Unc5h3* expression in the rhombic lip, ventricular zone and differentiating zone of the cerebellar primordium. *netrin1* expression is confined to the median sulcus and basal plate of the floor plate area. Scale bar: 700 μm (B) Midsagittal sections of E14.5 embryo showing *Unc5h3* expression in the cerebellum and roof of the mesencephalon while *netrin1* expression is restricted to the floor of the mesencephalon and fourth ventricle. Arrowhead marks the wider band of *netrin1* expression directly opposite the cerebellum. Scale bar: 700 μm. (C) Horizontal sections through the developing cerebellum and pontine area of E15.5 wild-type and *Unc5h3*^{rcm} mutant embryos showing *Unc5h3* and *netrin1* expression patterns. Arrowheads indicate ectopic *Unc5h3*-positive cells in lateral regions of the pontine area (pn) in mutant embryos. Scale bars: 700 μm. (Abbreviations: bp, basal plate; cb, cerebellum; cpm, cerebellar primordium; di, diencephalon; dz, differentiating zone; IV, fourth ventricle; lv, lateral ventricle; ms, median sulcus; md, medulla; me, mesencephalon; pn, pontine area; rl, rhombic lip; III, third ventricle; vz, ventricular zone.) Orientation: *r*, rostral; *c*, caudal).

Hagesteijn et al., 1992; Hamelin et al., 1993). Our expression results indicate that, in vertebrates, netrin1 may act as a chemorepellent for cells expressing the product of at least one member of the *unc-5* gene family, UNC5H3. We propose that neuroprogenitors in the wild-type cerebellum respond to the chemorepulsive cue from the extracellular ligand, netrin1, and are checked from moving into adjacent brain regions, hence forming the lateral rostral cerebellar domain (Fig. 7). However, in lateral regions of the developing *Unc5h3* mutant cerebellum, migrating neuroprogenitor cells that do not express a functional UNC5H3 receptor are unresponsive to netrin1 (or functionally related guidance cues), and thus migrate ectopically (Fig. 7). In support of this idea, we have further characterized the location of *netrin1*-expressing cells in regions consistent with such a signaling mechanism.

The chemorepulsive action of netrin1 during vertebrate embryonic development has been suggested by in vitro experiments. Trochlear motor axons are repelled by both netrin1-expressing COS cells and floor plate explants suggesting that netrin1 plays a role in the guidance of these neurons (Colamarino and Tessier-Lavigne, 1995). Furthermore, it has been suggested that netrin1 may function through a concentration-dependent inhibitory mechanism which, in vivo, may simply create an inhibitory barrier that prevents trochlear motor axons from approaching the ventral midline (Colamarino and Tessier-Lavigne, 1995). During cerebellar formation, netrin1 may also establish a similar exclusion zone that migrating *Unc5h3*-expressing neurons avoid.

UNC5H3/netrin1 signaling is unlikely to be the only molecular mechanism controlling cell movements within the cerebellum since many migrating cells remain within the normal cerebellar territory in mutant animals. Functional redundancy of UNC5H3 with other netrin1-binding family members may exist. Two additional vertebrate homologs of *unc-5*, *Unc5h1* and *Unc5h2*, have recently been described which share overlapping expression domains with *Unc5h3* in the cerebellar cortex (Leonardo et al., 1997). Although the function of these other receptors is currently unknown, they may operate in more central regions of the developing cerebellum but do not appear to compensate for the loss of UNC5H3 in lateral regions. Furthermore, *netrin1*-deficient mice might be expected to show similar cerebellar abnormalities as those in *Unc5h3* homozygotes, however, no obvious cerebellar phenotype has been reported (Serafini et al., 1996). This may indicate that other ligands in addition to netrin1, such as netrin2 or other netrin family members, activate the UNC5H3 receptor and hence compensate for its loss in *netrin1* mutants. The apparently normal development of many commissures and trochlear pathways in *netrin1*-deficient mice, and the repulsion of trochlear axons in vitro by *netrin1* mutant floor plates supports this idea (Serafini et al., 1996). In fact, co-expression of two apparently redundant netrins has been shown to occur at midline in *Drosophila* (Harris et al., 1996; Mitchell et al., 1996).

