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Summary

The cytoarchitecture of the hindbrain results from precise and co-ordinated sequences of neuronal migrations. Here, we show that reelin, an extracellular matrix protein involved in neuronal migration during CNS development, is necessary for an early, specific step in the migration of several hindbrain nuclei. We identified two cell populations not previously known to be affected in *reeler* mutants that show a common migratory defect: the olivocochlear efferent neurons and the facial visceral motor nucleus. In control embryos, these cells migrate first toward a lateral position within the neural tube, and then parallel to the glial cell processes, to a ventral position where they settle close to the pial surface. In *reeler* mutants, the first migration is not affected, but the neurons are unable to reach the pial surface and remain in an ectopic position. Indeed, this is the first evidence that the migration of specific hindbrain nuclei can be divided into two parts: a reelin-independent and a reelin-dependent migration. We also show that reelin is expressed at high levels at the final destination of the migratory process, while the reelin intracellular effector Dab1 was expressed by cell groups that included the two populations affected. Mice mutant at the Dab1 locus, called *scrambler*, exhibit the same phenotype, a failure of final migration. However, examination of mice lacking both reelin receptors, ApoER2 and VLDLR, did not reveal the same phenotype, suggesting involvement of an additional reelin-binding receptor. In the hindbrain, reelin signaling might alter the adhesive properties of efferent neurons and their ability to respond to directional cues, as has been suggested for the migration of olfactory bulb precursors.

Key words: Reeler mutant, Dab1, Hindbrain, Neuronal migration

Introduction

Migration of neuroblasts and neurons is an essential process in the development of the nervous system. Mechanisms for regulating neuronal migration include attraction, repulsion, or a change in cell adhesion properties (Marin and Rubenstein, 2003). One of the best-described examples of migration defects is the mouse autosomal recessive mutant *reeler*, characterized by ataxia, tremors and impaired motor coordination. Morphologically, the *reeler* mouse displays aberrant layer formation in the neocortex, hippocampus and cerebellum. This phenotype has been attributed to a mutation in the reelin gene (D'Arcangelo et al., 1995; Hirotsune et al., 1995). Reelin is a large extracellular matrix protein containing several EGF-like repeats. Several receptors have been described for reelin. The apolipoprotein E receptor 2 (ApoER2) and the very low-density lipoprotein receptor (VLDLR) can both bind reelin, and mice deficient for both proteins exhibit the same phenotype in the neocortex and the cerebellum as *reeler* mutants do. Another spontaneous mutation, *scrambler*, demonstrates the same layer disorganization as the *reeler* mutant does (Sheldon et al., 1997). *Scrambler* is a mutation of the gene disabled homolog 1 (*Dab1*), which encodes the Dab1 adaptor protein (Howell et al., 1997; Sheldon et al., 1997). Binding of reelin to its receptors results in the recruitment of Dab1 to a cytoplasmic docking site on the receptors. Phosphorylation of Dab1 on tyrosine residues is essential for its activity (Howell et al., 1997). The intracellular signaling pathways activated through Dab1 include non-receptor tyrosine and serine/threonine kinases (Bock and Herz, 2003).

The exact mechanisms involved in the *reeler*-like migration defects have not yet been elucidated. The aberrant layer formation documented in *reeler* and *scrambler* mutants may be related to defects in radial glia guided migration (Marin and Rubenstein, 2003). It has been shown that cortical neurons follow the long process of radial glial cells that extend from the lumen to the pial surface, performing a radial migration. Various lines of evidence indicate that the *reeler* phenotype could, at least partly, be due to impaired detachment of neuronal precursors from radial glial processes, to disorganization of radial glial scaffold, or to abnormal glial endfeet attachment at the pial surface (Tissir and Goffinet, 2003).

Defects in neuronal positioning in several other CNS regions

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have been observed in *reeler* mice. Study of migrating adult olfactory interneuron precursors have shown that reelin is necessary for the switch from tangential chain migration to radial individual migration in the olfactory bulb (Hack et al., 2002). In the spinal cord, a particular neuronal population that originates from the same precursor cells as motoneurons, the sympathetic preganglionic neurons, is ectopically positioned in the absence of reelin (Phelps et al., 2002).

The vertebrate hindbrain contains specialized nuclei with different functions and positions, which is the result of complex migrations that take place over a period of several days during embryonic development (Chandrasekhar, 2004). Most studies performed on hindbrain motoneuron nuclei in *reeler* hindbrain have been carried out after birth or in adult animals, except for the study of the facial nucleus phenotype described by Goffinet (Goffinet, 1984). Separate studies reported defects for the trigeminal, the cochlear nucleus, the nucleus ambiguous and the facial nuclei (Fujimoto et al., 1998; Goffinet, 1984; Martin, 1981; Terashima et al., 1993; Terashima et al., 1994). The common phenotype was a variable disorganization of the considered nucleus, the reason for this disorganization remaining unknown.

We used molecular markers that identify specific hindbrain neuronal populations in order to follow the migration and differentiation of these nuclei in the early development of the *reeler* mouse, as well as nuclei position in mutants for *Dab1* and reelin receptors. We demonstrate that ectopic positioning of hindbrain nuclei in mutant embryos is a more general deficiency of those nuclei that undergo radial migration, and includes facial visceral motoneurons and olivocochlear efferents. In all cases, the affected nuclei remain at the position where they normally switch from dorsolateral to radial migration. We conclude that the phenotype results from the impairment of the final step of migration where the neurons migrate ventrally along (or parallel to) radial glial fibers.

Materials and methods

Mice

Reeler-Orleans and *scrambler* mice were maintained on a BalbC background. *Reeler* homozygous mutant mice were identified by PCR genotyping according to Takahara et al. (Takahara et al., 1996). *Scrambler* mutants were bred as homozygous animals and no genotyping was necessary. *Vldlr* and *Apoer2* mutant mice were on a mixed C57Bl6/Sv129 background. Independently double-mutant embryos were obtained from H. Bock (Zentrum für Neurowissenschaften, Freiburg). *Reeler* mutants and *Vldlr/Apoer2* double-mutants were obtained by mating heterozygous animals (double-mutant *Vldlr*/*Apoer2* knockout embryos were generated by mating *Apoer2* heterozygous animals kept on a *Vldlr* knockout background), and genotypes were determined by PCR using the protocols described at the JAX mice site (The Jackson Laboratory, Maine, USA). Animals were housed under standard conditions with free access to water and food on a normal 12-hour light/dark cycle. Experiments were performed in accordance with the Principles for Laboratory Animals published by the European Ethical Committee. Control embryos in the same experiment were littermates of the mutant embryos.

In situ hybridization and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. In situ hybridization (ISH) on whole-mount preparation and cryostat sections were carried out as described by Carroll et al. (Carroll et al., 2001). ISH on vibratome sections has been described previously (Poluch et al., 2003). The following cDNA probes were used: islet 1 (Pfaff et al., 1996), c-Ret (Pachnis et al., 1993), Phox2b (Pattyn et al., 1997), Gata3 (Karis et al., 2001), Tbx20 (Kraus et al., 2001), Lhx4, ER81 (Gavalas et al., 2003), ApoER2 and VLDLR (Trommsdorff et al., 1999). Double in situ hybridization/immunohistochemistry were performed as described previously (Carroll et al., 2001), on floating vibratome sections. Indirect immunohistochemistry analyses were performed on vibratome sections using secondary peroxidase-conjugated antibody.

