Loss of projections, functional compensation and residual deficits in the mammalian vestibulospinal system of Hoxb1-deficient mice,

Hoxb1 and mammalian vestibulospinal system

Maria Di Bonito¹²³, Jean-Luc Boulland⁴⁵*, Wojciech Krezel⁶⁺, Eya Setti¹²³, Michèle Studer¹²³**, and Joel C. Glover⁴⁵**

¹University of Nice Sophia Antipolis, iBV, UMR 7277, 06108 Nice, France
²Inserm, iBV, U1091, 06108 Nice, France
³CNRS, iBV, UMR 7277, 06108 Nice, France
⁴Division of Physiology, Department of Molecular Medicine, University of Oslo, PB 1103 Blindern, 0317 Oslo, Norway
⁵Norwegian Center for Stem Cell Research, Oslo University Hospital
⁶IGBMC, CNRS UMR 7104, Inserm U 964, 1 rue Laurent Fries, BP 10142, 67404 Illkirch CEDEX, Strasbourg

DOI: 10.1523/ENEURO.0096-15.2015
Received: 21 August 2015
Revised: 2 November 2015
Accepted: 12 November 2015
Published: 24 November 2015

Funding: Norwegian Research Council; SouthEast Norway Regional Health Authority; Agence National Recherche [RO9125AA]. Telethon Foundation [TGM06AO3].

Conflict of Interest: Authors report no conflict of interest.

M.D.B., J.-L.B., W.K., M.S., and J.C.G. designed research; M.D.B., J.-L.B., W.K., E.S., M.S., and J.C.G. performed research; M.D.B., J.-L.B., W.K., M.S., and J.C.G. analyzed data; M.D.B., M.S., and J.C.G. wrote the paper.

Norwegian Research Council. SouthEast Norway Regional Health Authority. Agence National Recherche [RO9125AA]. Telethon Foundation [TGM06AO3].

corresponding authors

*J.L.B. and W.K. contributed equally to this work.

Correspondence should be addressed to Authors for correspondence: Joel C. Glover, Division of Physiology; Department of Molecular Medicine, University of Oslo; PB 1103 Blindern, 0317 Oslo, Norway. +47-98652457. joel.glover@medisin.uio.no; Michèle Studer, iBV - Institute of Biology Valrose; Inserm U1091, CNRS UMR7277, UNS, University of Nice Sophia Antipolis (UNS); Parc Valrose, 06108 Nice Cedex 2 – France. +33-492076419. michele.studer@unice.fr

Cite as: eNeuro 2015; 10.1523/ENEURO.0096-15.2015

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Accepted manuscripts are peer-reviewed but have not been through the copyediting, formatting, or proofreading process.

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Maria Di Bonito1,2,3, Jean-Luc Boulland4,5*, Wojciech Krezel4*, Eya Setti1,2,3, Michèle Studer1,2,3**, Joel C. Glover4,5**

(1) Univ. Nice Sophia Antipolis, iBV, UMR 7277, 06108 Nice, France; (2) Inserm, iBV, U1091, 06108 Nice, France; (3) CNRS, iBV, UMR 7277, 06108 Nice, France; (4) Division of Physiology, Department of Molecular Medicine, University of Oslo, PB 1103 Blindern, 0317 Oslo, Norway; (5) Norwegian Center for Stem Cell Research, Oslo University Hospital; (6) IGBMC, CNRS UMR 7104, Inserm U 964, 1 rue Laurent Fries, BP 10142, 67404 Illkirch CEDEX, Strasbourg

*contributed equally
**co-contributing authors

Authors for correspondence:

Joel C. Glover
Division of Physiology; Department of Molecular Medicine
University of Oslo; PB 1103 Blindern
0317 Oslo, Norway
joel.glover@medisin.uio.no
+47-98652457

Michèle Studer
iBV - Institute of Biology Valrose; Inserm U1091, CNRS UMR7277, UNS
University of Nice Sophia Antipolis (UNS); Parc Valrose
06108 Nice Cedex 2 - France
michele.studer@unice.fr
+33-492076419
**ABSTRACT**

The genetic mechanisms underlying the developmental and functional specification of brainstem projection neurons are poorly understood. Here, we use transgenic mouse tools to investigate the role of the gene *Hoxb1* in the developmental patterning of vestibular projection neurons, with particular focus on the lateral vestibulospinal tract (LVST). The LVST is the principal pathway that conveys vestibular information to limb-related spinal motor circuits, and arose early during vertebrate evolution. We show that the segmental hindbrain expression domain uniquely defined by the rhombomere 4 (r4) *Hoxb1* enhancer is the origin of essentially all LVST neurons, but also gives rise to subpopulations of contralateral medial vestibulospinal tract (cMVST) neurons, vestibulo-ocular neurons, and reticulospinal (RS) neurons. In newborn mice homozygous for a *Hoxb1* null mutation, the r4-derived LVST and cMVST subpopulations fail to form and the r4-derived RS neurons are depleted. Several general motor skills appear unimpaired, but hindlimb vestibulospinal reflexes, which are mediated by the LVST, are greatly reduced. This functional deficit recovers however during the second postnatal week, indicating a substantial compensation for the missing LVST. Despite the compensatory plasticity in balance, adult *Hoxb1* null mice exhibit other behavioral deficits that manifest particularly in proprioception and interlimb coordination during locomotor tasks. Our results provide a comprehensive account of the developmental role of *Hoxb1* in patterning the vestibular system and evidence for a remarkable developmental plasticity in the descending control of reflex limb movements. They also suggest an involvement of the lateral vestibulospinal tract in proprioception and in ensuring limb alternation generated by locomotor circuitry.
SIGNIFICANCE STATEMENT

The mammalian motor system is constructed from neuron groups that acquire specific functional identities in part through the action of patterning genes such as those in the *Hox* gene family. Here, we assess the role of the *Hoxb1* gene in the development of the murine vestibular system. Hoxb1 function is required to generate specific groups of vestibular neurons, in particular neurons that give rise to the lateral vestibulospinal tract (LVST). The lack of the LVST resulting from the absence of Hoxb1 function leads to an initial deficit in vestibulospinal reflexes, but these recover over the course of several days, indicating a pronounced functional compensation. Subtle behavioral deficits are maintained into adulthood, suggesting additional roles for the LVST in motor control, most notably in proprioception and interlimb coordination during locomotion.
INTRODUCTION

The vestibular system appeared early during vertebrate brain evolution, and connections from the vestibular nuclei to motoneurons in the brain stem and spinal cord are highly conserved within the vertebrate radiation (Diaz and Glover, 2002; Beisel et al., 2005; Duncan et al., 2012; Straka et al., 2014). Vestibular projections also appear early during brain development and are patterned by highly stereotyped blueprints of gene expression (Glover and Petursdottir, 1991; Diaz et al., 1998; Glover, 2000, 2003; Pasqualetti et al., 2007; Straka and Baker, 2013). Despite its conserved and stereotyped connectivity, the vestibular system exhibits marked adaptive plasticity in the face of sensorimotor mismatch, activity imbalances or outright damage (du Lac et al., 1995; Minor, 1998; Quinn, 1998; Raymond and Lisberger, 2000; Darlington and Smith, 2000; Yates et al., 2003; Glidden et al., 2005; Gittis and du Lac, 2006; Ronca et al., 2008; Cullen et al., 2009; Horak, 2009; Dutia, 2010; McElvain et al., 2010; Jamon, 2014; Shin et al., 2014). The contribution of the vestibular system to balance is also affected negatively by aging, resulting in vertigo, discomfort and falls in the elderly, associated with a high degree of morbidity and mortality (Ishiyama, 2009; Agrawal et al., 2013; Iwasaki and Yamasoba, 2014).