Abnormal migrations of granule and Purkinje precursors in *Unc5h3* mutants

Mutation of the *Unc5h3* gene results in both ectopic granule and Purkinje neurons within regions of the brain stem and midbrain. Although the presence of ectopic granule precursors appears to be the result of a continued subpial migration, which begins when cells first leave the rhombic lip, the abnormal movement of Purkinje cells is more difficult to explain. Unlike migration of the EGL, there is no previous evidence for similar

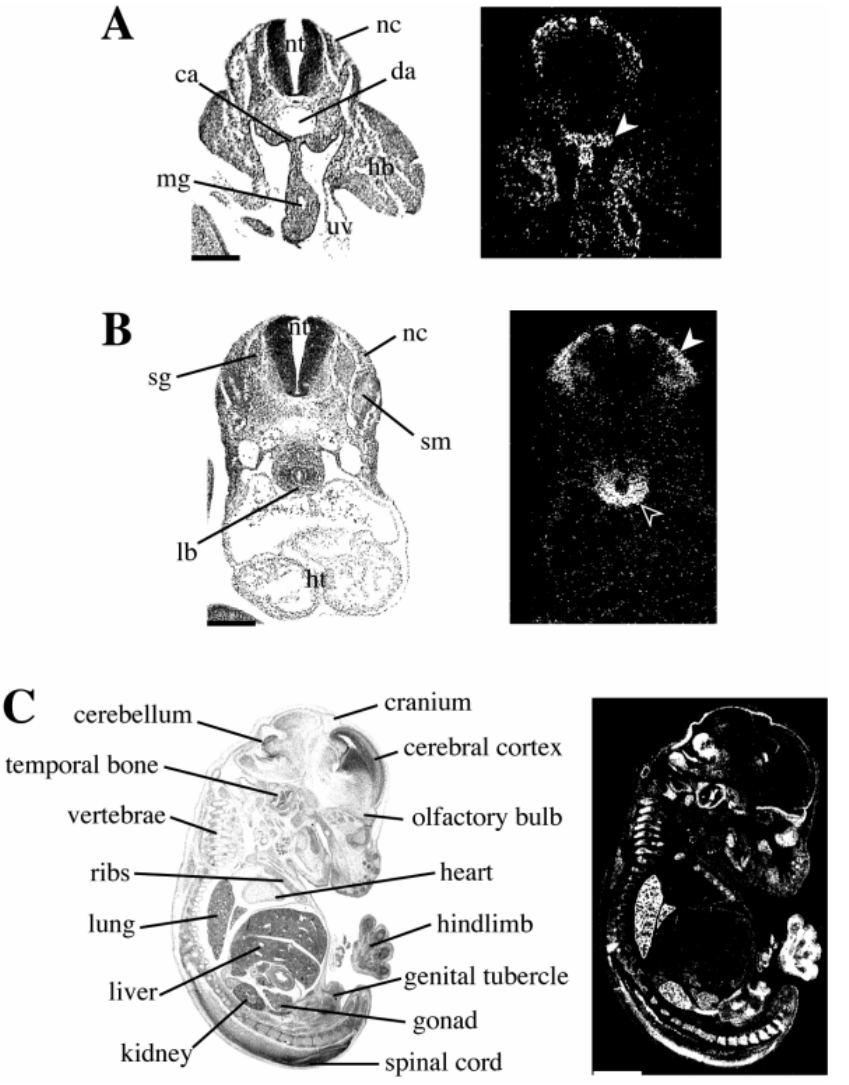

Fig. 6. *Unc5h3* expression domains exclusive of the CNS. Bright-field and corresponding dark-field images of E10.5 embryos in transverse section, showing the tail (A) and midtrunk region (B). Note *Unc5h3* expression in the coelomic angles (A, arrowhead), neural crest (B, white arrowhead) and lung bud (B, open arrowhead). Scale bars: A, 230 μm; B, 280 μm. (C) Corresponding bright-field and dark-field images of a parasagittal section at E14.5. Observe *Unc5h3* expression in major structures including cerebellum, lung, kidney, spinal cord, cartilage primordia of vertebrae and various other bones, gonad, genital tubercle and limb. Scale bar: 2.0 mm (Abbreviations: ca, coelomic angles; da dorsal aorta; ht heart; hb hindlimb bud; lb, lung bud; md, midgut; nt, neural tube; nc, neural crest; sm, somite; sg, spinal ganglion; uv, umbilical vein.)