The following antibodies were used for double immunofluorescence experiments on vibratome sections: mouse anti-islet 1 (39.4D5) and RC2 (Developmental Studies Hybridoma Bank). Secondary antibodies, FITC-conjugated anti-mouse IgG FC-fragment specific (Sigma F5897) and Cy3-conjugated anti-mouse IgM (Jackson Laboratories) were applied for islet 1 and RC2 labeling, respectively. Images were acquired with a Leica confocal microscope and Imaris-image processing software was used for further image analysis.

Retrograde labeling of cranial nerves

E11.5 and E12.5 embryos were dissected and fixed in 4% PFA. DiI injections were performed as previously described (Goddard et al., 1996). DiI was applied to the VII nerve or to the VIIIth ganglion of both sides of the embryos, and allowed to diffuse for 2 to 5 days. Alternatively, we injected DiI in one otocyst of the embryo to observe contralateral subpopulations. Hindbrains were then dissected and mounted in glycerol. In older embryos (E12.5), the brains were then embedded in 2% agarose and vibratome sectioned (60-80 μm). Image analysis was performed with either a standard Nikon epifluorescence microscope or a Biorad confocal microscope for optical sections.

Photoconversion of DiI was performed on vibratome sections as described previously (Singleton and Casagrande, 1996), then refixed in 4% PFA, dehydrated through ethanol series and stored at –20°C until ISH processing.

Results

Altered migration of branchial and visceral motoneurons in the reeler mouse

Hindbrain motoneuron precursors (MNPs) are born in the ventricular zone at E10.5. We used islet 1 as an early marker to follow MNP migration during hindbrain development. Islet 1 labels all cranial MNP, which includes branchial, visceral and somatic motoneurons (Pfaff et al., 1996). In wild-type embryos, branchial and visceral motoneurons migrated to a final ventral position near the pial surface, where they can be observed by whole-mount in situ hybridization (ISH) (Fig. 1A,C,E,G). Islet 1 labeling of cells deeper within the neural tube cannot be visualized by this technique after E12.5.

Going from rostral to caudal, the trigeminal branchiomotor (V), the cochlear efferent or olivocochlear (OC) nucleus, the facial visceral (FVM) and the facial branchiomotor (VII) nuclei can be observed (Fig. 1A).

We will describe first the migration of the different MNP throughout hindbrain development in wild type, and how the MNP are affected in *reeler* mutants (see Fig. 10 for a diagrammatic representation).

In control embryos, the position and the size of the V nucleus are conserved between E12.5 and E15.5 (Fig. 1A,C,E,G). In *reeler* mice, the V main nucleus is present at its normal position (Fig. 1B,D,F,H). At E12.5, the VII neurons form a continuous stream of cells migrating caudally close to the midline (Fig. 1A). Lateral to these migrating cells, a thin

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column of cells is labeled by islet 1 between the V and VII nuclei. Previous data suggest that these cells may represent at least three nuclei: the r3-born trigeminal neurons, OC efferents and FVM nuclei (Bruce et al., 1997; Pattyn et al., 2000) (Fig. 1A, arrows). These efferent neurons perform a lateral migration at r4 and r5 levels, followed by a ventral migration toward the pial surface between E11.5 and E14.5 (Fig. 1A,C,E, arrows; see Fig. 10). OC/FVM nuclei form a continuous cell population at E13.5 rostral to the VII nucleus (Fig. 1C, arrows) (Bruce et al., 1997; Fritzsch, 1999; Karis et al., 2001; Pattyn et al., 2000; Tiveron et al., 2003). In *reeler* mice, this column of islet 1-expressing cells appears reduced at E12.5 and is barely visible by E13.5 (Fig. 1B,D, arrow). At E14.5 and E15.5, no islet 1 labeling was observed at the ventral pial surface in this region, ruling out the possibility that the final migration process is simply delayed (Fig. 1F,H).

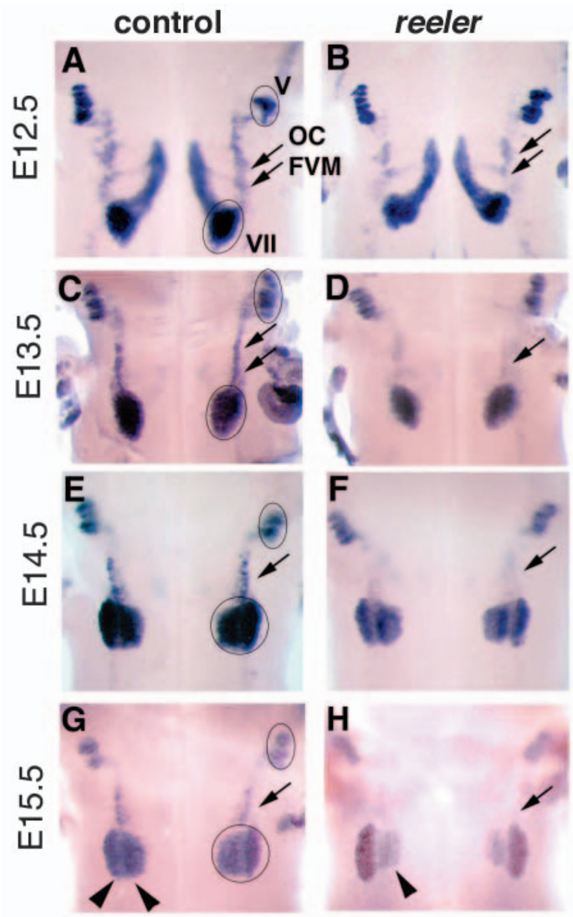

Fig. 1. Altered olivocochlear efferent and facial visceral motoneuron development in *reeler* mutant hindbrain. Motoneuron distribution was determined by using an islet 1 riboprobe on whole-mount preparations. Pial side view of flat-mounted control (A,C,E,G) and *reeler* (B,D,F,H) embryos at E12.5, E13.5, E14.5 and E15.5. (A) In control embryos at E12.5, islet 1 expression is observed in the Vth (trigeminal motor) and VIIth (facial) migrating nuclei, and in a discrete lateral column of cells (arrows) between these two nuclei that includes a sub-population of the trigeminal, the facial visceromotoneurons (FVM) and the olivocochlear neurons (OC). At this stage many facial motoneurons are still in the process of migrating caudally from their birthplace in rhombomere 4 to their settling position in rhombomere 6. (B) In *reeler* embryos, VII neuron migration is comparable to control, whereas the OC/FVM column is reduced in its rostrocaudal extension (arrows). (C) In control embryos at E13.5, islet 1 expression is clearly observed in the main Vth nucleus, in the OC/FVM motoneurons, and in the VIIth nucleus. (D) In *reeler* mutants, the OC/FVM group of motoneurons is barely detectable (arrow). (E-H) At E14.5 (E,F) and E15.5 (G,H), FVM (and/or associated OC) are not labeled by islet 1 at the pial side in *reeler* embryos (F,H; arrows). At this stage most of the migrations have already occurred or are in the final stages. By E15.5, the facial nucleus forms two lobes, medial and lateral, in control embryos (G, arrowheads). In *reeler* embryos (H, arrowhead), the medial lobe seems reduced when compared with the control.