Principal vestibular descending projections include the separate ipsilateral and contralateral medial vestibulospinal tracts (iMVST and cMVST) and the strictly ipsilateral lateral vestibulospinal tract (LVST) (Glover and Petursdottir, 1988; Diaz et al., 2003; Pasqualetti et al., 2007). Of these, only the LVST projects along the entire spinal cord, and it is pivotal in regulating the activity of trunk and limb musculature to counteract perturbations of body position (Shinoda et al., 1988; Kuze et al., 1999; Rose et al., 1999; Boyle, 2000; Backsai et al., 2002; Matesz et al., 2002; Kasumacic et al., 2010). A principal function is the activation of limb extensors and deactivation of limb flexors, asymmetrically around the body axis, to generate limb movements that maintain an upright body position when balance is lost (Wilson and Yoshida, 1969; Pompeiano, 1972).
Despite recent advances (Diaz et al., 1998; Pasqualetti et al., 2007; Kasumacic et al., 2010, 2015), we still lack a comprehensive understanding of how the LVST group arises developmentally, becomes specified to project along the LVST pathway and selectively innervates different populations of spinal neurons. Indeed, we have very little information about the specific spinal targets of the LVST and therefore about how the LVST exerts its effects.

One way to better understand the development and function of the LVST is to use molecular genetic approaches to interrogate LVST neurons about their origins, connections and physiological effects. The LVST group has been shown to derive predominantly from rhombomere (r)4 in both chicken and mouse (Diaz et al., 1998; Pasqualetti et al., 2007). Since the gene Hoxb1 is instrumental in establishing the identity of r4, Chen et al. (2012) used a Hoxb1 reporter mouse and a Hoxb1 null mouse to test the r4 origin of vestibulospinal neurons. Their results supported this origin and showed that inactivation of Hoxb1 led to a loss of vestibulospinal neurons. However, they did not assess whether this manipulation was specific to the LVST group. This is important because the cMVST group also has an r4-derived component, as may other vestibular and bulbospinal projection neuron groups (Auclair et al., 1999). Nor did Chen et al. (2012) investigate the functional effects that the loss of r4-derived vestibulospinal neurons elicited.

Here, we use a transgenic mouse that expresses Cre-recombinase under the control of the r4-specific enhancer element of Hoxb1 (b1r4-Cre mouse; Di Bonito et al., 2013a; Figure 1A) to make a comprehensive characterization of the contribution of r4 to the LVST, cMVST, iMVST and nearby vestibulo-ocular, vestibular efferent and reticulospinal neurons. We then test the dependence of these neuron groups on Hoxb1 function using a constitutive Hoxb1 null mutant mouse, and investigate the resulting behavioral effects. We provide novel evidence about the origins of these projection and efferent neurons and their dependence on Hoxb1 expression for normal development. Our results also shed light on the role of the LVST and the capacity for functional reorganization when
this important component of descending motor control is developmentally compromised.

METHODS

Animals

In the b1r4-Cre transgenic line (Di Bonito et al., 2013a), the Cre recombinase gene is expressed exclusively in rhombomere 4 under the control of the Hoxb1 r4 enhancer (Studer et al., 1994). The b1r4-Cre line was crossed with the ROSA26YY reporter line (Srinivas et al., 2001). In the double heterozygous b1r4-Cre/YFP mice, r4 and r4-derivatives are selectively labeled by YFP expression. Wild type mice generated from this crossbreeding were used as controls. The Hoxb1 null line (Di Bonito et al., 2013a) was crossed with the b1r4-Cre/YFP line to label r4 in the mutant background. A total of 61 mice were used in this study, including 11 b1r4-Cre/YFP embryos, 17 wild type postnatal mice, and 12 embryos and 21 postnatal mice from the Hoxb1 null x b1r4-Cre/YFP cross.

All mice were raised on a B6D2 genetic background and housed in groups of 2-4 animals/cage in 7am-7pm light/dark cycle with food and water freely available. Pregnancies were identified and timed by the presence of vaginal plugs. All efforts were made to minimize the number of animals used and their suffering in accordance with the European Communities Council directive 2010/63/EU for the care and use of animals. All procedures were conducted according to French ethical regulations and this project received the approval from the local ethics committee (CIEPAL NCE/2014-209).

Retrograde tracing with fluorescent dextran-amines

Pregnant dams were killed by cervical dislocation, subjected to a caesarean section, and E16.5 embryos with decidua were removed and submerged in ice-cold (4°C), oxygenated (95% O2 and 5% CO2), artificial cerebrospinal fluid (ACSF, containing in mM: NaCl 128, KCl 3, d-glucose 11 CaCl2 2.5, MgSO4 1,
NaH2PO4 1.2, HEPES 5 and NaHCO3 25). Embryos were then decapitated at low cervical levels and the brainstem was carefully dissected out. To maximize oxygenation the cerebellum was removed and the ACSF was exchanged every 10 min during the dissection.

To label vestibulospinal and reticulospinal neurons, we used the approach previously described by Glover (1995) and Kasumacic et al (2010). The spinal white matter at the level of the first cervical (C1) ventral root was cut unilaterally. The cut spanned the entire extent of the ventral and ventrolateral funiculi. Pre-made crystals of 3 kDa tetramethylrhodamine-conjugated dextranamines (RDA, Invitrogen, catalog number D-3308), alone or in combination (ratio 1:1) with biotin-conjugated dextran amine (BDA, Invitrogen, catalog number D-7135) were inserted into the cut. Four to 6 crystals inserted over a period of about 3 min ensured continuous exposure of the cut axons to high tracer concentration. Preparations were then incubated in the dark for a period of about 8 hours to allow retrograde transport of the tracers to both ipsilaterally- and contralaterally-projecting vestibulospinal and reticulospinal neurons.

To label vestibulo-ocular neurons, the same procedure was carried out after making a unilateral cut in the medial longitudinal fasciculus, approaching from the floor of the fourth ventricle, at the level of the pons/mesencephalon border, as described previously (Pasqualetti et al., 2007).

**Retrograding tracing with lipophilic dye**

Whole heads of P8 *Hoxb1 null* and control mice (n=3 for each genotype) were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The heads were dissected ventrally to expose the inner ear. Vestibular efferent neurons were retrogradely labeled by placing a crystal of the fluorescent carbocyanine dye DiI (Life Technologies, Molecular Probes, catalog number D-282) unilaterally into the inner ear, ensuring contact with the peripheral nerve branches. To allow the DiI to diffuse, preparations were incubated for 3 months in PBS containing 0.025% sodium azide; the first two months at 37°C and thereafter at RT to minimize tissue degradation. The brain
was then dissected free and vibratome-sectioned at 100 µm in the coronal plane, and the sections were mounted on slides for inspection under epifluorescence optics.

**Histology and microscopy**

YFP+ neurons and axons were visualized both by the intrinsic fluorescence of YFP and amplification using an antibody that recognizes both YFP and GFP (anti-GFP, Molecular Probes, catalog number A11122). Preparations were immersion fixed in 4% PFA in PBS, cryoprotected, frozen, and cryostat sectioned at 12µm in the sagittal plane. Sections were mounted on glass slides and incubated in the primary anti-GFP antibody at 1:500 overnight at 4°C. They were then rinsed and incubated for 1 hour at room temperature with either Alexa Fluor 488 (green)-conjugated goat anti-rabbit secondary antibody (Life Technologies, catalog number A11034) and Alexa Fluor 594 (red)-conjugated streptavidin (Invitrogen, Molecular Probes, catalog number S32356), to amplify the RDA/BDA labeling, or with a biotinylated secondary anti-rabbit antibody (Life Technologies, catalog number 32054). The slides incubated with fluorescent secondary antibodies were then rinsed and coverslimed for inspection under epifluorescence optics.