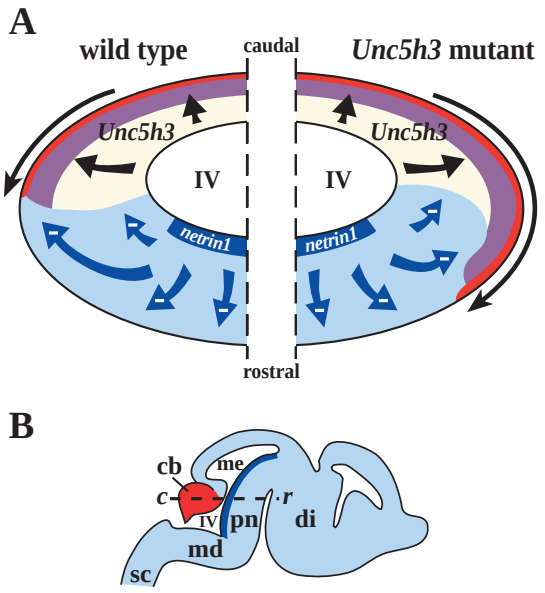

Fig. 7. The role of UNC5H3 and netrin1 in the establishment of the rostral cerebellar boundary. Schematic (A) represents a horizontal section through the embryonic cerebellar/pontine area and fourth ventricle (IV) as depicted by the dotted line (*c*-*r*) in the sagittal section through a late embryonic brain (B). The expression domains of *Unc5h3* in the cerebellum (in red, B) and *netrin1* in floor plate area (in blue) are shown (A and B). Radial Purkinje cell migrations (depicted by black arrows in bold) to form the Purkinje cell layer (in purple) appear normal in both wild-type and mutant cerebella. In lateral regions of the wild-type cerebellum, the tangential migrations (depicted by thin black arrows) of *Unc5h3*-expressing granule cells (in red) are checked by the chemorepulsive activity of netrin1 (blue arrows) and consequently the normal rostral cerebellar boundary is established. However, *Unc5h3*-defective granule and Purkinje cells migrate rostrally into adjacent brain regions in mutant mice. (Abbreviations: cerebellum (cb); diencephalon (di); fourth ventricle (IV); medulla (md); mesencephalon (me); pontine area (pn); spinal cord (sc). Orientation: *c*, caudal; *r*, rostral).

tangential movements of Purkinje cell precursors during normal cerebellar development. In *Unc5h3* homozygotes, Purkinje cell progenitors appear to be correctly positioned prior to the appearance and migration of granule progenitors and are detected in ectopic locations only after cells begin to migrate from the rhombic lip. Furthermore, at later stages of embryogenesis, ectopic granule precursors migrate rostrally in advance of Purkinje cells.

During normal cerebellar development, Purkinje and granule neurons become synaptic partners and their highly ordered alignment may involve some form of cell-cell communication. Interdependence between granule and Purkinje neurons during their terminal stages of differentiation has been previously noted (reviewed in Hatten and Heintz, 1995), however, little is known concerning the embryonic interactions of these cells and the mechanisms coordinating their positioning. Our data suggest that the movement of Purkinje cells may be subsequent and dependant on those of the granule cells. Moreover, these results indicate that communication may exist between embryonic Purkinje and granule cell precursors, which functions to establish their correct alignment.

The effect of the *Unc5h3* mutation on cerebellar size and foliation pattern (Ackerman et al., 1997) may also be secondary to that of abnormal cell migrations. In *Unc5h3* mutants, the displaced EGL in the pontine and midbrain regions continues to proliferate in a manner analogous to that seen in the normal cerebellum, ectopically producing a significant proportion of the future cerebellar mass. Cell proliferation within the normal territory of the cerebellum does not appear to compensate for this loss of ectopically positioned material and subsequently the cerebellum of *Unc5h3* homozygotes remains smaller compared to their wild-type counterparts. This reduction in the size of the mutant cerebellum may have an adverse effect on foliation. It has been suggested that cerebellar foliation is due to buckling forces produced by the more rapid expansion of outer cortical layers, including the EGL, over the surface of more slowly growing tissues underneath (Haddara and Nooreddin, 1966; Mares and Lodin, 1970). In the *Unc5h3* mutant cerebellum, smaller forces would be generated by a reduced EGL hence resulting in fewer folia.

In addition to its function in cerebellar development, the expression of *Unc5h3* in other cell types may implicate its involvement in other developing systems. Both primordial germ cells and neural crest tissue undergo vast migrations during embryogenesis (Le Douarin and Ziller, 1993; Rugh, 1990) and appear to express *Unc5h3* mRNA. Yet no obvious abnormalities have been detected during the development of these cells in *Unc5h3* homozygotes. Similarly, no apparent defects were found in other *Unc5h3*-expressing tissues in *Unc5h3* mutants. While these data may suggest that such *Unc5h3* expression is functionally redundant, the function of other members of the vertebrate UNC-5-like gene family which share similar expression patterns with *Unc5h3* (Leonardo et al., 1997) may be of more importance in the development of these tissues. Together with the data presented here, functional analyses of these additional family members will no doubt further our understanding of the molecular mechanisms involved in cell migration and positioning during embryonic development.

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