Facial (VII) motoneurons are born in r4 and form a stream of cells migrating caudally through r5 and into r6, before migrating ventrally toward the pial surface in r6 (Fig. 1A) (Garel et al., 2000). This process is complete by E15.5 (Fig. 1G). At this later stage, the VII nucleus comprises two lobes, a medial and lateral one (Fig. 1G, arrowheads). In *reeler* embryos, from E12.5 to E14.5, the VII nucleus is present in its normal position (Fig. 1B,D,F) whereas at E15.5, the medial lobe is reduced relative to the wild type (Fig. 1H, arrowhead). Using *Lhx4* and *ER81* transcription factor expression as markers of MN identities (Sharma et al., 1998; Gavalas et al., 2003), we observed that in *reeler* mice, the medial *Lhx4*+ cell

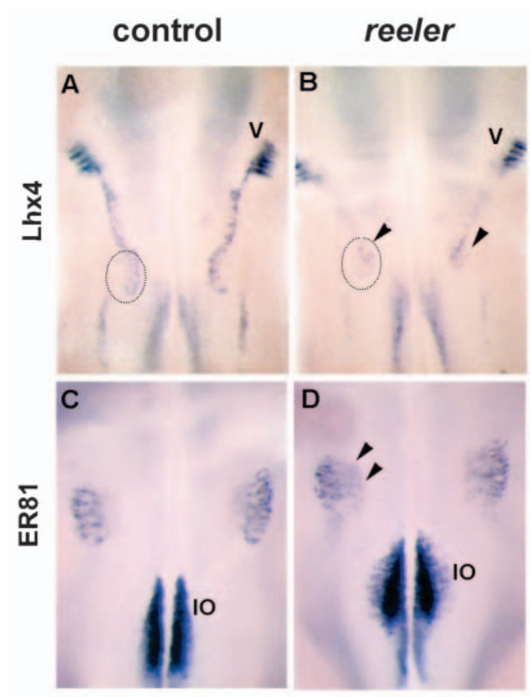

Fig. 2. Reduced and scattered expression of Lhx4 and ER81 by facial MN in *reeler* mice. The pial side of whole-mount E14.5 control (A,C) and reeler mutant (B,D) mice is shown. (A) Lhx4 is expressed in the trigeminal motor nucleus (V), a column of cells at the r4/r5 level, as well as in the medial part of the facial nucleus (circled), in control embryos. (B) In *reeler* mutants, the Lhx4-positive area in the facial nucleus is reduced in size (circle), and positive cells were observed deeper in the parenchyme (arrowheads). Most of the rostral column is not present at the pial surface. (C) In control embryos, *ER81* mRNA is expressed in cells in the most lateral part of the facial nucleus, and in the inferior olive precursors cells. (D) In reeler mutants, ER81 labeling is mainly localized in the lateral part of the facial nucleus (arrowheads), but in a more scattered pattern than in controls. IO, inferior olive.

group is reduced (Fig. 2A,B, arrowheads), whereas the lateral *ER81*+ cell group appears to be more scattered than in the wild type (Fig. 2C,D, arrowheads). We conclude that despite a global disorganization of the VII nucleus, the two populations of VII neurons are correctly specified.

Reelin is required for the final radial migration of FVM neurons to the pial surface

In whole-mount *reeler* preparations, no islet 1 labeling of OC/FVM nuclei was observed at the pial surface after E12.5. In order to assess the presence of islet 1-expressing cells through the thickness of the neural tube, we sectioned embryos after whole-mount islet 1 ISH.

In vibratome sections performed at the r5 level of E13.5 control embryos, the FVM appears as a compact group of islet 1-expressing cells close to the pial surface (Fig. 3A,F; arrow in 3A). In *reeler* hindbrain at the same rostro-caudal level, the FVM is absent from its normal position (Fig. 3B, circle), but a prominent group of ectopically positioned cells is present at the ventricular side (Fig. 3B, arrowed stippled circle). In addition, a more diffuse ectopic group is observed in an intermediate position (Fig. 3B, arrowhead). In cryostat sections, the same result was observed with ectopic groups of cells within the depth of the neural tube (Fig. 3G).

To further characterize the two ectopic clusters observed in *reeler* embryos, we used additional markers of MNP differentiation, Ret, Phox2B and neurofilament light chain (NF-L; Nefl – Mouse Genome Informatics). If MNP cells have a primary identity defect, they might not acquire the correct differentiation phenotype and/or perform the correct migration. The pattern of Ret expression at level r5 in control embryos (Fig. 3C) was comparable with that of islet 1 (Fig. 3A). In *reeler* mice, ectopic groups of cells were labeled by these three markers (Fig. 3D,E, stippled circles; Phox-2B and NF-L, data not shown), which suggests preservation of branchial or visceral motoneuron identity.

DiI injection into the VII nerve at E11.5 and E12.5 labels axons and cell bodies of the FVM and VII facial nucleus. We will focus mainly on the FVM, as VII motoneuron migration appears similar in wild type and *reeler* mutants until E13.5. Our results indicate that, in *reeler* hindbrain (Fig. 4B,D), as in control embryos (Fig. 4A,C), the FVM neurons are born normally in r5 and migrate laterally after exiting the ventricular zone. At E12.5, transverse sections of control embryos indicated that DiI-labeled cells have migrated ventrally toward the pial surface (Fig. 4E). However, in *reeler* mutants, these cells remained in a lateral and intermediate position (with respect to the dorsoventral axis) (Fig. 4F, stippled area). Although cell bodies are ectopically located in *reeler* mutants, their axons extend normally and exit the hindbrain at the correct position, concomitantly with the axons of facial motoneurons (Fig. 4B,D). Altogether, these results demonstrate that the FVM neurons remain in an intermediate position, which probably corresponds to the intermediate group of cells labeled by islet1 and c-Ret (Fig. 3).