The slides incubated with biotinylated secondary antibody were rinsed and incubated with biotinylated horseradish peroxidase (HRP) pre-incubated with avidin, and rinsed and carried through the HRP reaction using DAB as substrate as previously described (Di Bonito et al., 2013a), before being coverslimed for inspection under conventional optics.

In situ hybridization using a probe for mRNA of the transcription factor GATA3 was performed as previously described (Di Bonito et al., 2013a).

Digital photographic images were obtained at 5x magnification using a Leica DM 6000B microscope equipped with Leica DFC310 FX color camera and processed in Adobe Photoshop CS5 software using the Photomerge function to obtain a panorama of each brain section. In all images obtained with this system the RDA/BDA labeling was red, YFP labeling green, and double labeling appeared as varying shades of yellow to orange. In many cases, conjugated dextrans entered
the nucleus where the YFP was weaker than in the cytoplasm, so that some
double-labeled neurons appeared to have a red nucleus with surrounding yellow
to orange cytoplasm. To assist the color blind, images were then additionally
processed to replace red pixels with magenta pixels. Double-labeling thus
remained as yellow to orange. To assess double-labeling of individual neurons,
confocal z-stacks were obtained by laser scanning confocal microscopy at 40x
magnification using a Zeiss LSM 710 confocal. Neuronal cell bodies were
examined individually in the x-, y- and z-planes by ZEN software. False colors
were generated in all images to make RDA/BDA labeling magenta, YFP labeling
green, and double labeling thus appeared as a pale whitish hue, in contrast to the
yellow- to orange hues that obtained with the Leica DFC310 FX color camera
system.

Behavioral testing

Early postnatal period

Neonates and early postnatal mice have immature motor skills, particularly
because they are not capable of bearing their own weight until the second
postnatal week. For this reason, most adult behavioral tests cannot be used on
neonates and young mice without substantial modification. We therefore
developed specialized procedures and apparatus, and used these to test gender
balanced cohorts of Hoxb1 null (n=21) and wild type control mice (n=14) from
P5-P11. All tests were performed by an investigator blinded to the genotype of
the mouse.

Open field swimming test: To assess general locomotor ability, P5 mice were
placed in a water-filled rectangular pool (210mm x 165mm) and allowed to
swim freely while being video-filmed from above at 25Hz with a Nikon Micro-
Nikkor 55mm 1:3.5 objective. To avoid exhaustion and hypothermia, the
swimming time was limited to 20s and the water temperature was maintained at
about 36°C. Tracking for each mouse was done in ImageJ (Rasband, 1997) using
the Manual Tracking plugin (Cordelières, 2006). Analyses of trajectory, time
spent in the center or the periphery of the pool, number of rotations, and
minimum/maximum/average speeds was done using an in-house program coded in LibreOffice Basic (unpublished).

Vestibulospinal reflex test: To test the vestibulospinal reflex in P5-P11 mice, we engineered a small apparatus designed to rotate a mouse around its sagittal axis. In wild type mice, this rotation produces a compensatory extension of the hindlimb on the side towards which the mouse is turned, indicating an attempt to recover balance. The apparatus was composed of three different parts. The first was a Teflon mouse holder (available in multiple sizes to fit mice of different ages). The holder retains the mouse in a dorsoventral grip leaving the limbs free. The holder was affixed to the rotating part of the device at a right angle so that the sagittal axis of the mouse was aligned with the rotation axis. The rotation was generated by a spiral torsion spring. A stopper was used to maintain the spring in tension so that the removal of the stopper was followed immediately by rotation, which was limited to 90 degrees by a second stopper. Thus, all mice were subjected to a standardized rotation axis (roll axis), speed (1500 degrees/s = 26.2 rad/s) and angle (90 degrees). The rotating part was in turn affixed to a stand of a height that aligned the rotation axis with the center of the video camera objective (Nikon, Micro-Nikkor 55mm 1:3.5). Rotation of the mice and compensatory movements of the limbs were recorded at 200 frames per second (Integrated Design Tools, Inc.). Hindlimb extensions were scored visually as a clear change from an initial flexed position (both hindlimbs had to be flexed initially for the trial to be included) to an obviously extended position.

**Adults**

All tests were performed on gender-balanced cohorts of *Hoxb1 null* (n=10) and wild type control mice (n=7). Most mice were tested at 13-16 weeks of age, except for 2 mice in each group, which were tested at 28 weeks of age. Since performance of each older mouse did not differ from the rest of the group with corresponding genotype across all tests, they were not excluded from analyses, and results from mice at the two ages were pooled. All mice were tested in the
following battery of behavioral tests in the order described below, over a period of 4 consecutive days.

**Open field test:** Locomotor activity was tested simultaneously in two open field arenas (40x40x40 cm) made of Plexiglass and with white floors. The arenas were placed in a room homogeneously illuminated at about 150 lux. Each mouse was placed in the periphery of the arena and allowed to explore freely the arena for 30 min, with the experimenter hidden from the animal’s sight. Animal behavior was recorded with a video camera and analyses were performed post-hoc using EthoVisionXT® software (Noldus, USA). Behavioral parameters were calculated automatically with the exception of unsupported rearing (rearing on hindlimbs without support against the wall of the arena) and leaning (rearing on hindlimbs with support against the wall of the arena), which had to be identified by the investigator.

**Notched beam test:** Here we used a modified version of the notched beam paradigm, which was originally conceived to test motor coordination of the hindlimbs (Duchon et al. 2011; Waanders et al. 1989). Briefly, locomotion was assessed on a 1 meter long, 17 mm wide wooden beam with a flat upper surface engraved with regular, square, 17 mm deep, 17 mm wide and 17 mm long incisions spaced every 17 mm, with a box providing a secure hiding place at one end. During the habituation phase, each mouse was first placed in the box for 30 sec. It was then removed from the box and placed twice on the beam at increasing distances from the box and was allowed to traverse the beam to reach the box and hide in it for 30 sec. During the test, the mouse was placed at the end of the beam opposite the box, and the time to reach the box, the number of paw slips during the traverse and the number of hops (defined as synchronous movements of the two hindlimbs from one square to another) during the traverse were scored. The test was repeated 3 times at 30-60 sec intervals.

**Contact righting reflex:** The test was performed as described by Mandillo et al., (2008). Briefly, mice were placed in a narrow, 3 cm wide, clear Plexiglas cylinder and allowed to come to rest. The cylinder was then held in a horizontal position
and was rotated rapidly by 180° to bring the mouse to a supine position. The time required for the mouse to return to its original prone position was then measured.

Linear swimming test: The test was carried out in a 1-meter long Plexiglas tank (6 cm wide x 30 cm high) filled with water (20 cm deep) at a temperature of 21-23°C as described by Romand et al. (2013). Briefly, for habituation, animals were placed for 30 sec on a platform at one of the extremities of the tank and subsequently they were gently lowered into the water on the opposite end of the tank. The time required to reach the platform was measured, and body position and types of movements made during swimming were video-filmed and analysed.

Statistics
Differences of means were tested using the Student's t-test or the Mann Whitney U test, or using ANOVA on repeated measures (RMANOVA) when the evolution of performance within a group was analyzed over the course of test epochs. Post-hoc statistical comparisons of behavioral performance in Hoxb1 null and control mice were carried out using the protected least significant difference (PLSD) Fischer test as indicated in the corresponding figures.
RESULTS

Developmental origins of vestibular projection neurons and reticulospinal neurons

Previous fate-mapping studies in chicken and mouse embryos have assessed the rhombomeric origins of vestibular projection neurons through fate mapping using quail-chicken chimeras and transgenic mice, respectively (Diaz et al., 1998; Pasqualetti et al., 2007; Chen et al., 2012). However, in the mouse, putative r4 origins have been assessed only indirectly using either a r3-r5 lacZ reporter mouse (Pasqualetti et al., 2007) or a Hoxb1-GFP reporter mouse in which the GFP is initially expressed from r4 into the spinal cord and only later restricted to r4 (Chen et al., 2012). Furthermore, in neither the chicken embryo nor these two mouse lines were the origins of specific neuron subpopulations residing in r4 determined unambiguously, since these studies did not categorically identify neurons that were double-labeled by retrograde tracing and reporter gene expression or neurons devoid of reporter gene expression within r4. Here, by using b1r4-Cre/YFP mice, in which YFP staining is restricted to r4 from the onset of expression (Figure 1A; Di Bonito et al., 2013a), combined with RDA/BDA retrograde labeling, we have been able to provide an accurate and comprehensive assessment of the contribution of r4 to vestibular projection neurons and reticulospinal neurons. We retrogradely labeled vestibulospinal and reticulospinal neurons (n=5 preparations) or vestibulo-ocular neurons (n=3 preparations) unilaterally to distinguish the different groups that project ipsilaterally and contralaterally.

LVST neuron group. Using the b1r4-Cre mouse line (Figure 1A-C), we could show that essentially all LVST neurons originated from r4, including those located in r3 and r5 that migrate there from r4 prior to E16.5 (Figure 1D, E). RDA/BDA-labeled, YFP+ axons could be followed from the LVST neuron group towards the spinal cord along the trajectory of the LVST (Figure 1C; arrowheads in Figure 1G). Because retrograde labeling is not 100% efficient, we cannot rule out that a few LVST neurons do not originate from r4, but the overwhelming majority clearly does.
Reticulospinal neurons. Reticulospinal neurons are located in and presumably derive from multiple rhombomeres in the mouse, including r4 (Figure 1C; Auclair et al., 1999). However, rhombomere-specific fate mapping of the reticulospinal neurons has not been performed in rodents. Here, we could determine that a subpopulation of ipsilaterally projecting reticulospinal neurons in r4 and r5 derives from r4 (Figure 1F-H). Thus, at least some reticulospinal neurons acquire their locations through inter-rhombomeric migration.

iMVST and cMVST neuron groups. None of the iMVST neurons derives from r4, consistent with their origin from r6 (Figure 2A; Diaz et al., 1998; Pasqualetti et al., 2007). The cMVST neurons that reside in r4 clearly originated from r4 (Figure 2B), whereas the cMVST neurons in r5 clearly did not (Figure 2C). Thus, the location of these two cMVST subpopulations in adjacent rhombomeres reflects primarily differential rhombomeric origins and not migration from a single rhombomere, as is the case for the LVST neurons.

Vestibulo-ocular neuron groups. Four different vestibulo-ocular (VO) neuron groups project to the trochlear and oculomotor nuclei in both the chicken and the mouse, and these derive from regions either rostral (iR-VO group, cR-VO group) or caudal (iC-VO group, cC-VO group) to r3 (in the mouse, Pasqualetti et al., 2007) or r4 (in the chicken, Diaz et al., 1998). In addition, there are scattered ipsilaterally projecting VO neurons lying in r3 (mouse) or r4 (chicken) between the iR-VO and iC-VO groups. Since it has not been determined whether these scattered neurons belong to the iR-VO or iC-VO groups, or whether they represent a separate, sparse group, we refer to them simply as “scattered ipsi VO neurons”. In the mouse, the iC-VO group is noteworthy because after first appearing (by E11.5), it dwindles markedly in number with subsequent development (through as yet undetermined mechanisms) such that by E16.5 only scattered iC-VO neurons remain in r4 and r5 (Pasqualetti et al 2007). Thus, the VO projection neurons in the E16.5 mouse present as three distinct groups, the cR-VO, cC-VO and iR-VO groups, along with a “tail” of ipsilaterally projecting VO neurons that comprises the scattered ipsi VO neurons in r3 and the residual iC-VO neurons in r4 and r5.
Here, we could show that some of the scattered ipsi VO neurons in r3 and some of the residual iC-VO neurons in r4 originated from r4 (Figure 2D). We also determined that no cR-VO (data not shown), iR-VO (Figure 2E) or cC-VO neurons (Figure 2F, G) originated from r4, although some cC-VO neurons migrate into r4 (Figure 2F). Thus, although minor interrhombomeric migration to or from r4 occurs, this contributes relatively little to the patterning of the VO projection neuron groups.

**Loss of the LVST group, the r4-derived portion of the cMVST group and some r4-derived reticulospinal neurons in Hoxb1 null mice**

Next, we used retrograde labeling in the Hoxb1 null mouse (labeling vestibulospinal and reticulospinal neurons unilaterally: n=3; labeling vestibulo-ocular neurons unilaterally: n=3) to test the dependence of r4-derived neuron populations on Hoxb1 expression. First, we demonstrated that the LVST neuron group was severely depleted if not absent in E16.5 Hoxb1 null embryos (Figure 3F-H). This is in accordance with the finding by Chen et al. (2012) that vestibulospinal neurons in the lateral vestibulospinal nucleus are lost in a different Hoxb1 null mouse strain. No retrogradely labeled YFP+ neurons were found in r3, r4 or r5 in the region that normally contains the LVST group (Figure 3A-C, F-H), and there was no sign of YFP+ axons along the trajectory of the LVST. A few retrogradely labeled YFP-negative neurons were present in the area normally occupied by the LVST (arrowheads in Figure 3F-H). These, however, numbered only a few tens (Figure 3F-H shows an example where they are particularly numerous), whereas the LVST group normally contains many hundreds and perhaps well over a thousand neurons. The identity of these non-r4-derived neurons remains unclear.

The r4-derived portion of the cMVST group was also missing, leaving only the r5-derived portion (Figure 3D,E,I,J). Thus, all r4-derived vestibulospinal neurons were either lost or transformed to another phenotype in the absence of Hoxb1, as previously shown for other r4-derived structures (Di Bonito et al., 2013a, b).
expected, the iMVST (data not shown), as well as the r5-derived portion of the cMVST group, neither of which originate from r4, were unaffected in the Hoxb1 null mice.  

In contrast to the essentially complete absence of r4-derived vestibulospinal neurons, the r4-derived population of reticulospinal neurons was depleted, but not absent, in both r4 and r5 of Hoxb1 null mice (Figure 3K-V). Similarly, we still found r4-derived scattered ipsi VO neurons in r3 and iC-VO neurons in r4 of the mutant mouse (examples indicated by arrowheads in Figure 4A-F). Thus, not all r4-derived vestibular projection neurons are lost when Hoxb1 function is eliminated. As expected, the cR-VO and cC-VO groups, which do not derive from r4, were not affected (data not shown).  

We nevertheless found that a sizeable group of r4-derived YFP+ neurons had migrated into r3 in the region of the iR-VO group in the Hoxb1 null mutant (Figure 3I,J and Figure 4J-L). None of these, however, projected to the spinal cord, indicating that they had a non-vestibulospinal phenotype (Figure 3I,J). Indeed, some of them could be retrogradely labeled from the ascending MLF, indicating a vestibulo-ocular (or potentially vestibulothalamic) phenotype (Figure 4J-L). It remains to be determined whether these neurons represent an overproduction of the scattered ipsi VO and/or iR-VO neurons or LVST neurons that have been transated to another phenotype.  