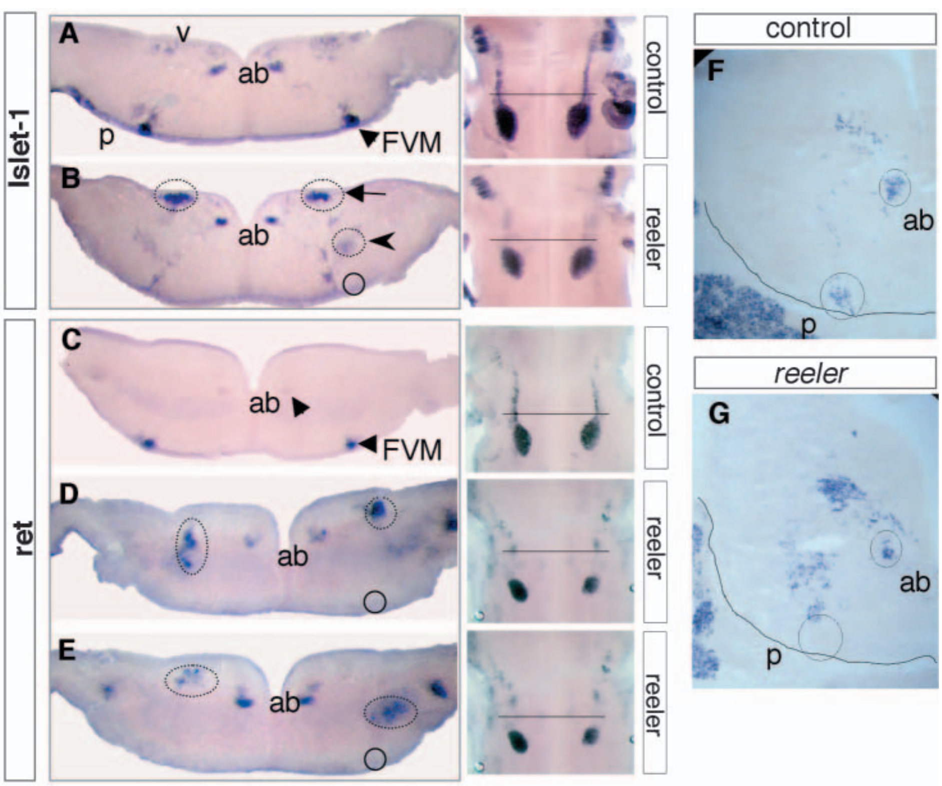

Fig. 3. Ectopically positioned motoneurons at r5 level in *reeler* mutants. (A,B) Vibratome sections after whole-mount in situ hybridization with an islet 1 riboprobe at E13.5. Level of the vibratome section is indicated on the whole-mount-stained hindbrain to the right. (A) At r5 level, a compact group of cells is clearly detectable at the pial surface in control embryos (arrow, FVM). (B) In *reeler* mutants, a strong islet 1 labeling is observed at the ventricular side of the hindbrain (arrow) and a more diffuse group of islet 1-positive cells is localized in a more medial position (arrowhead). Abducens nucleus (ab) is labeled near the ventricular zone. Note the absence of islet 1-positive cells at the pial surface in *reeler* mutants (circle). (C-E) Vibratome sections after whole-mount in situ hybridization with a *Ret* riboprobe on control and *reeler* embryos at E12.5 indicates that ectopic r5 MNs retained FVM markers. (C) Ret is expressed at the end of FVM migration in controls (arrowhead) and in the abducens nuclei near the floor plate (arrow). (D,E) In *reeler* hindbrains, ectopically positioned Ret-positive cells are observed at levels r4/r5 near the ventricular zone and at intermediate levels (dashed circles), but are absent at the pial surface (circle). v, ventricular surface; p, pial surface. (F,G) Cryostat sections at r5 level of E13.5 embryos labeled with an islet 1 riboprobe. (G) Islet 1 labeling is comparable to results obtained from vibratome sections in *reeler* mutants: a lack of pial surface labeling (stipple circle), and ectopic groups of cells within the neural tube and close to the ventricular side.

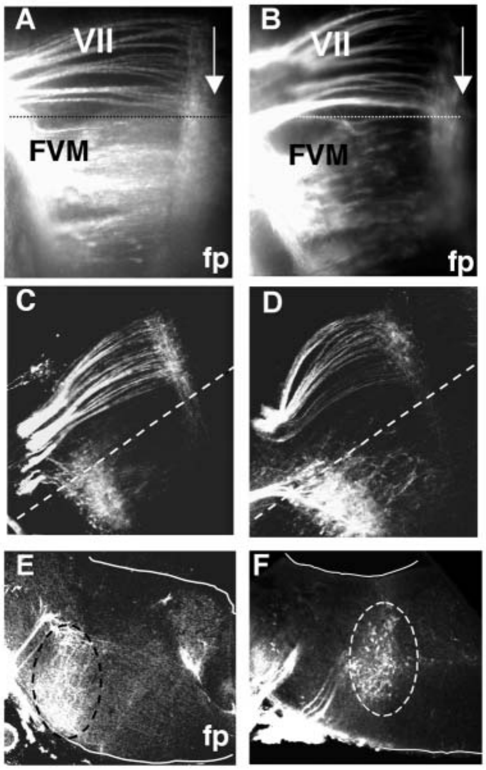

Fig. 4. Aberrant migration of facial visceral motoneurons in *reeler* mutants. Facial branchial (VII) and visceral motoneurons (FVM) were retrogradely labeled with DiI by injection from the VIIth nerve root at E11.5 and E12.5. Embryos were flat mounted at E11.5 (A,B) and E12.5 (C,D). (A,B) At E11.5, characteristic axon fasciculation and caudal migration of facial motoneurons were observed at the r4 level. White arrow indicates caudally migrating facial motoneurons. In r5, FVMs were migrating from the ventricular zone to a lateral position both in control embryos and in *reeler* mutants. (C,D) At E12.5, FVMs are organized in a compact group caudal to the axon exit point in control and *reeler* embryos. (E,F) Transverse vibratome sections of embryos in C and D indicated that FVMs have migrated ventrally toward the pial surface in controls (dashed circle in E). In *reeler* mutants, FVM neurons remained in a more mediolateral position (circled in F). C-F are confocal images. fp, floor plate.

Impaired ventral migration of olivocochlear precursors with normal vestibular efferent location in *reeler* mice

We then investigated the position of OC precursors in *reeler* mutants. The vestibular (VEN) and OC efferent nuclei are born in r4 at E10.5 as inner ear efferents, and perform a lateral migration in r4. By E12.5, this population segregates into two groups, one of which, the VEN, migrates to a dorsal position while the other, the OC efferents, migrates ventrally (Fritzsch, 1999) (see Fig. 10). Both migrations are complete by E14.5.

Using markers of VEN and OC nuclei, Gata3 and Tbx20, we compared the position of the two nuclei in control and *reeler* mice (Karis et al., 2001; Tiveron et al., 2003; Kraus et al., 2001). In control hindbrain, Gata3 labeled the reticular formation in a characteristic ‘butterfly shape’, as well as the OC neurons, which form a dense column of Gata3-expressing cells at the pial surface (Fig. 5A, arrows in 5C,E). In *reeler* embryos, reticular formation labeling was identical to control embryos but the OC precursor column was barely visible at the pial surface except for a small group of Gata3-positive cells observed at the edge of the reticular formation (Fig. 5B,F, unfilled arrowhead). In vibratome sections of the same embryos, at r4 and r5 levels, ectopic groups of Gata3-positive cells were observed (Fig. 5D,F, black arrowheads). Similar results were obtained on cryostat sections (data not shown).

Further confirmation of an OC migration defect was obtained using the marker Tbx20. After retrograde labeling from the VIIIth right ganglion and photoconversion, Tbx20 ISH was performed in control and *reeler* vibratome sections at E12.5. Photoconversion brown precipitate was observed at the VII/VIIIth nerve and provides a landmark to superimpose the ISH and fluorescent pictures. In control sections, *Tbx20* was expressed in VII motoneurons at the ventricular zone and by OC neurons at the pial surface (Fig. 5G,H, circle). On sections from *reeler* mice, an ectopically positioned compact group of cells was observed in a dorsal position (Fig. 5I,K, circle). This group was never observed in wild-type embryos. Comparison with the DiI-labeled cells indicates that the ectopic Tbx20-positive cells are scattered along the migratory pathway, at a position where the cells should switch migration to a ventral direction (Fig. 5I-L). These results suggest that OC neurons remain in a dorsal position instead of migrating to the pial surface. The failure of OC ventral migration might imply an inability of the VEN and OC to segregate at the end of their lateral migration. As seen with the Gata3 labeling, faint Tbx20 labeling was observed toward the pial surface (Fig. 5I, unfilled arrowhead).

In order to assess VEN and OC positions in *reeler* mutants, we performed DiI-retrograde labeling from the cochlear and VIIIth ganglion (injected both sides, Fig. 6A,B) in wild type and *reeler* mutants at E12.5. In Fig. 6A, transverse sections of control hindbrain showed that VEN was visible in a dorsal position near the ventricular zone, with the OC group in a ventral location. In *reeler* hindbrain, VEN was positioned normally; however, OC cells were found in an ectopic position, close to the VEN (Fig. 6B).