**Loss of vestibular efferent neurons in Hoxb1 null mice**  

Another population of vestibular neurons is the group of cholinergic vestibular efferent neurons that innervates hair cells and calyx afferent endings in the vestibular end organs in the inner ear (Highstein 1991). Vestibular efferent neurons, similar to cochlear efferent neurons, originate from ventral r4 and express high levels of Gata2 and Gata3 (Tiveron et al., 2003). No Gata3 expression was detected in the vestibular efferent neuron domain in E14.5 Hoxb1 null mice (n=3; Figure 5A, A'), commensurate with an absence of inner ear efferent neurons (Di Bonito et al., 2013a). To confirm the absence of vestibular
efferents at postnatal stages when connectivity is established, DiI crystals were
inserted into the inner ears of wild type (n=3) and *Hoxb1 null* mice (n=3) at P8.
No efferent neurons were retrogradely labeled at this stage in the mutant mice
(Figure 5C, C'), confirming their absence and excluding the presence of any other
ectopic source of efferent input to the inner ear. Thus, vestibular projection
neurons and vestibular efferent neurons derived from r4 are critically dependent
on the expression and function of *Hoxb1*.

**Behavioral deficits and compensation in *Hoxb1 null* mice during the early
postnatal period**

To investigate whether and how the deficits in the vestibular system observed in
*Hoxb1 null* mice affect general motor behavior and balance, we followed a group
of *Hoxb1 null* mice from early postnatal stages to adulthood.

Testing general motor capability in early postnatal life is challenging because
during the first postnatal week rodents are not strong enough to bear their own
weight with extended limbs (Muir 2000), and motor function develops gradually
thereafter (Geisler et al., 1993; Brocard et al 1999; Clarac et al., 2004; Lelard et
al., 2006). Thus, specially designed tests need to be employed. Here, we used an
open field swimming test to first assess general motor capacity, and a specially
designed body rotation device to test specifically the vestibulospinal reflex as it
manifests in the hindlimbs (Figure 6A-G).

As assessed by the open field swimming test at P5, general motor capacity was
not affected in any decisive way. *Hoxb1 null* mice (n=9) did not differ from
control mice (n=6) in average or maximum speed of swimming, the proportion
of time spent swimming in the most central part of the pool versus the
periphery, nor the number of rotations performed during the swimming
trajectory (Mann-Whitney U-test, U=16, p=0.12; Figure 6A-C, E). *Hoxb1 null* mice
did, however, swim for moderately longer overall distances than control mice
during the 20 second swimming session (Mann Whitney U-test, U=12, p=0.04;
Figure 6D). It is not clear what caused this difference, but it runs counter to any
gross motor deficit.
In contrast, the vestibulospinal reflex in the hindlimbs was strongly depressed in Hoxb1 null mice relative to control mice when tested at P5 (n=9 Hoxb1 null mice, n=10 wild type control mice, Mann Whitney U-test, U=14, p=0.0047) and P7 (n=21 Hoxb1 null mice, n=14 wild type control mice, Mann Whitney U-test, U=55, p=0.0008; Figure 6F, G). The extension of the ipsilateral leg typically elicited in control mice by a unilateral 90° rotation in the longitudinal axis was elicited only about half as often in Hoxb1 null mice at these stages. Strikingly, the rate recovered to control levels by P11, indicating a substantial compensation (n=8 Hoxb1 null mice, n=4 wild type control mice, Mann Whitney U-test, U=19, p=0.27; Figure 6G).

Hoxb1 null mice display perturbed motor behaviors as adults

To investigate whether the compensation in the vestibulospinal reflex observed by P11 was maintained in adult Hoxb1 null mice, we carried out a series of behavioral tests, sensitive to potential deficits in balance, motor coordination and proprioception, on a subgroup of the control and Hoxb1 null mice previously tested at early postnatal stages. The tests included the contact righting test, the open field test, the linear swimming test and the notched beam test which are all presented for n=10 Hoxb1 null mice and n=7 wild type control mice (Figures 7, 8).

Focusing initially on potential perturbation of the vestibular system, we noted that the righting reflex assessed by the contact righting test, known to be a sensitive measure of equilibrium, was not impaired in Hoxb1 null mice (Students t-test, p>0.4 for each trial; Figure 7A). Nor did Hoxb1 null mice display head tilting or circling behaviors frequently associated with different types of vestibular dysfunctions (not shown). Thus, the compensation in the vestibulospinal reflex seen during the second postnatal week evidently translated into a generally normalized capacity for vestibular function in the adult.
In addition, we found no significant difference in distance covered in the open field test over the entire test period (Hoxb1 null: 49.5±5.7m/30 min versus control: 50.3±7.6m/30 min; Students t-test, p=0.9; Figure 7B), but there was a tendency for Hoxb1 null mice to rear on their hindlimbs less often than control mice (Hoxb1 null: 94.9±12.8 rears/30 min versus control: 125.0±24.2 rears/30 min; Students t-test, p=0.10; Figure 7C). In a more detailed analysis, we distinguished between rearing without leaning against the wall of the arena (hereafter called unsupported rearing) versus rearing while leaning against the wall (hereafter called leaning). Unsupported rearing was significantly less frequent in Hoxb1 null mice relative to control mice over the entire test period (Hoxb1 null: 15.4±4.2 unsupported rears/30 min versus control: 43.4±13.4 unsupported rears/30 min; Students t-test, p=0.03; Figure 7D), whereas there was no difference in the frequency of leaning (Hoxb1 null: 79.5±10.9 leans/30 min versus control: 92.8±15.6 leans/30 min: Students t-test, p=0.4 for each time bin; Figure 7E). The frequency of unsupported rearing was significantly lower in each time bin during the test period (Students t-test, p=0.1, p=0.03 and p=0.02 for 0-10, 10-20 and 20-30 time bins, respectively; Figure 7D), and was not correlated with reduced time spent in the central part of the arena, which did not differ significantly during the test period (Students t-test, p>0.2 for each time bin; Figure 7F). Thus, the difference in unsupported rearing of Hoxb1 null mice cannot be attributed to perturbed emotional processing (for example higher anxiety, motivating them to stay away from the most open area of the arena and close to the walls). It is more likely due to a more subtle deficit in balance that is especially evident when the entire weight of the body is carried by the hindlimbs.

To assess motor capability with less dependence on weight-bearing equilibrium, we performed the linear one meter swimming test, which elicits non-weight-bearing locomotion in which hindlimbs drive forward propulsion while forelimbs are held in a static flexed position (Figure 8A-E). Hoxb1 null mice displayed the same body position and head orientation during swimming as control mice (Figure 8A,B), but instead of continuously using alternating hindlimb movements, they occasionally extended their hindlimbs synchronously
(Figure 8B and Video 1, Video 2). Interposition of synchronous hindlimb movements was significantly more frequent in Hoxb1 null than control mice (RMANOVA, F[1,15]=10.5, p<0.005 for main effect of the Hoxb1 null mutation; Figure 8C). Since these synchronous hindlimb movements interrupted the smooth forward motion of swimming, their generation is likely related to the tendency for Hoxb1 null mice to take longer to swim the 1 meter distance, a time that became statistically significant on the last trial (Hoxb1 null: 3.8±0.3 sec versus control: 5.4±0.9 sec; p=0.03; Figure 8D).

Also in support of a motor deficit not related only to balance per se, Hoxb1 null mice used significantly more time to traverse the one meter notched beam (Figure 8E-G), a difference evident in all three trials, as documented by RMANOVA (F[1,15]=6.0, p=0.03 for main effect of Hoxb1 null mutation) and post-hoc analyses. These longer times were associated with an increased number of slips during stepping across each notch (RMANOVA, F[1,15]=11.2, p=0.004 for main effect of the Hoxb1 null mutation; Figure 8H). We also assessed hindlimb coordination during the traverse of the notched beam, and found that although Hoxb1 null mice tended more often than wild type control mice to display hopping when moving from one step of the beam to the next, with synchronous extensions of both hindlimbs analogous to the synchronous strokes exhibited during swimming (Figure 8G, I; Video 3, Video 4), this difference did not reach significance (F[1,15]=1.1, p=0.3 for main effect of the Hoxb1 null mutation).

Thus, despite the substantial early compensation in vestibular function, Hoxb1 null mice exhibit abnormalities in proprioceptive and locomotor behavior as adults, which are likely to be due to either the loss of the LVST or the deficit in reticulospinal projections, or both.
DISCUSSION

General summary
Using a combination of retrograde labeling and genetic fate mapping, we here provide an extensive characterization of the contribution of r4 to the vestibular system, and in particular to specific populations of vestibular projection neurons. We also demonstrate a contribution of r4 to the reticulospinal system, which, though minor, is important when considering the physiological and behavioral effects of perturbing r4 development. We demonstrate that inactivating Hoxb1, a master gene involved in imparting r4 identity (Studer et al., 1996, Di Bonito et al., 2013a, b), leads to the absence of several r4 derivatives (including vestibular efferent neurons and specific subpopulations of vestibulospinal neurons), and partially affects other r4 derivatives (including reticulospinal and vestibulopapular neurons). We also make the first assessment of the behavioral effects that result from this genetic perturbation of r4.

Segmental patterning of vestibular projection neurons: the role of r4 and Hoxb1
Where earlier studies have only been able to presume the relationship between neuron groups and the r4 boundaries due to technical limitations in fate mapping, we have been able to define explicitly which neurons derive from r4 and which do not. Thus, we can now say with certainty that the vast majority, and possibly all, LVST neurons derive from r4, as does a specific subpopulation of cMVST neurons. We also have strong evidence that some iC-VO neurons originate in r4, as do the scattered ipsi VO neurons in r3. Interestingly, our study has identified a population of reticulospinal neurons that derives from r4, some of which migrate into r5. The functional role of this particular reticulospinal neuron subpopulation remains to be defined. Finally, r4 also gives rise to the vestibular efferent neurons, but these represent a quite different category of neurons from the vestibular projection neurons, as they are more closely related to motoneurons. They originate from a more ventral domain, extend their axons to the periphery, utilize acetylcholine as principal neurotransmitter, and modulate the contractile state of their target hair cells.
This places the contribution of r4 to the patterning of vestibular projection neurons into relief. Rhombomere 4 lies about midway along the rostrocaudal extent of the vestibular nuclear complex and represents in several respects a key domain in patterning vestibular projection neurons. It gives rise to the principal vestibulospinal neuron group, the LVST, but to few vestibulo-ocular projection neurons. The other vestibulospinal neuron groups, coursing in the MVST, derive predominantly from the more caudal r5 and r6, although a portion of the cMVST group derives from r4. Thus, within the context of vestibular projection neurons, r4 is primarily related to vestibulospinal specification, whereas vestibulo-ocular projection neurons derive predominantly from more rostral and more caudal rhombomeres (Figure 9).

The lack of LVST neurons and r4-derived cMVST neurons in the Hoxb1 null mutant (Figure 9) suggests either an outright failure of their generation from progenitor cells, their subsequent early death, or their differentiation to another, non-vestibulospinal phenotype. Since loss of Hoxb1 function prevents the maintenance of r4 identity (Studer et al., 1996; Gavalas et al., 1998; Weicksel et al., 2014) and mainly transfates r4 into r3 (Di Bonito et al., 2013a, b), the differentiation of r4-derived neurons into r3-related fates would be expected. As noted, a small number of non-r4-derived spinally-projecting neurons was present in the LVST domain in the Hoxb1 null mutant. Their origin and function remains to be determined, but they could represent some MVST neurons that have attained abnormally rostral positions.

On the other hand, vestibulo-ocular neurons and a minor population of reticulospinal neurons derived from r4, are not wholly eliminated in the Hoxb1 null mutant. These findings prompt several hypotheses: (i) vestibulo-ocular and reticulospinal neurons are less dependent than vestibulospinal neurons on Hoxb1 expression, (ii) differentiation and maintenance of vestibular projection neurons is characterized by a hierarchy wherein vestibulo-ocular neurons represent a default phenotype that is overridden by the function of Hoxb1 to create a vestibulospinal phenotype, and (iii) r3-like vestibulo-ocular and
reticulospinal neurons are generated in r4 in the absence of Hoxb1, as is the case with other r4-derived neuronal populations such as facial branchial motoneurons, auditory neurons and trigeminal neurons (Studer et al., 1996; Di Bonito et al., 2013a, b). This last hypothesis would also explain why LVST and cMVST neurons, which normally originate from r4 but not r3, fail to form in Hoxb1 null mice (the mutant r4 becomes r3 which does not give rise to vestibulospinal neurons), whereas vestibulo-ocular and reticulospinal neurons, which normally originate from both r3 and r4, are still generated from r4 in the Hoxb1 mutant (the mutant r4 becomes r3 which does give rise to these neuron types). To ultimately demonstrate transfating, we would need rhombomere- and group-specific markers of the vestibular projection neurons, which are not yet available.

The LVST group as a developmental and functional entity

Earlier studies have highlighted the fact that the LVST neuron group and the lateral vestibular nucleus (LVN) are not equivalent, as many vestibulospinal neurons projecting in the MVST are located in the ventral LVN (reviewed in Akaike (1983). The non-congruence of the LVST neuron group and the LVN has been demonstrated explicitly in the chicken embryo by direct comparison of hodological and cytoarchitectonic domains, which has shown that also parts of the cMVST, iR-VO and cC-VO neuron groups (and potentially other vestibular projection neuron groups) lie within the LVN (Diaz et al., 2003). More recently, this lack of congruence has been noted in tracing studies of the LVST in the adult mouse (Liang et al., 2014). Although a common practice in the literature, we strongly recommend that in the future the source of the LVST be denoted not as the LVN, but rather as the LVST neuron group, which is correct and precise.

That the LVST group derives essentially in its entirety from r4 implicates Hoxb1 as a core molecular component in the genetic program for LVST specification. Nevertheless, some LVST neurons migrate from r4 into r3 and r5, raising the possibility that these subpopulations differ functionally from the main subpopulation that remains in r4 (Figure 9). This could be related to the
topographic organization of the LVST and its spinal targets, in which the most rostral and caudal LVST neurons connect respectively to cervical and lumbar spinal levels (Shamboul, 1980). Numerous studies have shown that the LVST neurons exhibit further heterogeneity in their size, clustering and dendritic arbors (see for example Glover and Petursdottir, 1988; Liang et al., 2014), indicating that their r4-specific origin does not impart a monolithic pattern of differentiation.

Of the three vestibulospinal neuron groups, only the LVST group projects beyond cervical and upper thoracic segments (Wilson and Peterson, 1978; Kasumacic et al., 2010), and activity in the LVST is known to activate primarily extensor muscles. Thus, it is thought that the vestibulospinal reflex in the hindlimbs is elicited predominantly by impulses traveling in LVST axons that synapse on extensor motoneurons, or interneurons immediately presynaptic to these, in the lumbar spinal cord. Numerous studies have investigated the behavioral effects of peripheral vestibular lesions, but to our knowledge this is the first time the central source of the LVST has been eliminated bilaterally, certainly using non-surgical techniques. Using a dedicated vestibulospinal reflex test device for young mouse pups, we show that the bilateral lack of the LVST greatly diminishes the fidelity of the vestibulospinal reflex in the hindlimbs.