Previous studies described two OC groups: a larger ipsilateral-projecting cluster, which represents the lateral OC (LOC), and a smaller population of contralateral-projecting OC, which represents the medial OC (MOC) (Fritzsch, 1999; Simmons, 2002). Unilateral labeling (Fig. 6C,D) was performed to distinguish LOC from MOC populations in *reeler* mutants (n=4). We observed contralateral and ipsilateral cells in a dorsal position. According to Fritzsch (Fritzsch, 1999), contralateral-labeled cells should represent vestibular and MOC neurons, suggesting ectopic dorsal location of the MOC in *reeler* mice. However, labeling of a few ipsilateral cells in a ventral position (Fig. 6D, arrow) suggests that the LOC population might be only partially affected.

Our data indicate that the OC precursor cells are specifically affected in *reeler* mice. They share a common migratory step with the FVM neurons: a final ventral migration parallel to radial glia fibers toward the pial surface. Both populations, OC and FVM neurons, failed to perform their final radial migration in the absence of reelin signaling.

Reelin and Dab1 are expressed early in hindbrain development

Reelin and Dab1 are expressed in the early steps of hindbrain development (Carroll et al., 2001). As we observed specific

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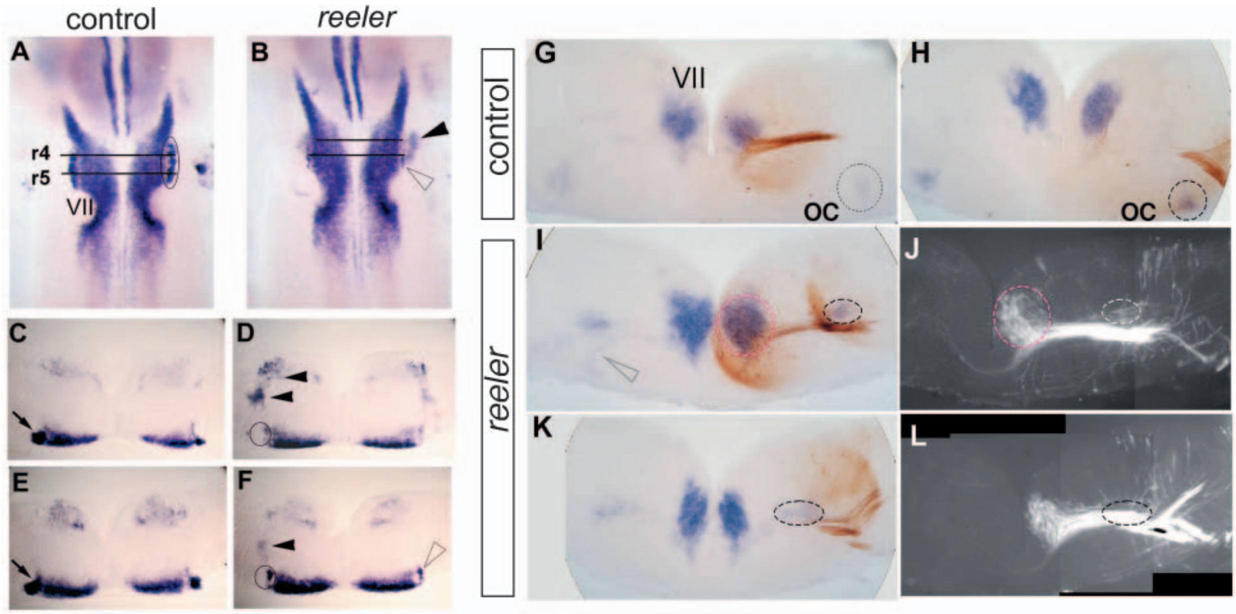

Fig. 5. Positioning of olivocochlear (OC) precursors is affected in *reeler* mice. (A) In control embryos at E12.5, Gata3 is expressed in the reticular formation at levels r4-6 and in a dense column of cells positioned rostrally to the facial nucleus and close to the pial surface (circled). (B) In *reeler* mutants at E12.5, the Gata3 expression pattern was similar to controls except for the dense column of cells (black arrowhead) which was localized on the ventricular side, as visualized in vibratome sections of the same embryos (C-F). Results corresponding to two different levels (r4, C,D; and r5, E,F) are shown. (C,E) A compact group of cells is observed in control embryos at the edge of the reticular formation (arrow). (D,F) In *reeler* embryos this compact group was absent (circles), but cells were observed in a dorsal position (arrowheads). At the r5 level, Gata3 labeling is observed in a small group of cells at the pial surface (unfilled arrowhead, F; compare with B). (G-L) Retrograde labeling from the VIIIth ganglion (right side) and photoconversion of the DiI signal (brown precipitate) were performed and subsequently followed by Tbx20 ISH (blue precipitate). Photoconversion provided a strong labeling at the VII/VIIIth nerve bundle on two consecutive sections from control embryos (G,H) and reeler mutants (I,K). Using this landmark, we were able to superimpose the ISH (I,K) and fluorescent pictures (J,L). (G,H) At E12.5, Tbx20 is expressed by facial branchial motoneurons (VII), vestibular (VEN) and OC efferents. OC neurons are localized in a ventral position as a compact group (circled). (I,K) In *reeler* mutants, Tbx20 is strongly expressed in facial motoneurons (red circle) and in a compact group of cells in a dorsomedial position (black circle). No labeling was observed at the ventral side, except for few scattered cells (I, unfilled arrowhead). Comparison with DiI labeling (compare I with J and K with L) indicated that ectopic Tbx20-positive cells accumulate at the position where they should migrate ventrally (dashed circles).

migration defects in r4 and r5, we examined in detail reelin and Dab1 expression patterns in the r4-r6 area (Fig. 7). Using whole-mount ISH, we observed an increase in reelin expression from E11.5 (Fig. 7A) to E12.5 (Fig. 7B), with a strong expression localized in r6, close to the pial surface. We performed reelin and *Dab1* ISH on serial vibratome sections. We observed areas with strong reelin expression at r4, r5 and r6 levels, close to the pial surface (Fig. 7D,G,J). Dab1 was strongly expressed in a region that includes the OC cells in r4 (Fig. 7E), the FVM in r5 (Fig. 7H) and the migrating branchial motoneurons in r6 (Fig. 7K). In order to correlate reelin expression and motoneuron position, combined reelin ISH followed by islet 1 immunohistochemistry was performed. The settling position of islet-positive motoneurons showed strong reelin expression; whereas the tangentially migrating motoneurons are found in a reelin-negative area (Fig. 7C,F,I).

Hindbrain radial glia development appears normal in *reeler* mice

As the final dorsoventral migration of FVM and OC neurons is a radial migration, and as reelin has been implicated in radial glia process development in neocortex and dentate gyrus, the integrity of radial glial cells in the hindbrain was explored using RC2 as a marker of radial glia cells. Double immunolabeling was performed with islet 1 and RC2 at r4 level from E12.5 control and *reeler* embryos. No gross abnormalities were noticed, neither in the length of processes nor in end-feet attachment (Fig. 8A,B).

Trajectories of islet 1-positive cell body translocation are clearly distinct from radial glial alignment (Fig. 8E,F, arrows), strongly suggesting two successive migrations for the affected neurons: a primary tangential or lateral migration, perpendicular to radial glia cells, and a secondary radial or ventral migration, parallel to radial glia processes.