Using non-weight-bearing swimming to test general motor capability in the Hoxb1 null mice at the same age when the vestibulospinal reflex is profoundly affected (P5) revealed no obvious differences from controls in a number of variables, including maximum speed, average speed, and number of turns. Moreover, the total trajectory traversed was about 20% higher in the Hoxb1 null mice. Thus, the bilateral loss of the LVST had little effect on general motor capability within the limits of the behavioral tests that we used, suggesting that the LVST is primarily concerned with weight bearing motor functions and particularly with the limb extension that typifies the vestibulospinal reflex.
 Recovery of the vestibulospinal reflex

Though greatly diminished at P5, the vestibulospinal reflex in the hindlimbs gradually recovered over the ensuing week. Several possible explanations exist, including the growth of the iMVST or cMVST beyond their normal longitudinal span, a vestibulospinal function for the few non-r4-derived spinally-projecting neurons seen in the LVST domain or a greater recruitment of vestibulo-reticulospinal connections into the reflex pathway, an explanation that we consider the most probable. Though intrinsically slower than the direct vestibulospinal pathway, connections from vestibular nuclei (including the ventral part of the LVN where many LVST neurons reside) to the medial reticular formation are thought to provide a parallel pathway to the spinal cord (Peterson and Abzug, 1975). Since reticulospinal projections are also well developed by birth in the mouse (Leong et al., 1984; Auclair et al., 1999), they could clearly provide an existing substrate for transmitting vestibular signals to the lumbar cord even in the absence of the LVST.

Motor deficits in the adult – possible additional roles for the LVST in regulating motor function

Despite profound compensation of the vestibulospinal reflex during early postnatal life, other functional deficits in the motor system were evident in the adult. Most of these did not appear to affect balance or equilibrium per se, but rather the coordination of limb movement that is required to perform more demanding motor tasks. Adult Hoxb1 null mice had a normal righting reflex and did not exhibit abnormal circling behavior, suggesting that the early compensation seen for the vestibulospinal reflex was persistent, bilateral and effective in a more general vestibular context. They did however show a decrease in unsupported rearing in the open field, suggesting that the compensation was not sufficient to provide effective balance in all situations.

Nevertheless, the most obvious behavioral deficits were less balance-related. For example, during swimming, adult Hoxb1 null mice were more likely to employ synchronous hindlimb kicks than control mice. There was a tendency for synchronous activation of the hindlimbs and the forelimbs to be more prevalent...
in *Hoxb1* null mice when navigating the notched beam as well. This interesting phenotype suggests a link to the spinal locomotor network, which typically generates alternating hindlimb movements in adults (Kiehn, 2006), but during situations in which the balance between excitation and inhibition between left and right sides is altered (Restrepo et al., 2011) or when specific elements of the locomotor central pattern generator are perturbed genetically (Lanuza et al., 2004; Crone et al., 2008; Zhang et al., 2008; Talpalar et al., 2013; Shevtsova et al., 2015). The other deficit that was particularly evident was the increased number of slips made by *Hoxb1* null mice on the notched beam, which most likely contributed to the increased time required to traverse the beam. This phenotype could reflect a variety of potential deficits, including in the dynamic coordination of hindlimb muscles imposed by the spinal locomotor network but also in the various mechanoreceptive and proprioceptive inputs that mold locomotor output according to the demands of the substrate.

Although both the swimming and the notched beam tests were originally conceived to evaluate limb coordination, they could also be affected by perturbed balance and equilibrium, or by proprioceptive deficits, as has been described previously for the notched beam test (Shelton et al., 2008). Moreover, we have shown that another descending input, from the small number of r4-derived reticulospinal neurons, is also depleted in the *Hoxb1* null mice. Thus, we cannot rule out that the non-balance-related motor deficits result from perturbation of this pathway instead of from the loss of the LVST. Nevertheless, in a recent report using transsynaptic rabies virus tracing, Bourane et al., (2015) provide evidence for synaptic connections between the vestibular nuclei and a set of dorsal horn interneurons involved in light touch and fine motor control. Thus, we propose that the non-balance-related motor deficits in adult *Hoxb1* null mice might reflect two additional roles for the LVST: 1) providing a descending channel for introducing bias in the activation of the two hindlimbs by the spinal locomotor network, thus contributing to alternation as opposed to co-activation, and 2) regulating local sensorimotor circuits in the spinal cord according to the dynamic balance of extensor and flexor muscle activity required to move the
hindlimbs in precisely coordinated patterns. Thus, instead of operating solely within the realm of balance and equilibrium, the LVST may provide a rapid channel for fine-tuning extensor muscle activity in a more global context.
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Figure Legends

Figure 1. Fate mapping of LVST neurons and reticulospinal neurons.

A) The b1r4-Cre line, which expresses the Cre recombinase under the control of the Hoxb1 r4-enhancer, is crossed with the ROSA26YY reporter line to label r4 derivatives by YFP expression. B, C) Schematic diagrams of E16.5 brain in the parasagittal plane illustrating the location of YFP-positive r4 and r4-derivatives (green) and the domains of the LVST group and the main population of ipsilaterally projecting reticulospinal (RS) neurons (gray). Magenta arrowhead in (C) indicates the tracer application site used to retrogradely label vestibulospinal and reticulospinal neurons. D, E) All LVST neurons, including those in r3 and r5, derive from r4. The first panel in each horizontal series is a low magnification image, with a white square indicating the region shown in the subsequent images. F-H) A population of ipsilaterally projecting RS neurons in r4 and r5 derives from r4. Arrowheads in G indicate LVST axons projecting just dorsal to the RS neurons. RDA/BDA labeling is depicted by magenta, YFP immunolabeling by green, and double labeling appears as varying hues of yellow and orange. Note that in this and subsequent figures some double labeled neurons appear to have a magenta nucleus with surrounding yellow to orange cytoplasm; this is because the RDA/BDA can partition into the nucleus where the YFP is weak. Abbreviations: Tel (telencephalon), Di (diencephalon), Mes (mesencephalon), cb (cerebellum), SC (spinal cord), vnl (ventral nucleus of lateral lemniscus), facial (VIIth cranial facial motor nucleus), LVST (lateral vestibulospinal tract group), RS (reticulospinal), RDA (tetramethylrhodamine-conjugated dextran amine), YFP (Yellow Fluorescent Protein). Scale bars: 200µm.

Figure 2. Fate mapping of other vestibulospinal neurons and of vestibulo-ocular neurons.

A) No iMVST neurons derive from r4. B, C) cMVST neurons located within r4 derive from r4, whereas those located in r5 do not. The first image in each horizontal sequence is at low magnification, and the subsequent images show higher magnification. RDA/BDA labeling is depicted by magenta, YFP
immunolabeling by green, and double labeling appears as varying hues of yellow and orange. To the right are shown two schematic diagrams. The upper one shows the location of YFP+ r4 and r4-derivatives (green) and the domains of the iMVST and cMVST groups (grey) in a parasagittal view of the brainstem. The magenta arrowhead indicates the site of tracer application used to retrogradely label vestibulospinal neurons. The lower one shows a dorsal view of the brainstem, indicating the rhombomeric domain (r4 in green) and the locations and rhombomeric origins of the 3 vestibulospinal groups. Magenta indicates a non-r4 origin and yellow (double labeled YFP and RDA) indicates an r4 origin. D, E) Residual iC-VO neurons in r4 and some scattered ipsi VO neurons in r3 derive from r4 (D), but no iR-VO neurons derive from r4 (E). F, G) No cC-VO neurons derive from r4, although a subpopulation migrates into r4 (F). To the right are shown two schematic diagrams. The upper one shows the location of YFP+ r4 and r4-derivatives (green) and the domains of the vestibulo-ocular groups (grey) in a parasagittal view of the brainstem. The magenta arrowhead indicates the site of tracer application used to retrogradely label vestibulo-ocular neurons. The lower one shows a dorsal view of the brainstem as above, indicating the rhombomeric origins and eventual locations of the vestibulo-ocular groups. Magenta indicates a non-r4 origin and yellow (double labeled YFP and RDA) indicates an r4 origin. Abbreviations: LVST (lateral vestibulospinal tract group), iMVST (ipsilateral medial vestibulospinal tract group), cMVST (contralateral medial vestibulospinal tract group), iC-VO (ipsilateral caudal vestibulo-ocular group), iR-VO (ipsilateral rostral vestibulo-ocular group), cC-VO (contralateral caudal vestibulo-ocular group), cR-VO (contralateral rostral vestibulo-ocular group), RDA (tetramethylrhodamine-conjugated dextran amine), YFP (Yellow Fluorescent Protein). Scale bars: 200µm.