Hindbrain *reeler*-like phenotype displayed by *scrambler* mutants but not by Reln receptor mutants

Genetic and biochemical evidence has placed reelin, the ApoER2/VLDLR receptors and Dab1 in a common signaling pathway that involves the binding of reelin to ApoER2/VLDLR receptors and then the recruitment and phosphorylation of the intracellular adaptor molecule Dab1 (Herz and Bock, 2002). Because in the brain and spinal cord, mutants for reelin receptors and Dab1 display comparable phenotypes to *reeler* mutants, we investigated whether the components of this pathway were involved in the phenotype observed in the hindbrain.

We analyzed hindbrain motoneuron migration in *scrambler*

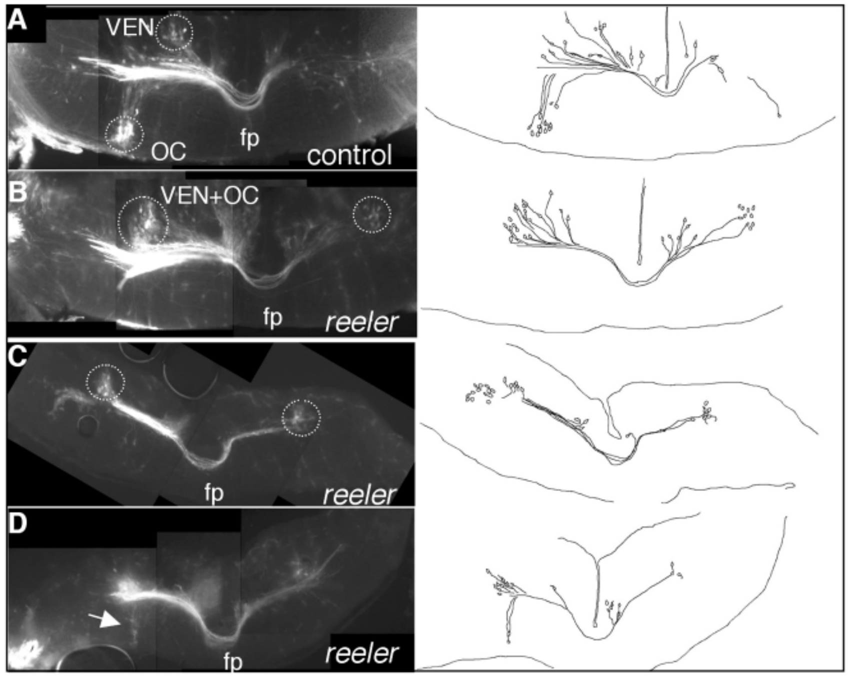

Fig. 6. Normal vestibular efferent location but abnormal medial and lateral olivocochlear efferent neuron localization in the *reeler* mutant. (A,B) Retrograde labeling of the OC and VEN from the VIIIth ganglion (bilateral injections). Drawings of labeled cells with clear labeled projections and which are not VII precursor cells, were done to clarify injection interpretation. (A) The OCs were identified in a ventral position in control mice. (B) These cells were located ectopically, in close association with VEN, in *reeler* mutants (circled). (C,D) Retrograde labeling of the OC and VEN from the otocyst (left side) in reeler mutants, to label LOC and MOC cells. Dorsally located cells were labeled on contralateral and ipsilateral sides, which indicated that MOC are affected in *reeler* mutants (C). A few ipsilateral cells close to the pial surface were labeled on the ipsilateral side only (arrow, D), which should reflect LOC cells migrating to the pial surface. LOC is the major component of the OC precursors, this observation might reflect subpopulation migratory properties, independent of reelin. fp, floor plate; LOC, lateral olivocochlear efferents; MOC, medial olivocochlear efferents; OC, olivocochlear efferent; VEN, vestibular efferent nucleus.

mutants by islet 1 ISH on E15.5 embryos. A comparable phenotype to that of *reeler* was observed in *scrambler* mutants: namely an absence of islet 1 labeling at the position of OC/FVM at the pial surface and a disorganization of the VII nucleus cells (Fig. 9C). Ectopic groups of cells were observed deeper within the neural tube in vibratome sections (data not shown).

We then studied the potential involvement of the reelin receptors, VLDVR and ApoER2, in hindbrain motoneuron migration by carrying out islet 1 ISH on VLDLR/ApoER2 double-mutant hindbrains at E12.5, E14.5 (data not shown) and E15.5 (Fig. 9D). The overall pattern was similar to wild-type mice. Islet 1 staining was observed, albeit with weaker intensity, in the expected position of the OC/FVM nuclei at the pial surface (Fig. 9D), comparable to that of control embryos. As the lack of *reeler*-like phenotype was an unexpected result, we examined receptor expression pattern at E12.5 in vibratome sections. We observed that the ApoER2 receptor was strongly expressed throughout the neural tube (Fig. 9F), whereas VLDLR receptor expression was weak (Fig. 9G). Neither of the two expression profiles suggests a specific expression of the receptors in the nuclei affected in *reeler* mutants.

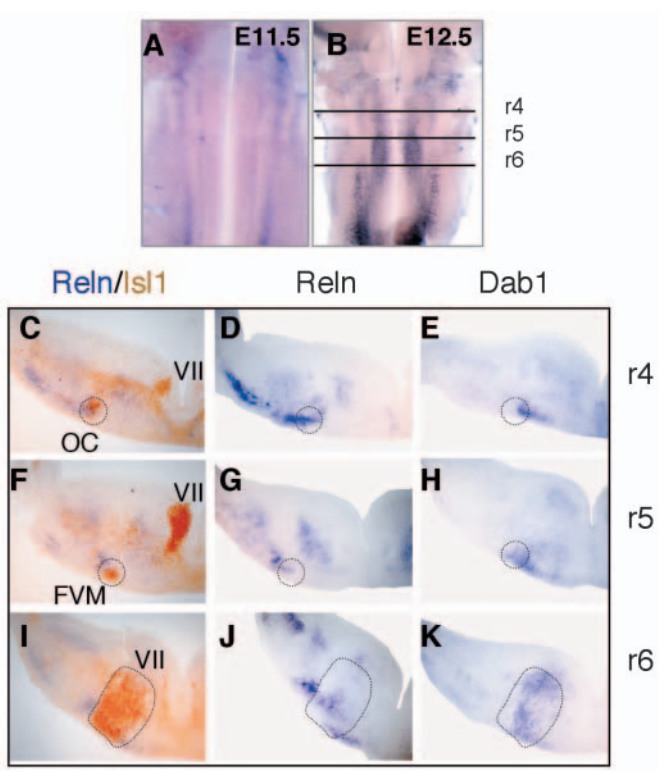

Fig. 7. Relationship between reelin pathway elements and hindbrain neuron ventral migration. Whole-mount ISH for reelin expression revealed an increase in reelin expression between E11.5 and E12.5 (compare A with B). (C-K) Combined reelin ISH followed by islet 1 immunohistochemistry (C,F,I) were performed concomitantly with reelin (D,G,J) and Dab1 (E,F,K) ISH in adjacent vibratome sections at E12.5. (C,D) At r4 level, islet 1-positive OC cells are observed at the pial surface, in close association with a highly reelin-positive area. The circle represents an equivalent position on adjacent sections. (F) At r5 level, islet 1-positive VII motoneurons undergoing caudal migration can be seen near the midline. (G) Reelin-expressing cells are found in several areas with a strong expression near the pial surface. Islet 1-positive FVM cells are observed in a position close to this reelin-positive region (F). (I) At r6 level, VII motoneurons are in the final migratory step toward the pial surface. This shows that the final position of the neurons is a reelin-positive area (J), whereas longitudinal migrating VII motoneurons are reelin negative (F,G). Dab1 is strongly expressed in a region that includes the OC cells in r4 (E), the FVM in r5 (H), and the migrating branchial motoneurons in r6 (K).

Discussion

In the present study, we analyzed the role of reelin signaling in the positioning of identified hindbrain nuclei. Hindbrain

Development

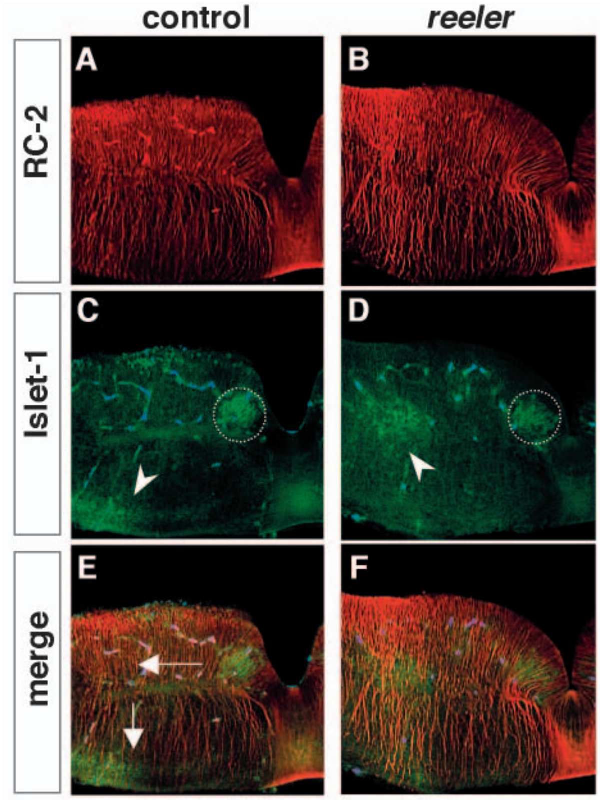

Fig. 8. Normal radial glia development in *reeler* mutant. RC2 (red) and islet 1 (green) labeling in r4 vibratome sections at E12.5. RC2 staining was comparable between control (A) and *reeler* (B) embryos. Islet 1 labeling indicated the OC ventral position in controls (arrowhead, C) and the ectopic location in *reeler* mutants (arrowhead, D), together with the facial ventricular location (dashed cicles, C,D). Note the distinct trajectories of the radial glia fibers and the lateral position of the ectopic OC nucleus (arrows, E,F). Autofluorescence of blood vessels was represented in blue (C,D) or pink (E,F).

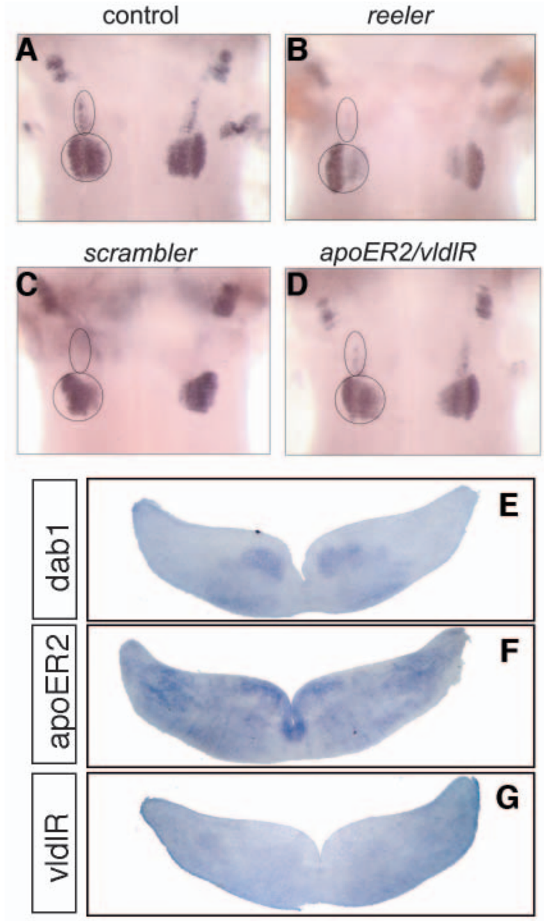

Fig. 9. Hindbrain *reeler*-like phenotype for *scrambler* mutant but no hindbrain phenotype for Reln receptors mutants. (A) At E15.5, islet 1 labels trigeminal motor, OC/FVM and facial motoneurons in control mice. (B,C) Islet 1-labeled OC/FVM cells are absent at the pial surface in *reeler* (B) and *scrambler* (C) mice. Abnormal organization of the VII nucleus is seen in *reeler* and *scrambler* mutants (lower circle). In double mutants for Reln receptors, ApoER2 and VLDLR, the Isl1 expression pattern is similar to that in control embryos, in particular, the OC/FVM group of cells can be identified at the pial surface (upper circle, D). (E-G) Expression patterns of Dab1, ApoER2 and VLDLR were examined at E12.5 in consecutive vibratome sections through r5 using ISH. Dab1 expression is observed in facial precursors close to the ventricular region (E). ApoER2 was strongly expressed within the neural tube (F); however, VLDLR was only faintly expressed (G). No specific localization in relation to the OC/FVM and facial precursor positions was observed.

MNPs undergo characteristic migrations. The branchial and visceral motor neurons arise from specific rhombomeres where they receive precise signals necessary for their specification (Gavalas et al., 2003; Goddard et al., 1996; Studer et al., 1996). They undergo multiple migrations, which end with the settling of the neurons at the pial surface of their rhombomere of birth (Fig. 1A, Fig. 9). Previous data on the *reeler* hindbrain phenotype mostly described a partial disorganization of hindbrain nuclei at late developmental stages or in adult animals, except for a partial abnormal location of the facial nucleus, which was first described by Goffinet (Goffinet, 1984), as early as E13. By studying early migration events in mouse hindbrain development, we sought to elucidate whether and at what point reelin signaling intervenes in these migration processes. We demonstrate that an additional motoneuron population is affected in the absence of reelin signaling; we show defects in migration of the FVM, in addition to the facial nucleus and OC efferents. The absence of reelin leads to a common migration defect, a failure of ventral migration parallel to radial glial processes.

Despite their ectopic positioning, the affected nuclei expressed the appropriate molecular markers. Thus, the migration defect is not due to re-specification of these cell types. Our results show for the first time that the migration of several types of hindbrain neurons is separated into two major distinct steps: a dorsolateral reelin-independent migration and a ventral reelin-dependent migration.

Normal cell specification but perturbed organization of the facial nucleus

It has previously been shown that the facial nucleus, even though in the correct rostro-caudal position, is abnormally shaped in *reeler* neonates (Goffinet, 1984). We observed reelin expression in the area of settlement of the facial nucleus, and Dab1 expression by most of the facial motoneurons. Our results show that in *reeler* and *scrambler* mice, the facial nucleus performs a normal tangential migration and that the last phase of migration is affected. This defect is apparent for the medial lobe of the nucleus at E14.5; however, motoneuron identities are conserved as deduced from Lhx4 and ER81 expression analysis. These results fit with the observations of Terashima et al., who showed that the muscle targets of this nucleus were correctly innervated in *reeler* mice (Terashima et al., 1993). Dab1 mutants display a hindbrain *reeler*-like phenotype, and the compound Dab1/p35 mutants exhibit a complete failure of facial neuronal caudal migration (Ohshima et al., 2002). p35 is the activator of Cdk5, which is involved in cortical neuron migration (Ko et al., 2001). These results, and the study by Beffert et al. on cortical development, suggest that Cdk5 and reelin act through parallel pathways that might share common effectors in the regulation of facial nucleus migration (Beffert et al., 2004; Ohshima et al., 2002).

Several factors are known to play a role in regulating facial motoneuron migration. Hoxb1 determines the primary specification of the inner ear efferents, facial motoneurons and their caudal migration (Goddard et al., 1996; Studer et al., 1996). The influence of different additional signals is necessary for the facial complex migration. Nkx6.1 is required for facial motoneuron migration into r5 and r6. Premature turning and migration arrest in Nkx6.1 mutants is associated with ectopic expression of cell surface receptors such as Ret and Unc5h3 (Muller et al., 2003). Double Nkx6.1/Nkx6.2 mutants show a complete lack of migration (Pattyn et al., 2003). How reelin signaling interacts with the other signals and how only a subset of facial MNP respond to reelin signaling remain to be clarified.

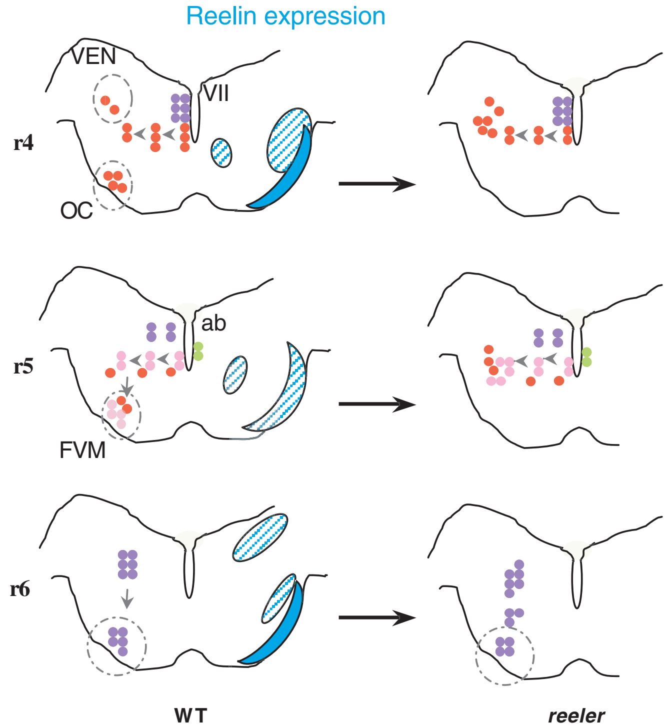

Fig. 10. Scheme showing migration defects in the *reeler* hindbrain. Neuronal population migration is indicated at r4, r5 and r6 levels on the left side of transverse sections of E12.5 control hindbrain. The affected populations are colour coded: VEN/OC, red; FVM, pink; facial branchial motoneurons, light purple; abducens, green. On the right side of the section, reelin expression is indicated. High expression is shown in blue, whereas light expression is represented with blue stripes. Migration defects observed in *reeler* mutants are illustrated on the right part of the figure. Ventral migration of the OC and FVM neurons is arrested in an intermediate position in *reeler* mice.

Reelin signaling is necessary for final ventral migration of FVM and OC neurons

Not much is known about the molecular mechanisms involved in the migration of visceral motoneurons and cochlear efferent neurons. Previous descriptions of FVM and OC migration during hindbrain development have reported the presence of cochlear efferents in a ventral position at E12.5 in r4 and r5, where they settled in a location medial to FVM. (Auclair et al., 1996; Bruce et al., 1997; Fritzsch and Nichols, 1993). Our results suggest that two groups of cells are intermingled at r5 level in wild-type embryos: namely OC and FVM precursor cells (Fig. 10).

Inner ear efferent cells, which have an r4 origin, split into two groups (VEN and OC) with distinct migratory behaviors. A recent study of their development and migration indicated that the correct migration of VEN neurons into a lateral position depends on the maintenance of Mash1 expression, whereas OC migration is Mash1 independent (Tiveron et al., 2003). In addition, EphB2 has been shown to be essential for VEN contralateral axonal projections and axon guidance, and is necessary for normal vestibular function (Cowan et al., 2000). In *reeler* mice, we observed a defect in the ventral migration of the OC neurons, which remain in a dorsolateral position near the VEN population, which is itself unaffected. As is the case for FVM neurons, the OC population appears to stop at the point where they should separate from the VEN and start their radial migration. OC neurons are characterized as lateral and medial OC (LOC and MOC) according to their migration position near the pial surface and their projections. VEN and MOC have contralateral projections toward the periphery; VEN remain in a dorsal position, whereas MOC settle in a ventral position. We were not able to demonstrate whether OC subpopulations were affected equally because a few cells were still able to migrate ventrally in *reeler* mutants. However, as we only observed these cells on the ipsilateral side after retrograde labeling, the LOC might be less affected. Detailed analyses at different stages of development are needed to clarify this question.

Our data demonstrate that a general migratory step, the radial migration toward the pial surface, shared by most hindbrain branchial and visceral motoneuron-derived efferents, as well as by OC efferents, is under the control of the reelin signaling pathway, and indicate a possible common ontogenetic origin of these three cell groups.

Reelin signaling in the hindbrain: comparison with other reelin-dependent migrations

The absence of reelin seems to have opposing effects in different situations. In the spinal cord, reelin absence allows preganglionic neurons to migrate ectopically along radial glia toward the central canal; in the olfactory bulb, absence of reelin causes accumulation of tangentially migrating precursors where they should disperse into the bulb; in the *reeler* cortex, neurons fail to detach from their glial guides (Tissir and Goffinet, 2003). In spite of apparent contradictions, a common link is a change in migratory behaviour, either an arrest or a direction switch. Our results for the hindbrain show that reelin intervenes where neurons change migration direction. In conclusion, our data support the hypothesis that reelin acts as a signal that renders neurons competent to change migration behaviour.

The lack of a *reeler*-like phenotype in the ApoER2/VLDLR mutants is intriguing. In the neocortex and dentate gyrus, radial glial cells express ApoER2/VLDLR receptors, and reelin is involved in radial glial process elongation and maturation (Graus-Porta et al., 2001; Hartfuss et al., 2003). ApoER2/VLDLR double mutants display a *reeler*-like phenotype in the neocortex (Trommsdorff et al., 1999). We did not observe any major defect in radial glial processes; although we cannot exclude that radial processes are affected in the *reeler* hindbrain (e.g. by changes in brain lipid-binding protein content), it seems unlikely that radial glial cells are the direct target of reelin signaling in this case.

Yip et al. described recently the same phenotype as *reeler* in the spinal cord of *scrambler* and ApoER2/VLDLR double mutants. However, although ApoER2 and VLDLR expression is observed in preganglionic motoneurons (Yip et al., 2004), we could not correlate expression of these receptors with any of the affected nuclei in the hindbrain. This suggests the involvement of another possible receptor, such as integrins or CNRs. Analysis of CNR expression (Carroll et al., 2001) in the hindbrain might fit with a potential receptor role for reelin, although their capacity for Reln binding is still controversial (Jossin et al., 2004). β1 integrin is another potential candidate (Dulabon et al., 2000) and further investigation in this direction is necessary.

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