Figure 3. Loss of vestibulospinal neurons and depletion of reticulospinal neurons in the Hoxb1 null mutant. A-J) Loss of vestibulospinal neurons. Comparison of the two vestibulospinal groups that derive wholly or partly from r4 in the wild type (LVST, A-C; cMVST, D,E) and the Hoxb1 null mutant (F-J) shows a complete absence of r4-derived LVST and cMVST neurons. The white arrowheads in D indicate examples of r4-
derived cMVST neurons. Those in F-H indicate examples of non-r4-derived spinally projecting neurons in the *Hoxb1 null* mutant in the area where the LVST is normally located. The green arrowheads in I and J indicate non-spinally projecting r4-derived cells that migrate into r3 within the vestibular nuclear complex in the *Hoxb1 null* mutant. (K-P) Depletion of r4-derived reticulospinal neurons. Reticulospinal neurons derived from r4 (white arrowheads) are more numerous in the wild type (K-M) than in the *Hoxb1 null* mutant (N-P). RDA/BDA labeling is depicted by magenta, YFP immunolabeling by green, and double labeling appears as varying hues of yellow and orange. Q-V) Examples of confocal z-stacks to demonstrate double labeling, in this case of two reticulospinal neurons. Each panel shows a z-stack viewed in the x-y plane and from the x-z and y-z faces, with x and y transects intersecting at a reticulospinal neuron that is double labeled (one in Q-S, another in T-V). In each row of panels, the right panel shows only RDA (magenta), the middle panel shows only YFP (green) and the left panel shows a merge of the two (note that here the magenta and green combine to create a pale white, as opposed to the yellow/orange that depicts double labeling in the panels above; see Materials and Methods). Abbreviations: LVST (lateral vestibulospinal tract group), cMVST (contralateral medial vestibulospinal tract group), RS (reticulospinal neurons). Scale bar: 200µm (A-P); 20µm (Q-V).

Figure 4. Effects of *Hoxb1 null* mutation on vestibulo-ocular neuron groups. A-F) No loss of iC-VO or scattered ipsilateral VO neurons in the *Hoxb1 null* mouse. A-C are a series of parasagittal sections in a wild type (wt) mouse embryo showing the presence of the few iC-VO neurons in r4 and scattered ipsilateral VO neurons in r3. Some of each of these can be seen to be r4-derived (examples indicated by white arrowheads). D-F are a similar series of sections in the *Hoxb1 null* mutant, in which the situation is similar to that in the wild type: there is no indication that iC-VO or scattered ipsilateral VO neurons are lost, and some of each are r4-derived. G-L) Ectopic presence of r4-derived neurons in the region containing the iR-VO group. G-L present a series of parasagittal sections through the region containing the iR-VO group in wild type (G-I) and *Hoxb1 null* (J-L) mice. In this region, *Hoxb1 null* mutants have ectopic r4-derived neurons.
(examples indicated by white arrowheads) not seen in wild type mice. RDA/BDA labeling is depicted by magenta, YFP immunolabeling by green, and double labeling appears as varying hues of yellow and orange. Abbreviations: iC-VO (ipsilateral caudal vestibulo-ocular group), iR-VO (ipsilateral rostral vestibulo-ocular group). Scale bar: 200µm.

Figure 5. Loss of vestibular efferent neurons in the Hoxb1 null mutant. In situ hybridization for Gata3 transcripts (A, A’) and Hoxb1-driven expression of YFP (B, B’) in wild type (wt) and Hoxb1 null mouse embryos. Gata3 in situ hybridization in wild type mice labels the vestibular efferent neurons (VEN) in the specific region of r4 where they differentiate (arrows, A and B). In Hoxb1 null mice this region is devoid of Gata3 expression (*, A’). Application of DiI to the peripheral nerves in the inner ear at P8 retrogradely labels vestibular efferent neurons in wild type mice (VEN, C) but not in Hoxb1 null mice (*, C).

Figure 6. Behavioral effects of the Hoxb1 null mutation in young mice. A-E. Open field swimming test. A. Typical swimming trajectories of wild type (blue) and Hoxb1 null mice (red). B. Comparison of maximum and average swimming speeds. C. Comparison of time spent within the central region (light grey in A) and peripheral region (dark grey in A). D. Comparison of average total swimming distances (trajectory). E. Comparison of average number of rotations. F,G. Hindlimb vestibular reflex test. F. An example of the test. A wild type mouse pup mounted in the rotation device is shown from the rear. At rest (top panel) the pup holds its hindlimbs in a flexed position, and the tail straight back. Red arrow indicates right hindlimb. No movement is detected at the midpoint of the rotation (middle panel), but by full rotation (bottom panel), a marked extension of the right hindlimb has been elicited, and the tail has deviated to the same side and upwards. G. Comparison of average response rate in the hindlimb vestibular reflex test from P5 to P11. Error bars represent * p<0.1, **** p<0.005, ***** p<0.001.

Figure 7. Behavioral effects of the Hoxb1 null mutation in adult mice: righting reflex and open field test. A. Comparison of time required for the righting reflex
in wild type (blue) and *Hoxb1 null* (red) mice. B-F. Open field behavior. Naive

*Hoxb1 null* mice and their littermate controls were tested during 30 minutes in the open field and total distance was scored for three consecutive 10min time epochs for distance covered (B), total rearing events (C), unsupported rearing (D), leaning (rearing supported against the wall of the arena, E) and time spent in central zone of the arena (F). Error bars represent SEM. Significant differences with respect to control mice were calculated using the PLSD Fischer test. *

*p<0.05.*

Figure 8. Behavioral effects of the *Hoxb1 null* mutation in adult mice: linear swimming test and notched beam test. A-C. Linear swimming test. A, B. Examples of alternating hindlimb swimming movements in wild type mice (A) and synchronous hindlimb swimming movements in *Hoxb1 null* mice (B). Green and red traces delineate respectively the hindlimb closest to and furthest from the camera. C. Comparison of average number of synchronous hindlimb strokes. D. Comparison of average time required to swim the total distance of 1 meter. E-I. Notched beam test. E. Comparison of average time required to traverse the beam. F, G. Examples of alternating and synchronous locomotor movements in wild type and *Hoxb1 null* mice, respectively. H. Comparison of number of slips made while traversing the beam. I. Comparison of number of hops made while traversing the beam. Error bars represent SEM. Significant differences with respect to control mice were identified using PLSD Fischer test: *, *p<0.05*; **, *p<0.01.*

Figure 9. Summary of segmental origins of vestibular projection neurons, vestibular efferent neurons and reticulospinal neurons in the wild type (top) and of the phenotype of the same neuron populations in the *Hoxb1 null* mutant (bottom).

Video 1. Swimming exhibited by a wild type mouse.

Video 2. Swimming exhibited by a *Hoxb1 null* mutant mouse.
Video 3. Traversal of the notched beam by a wild type mouse.

Video 4. Traversal of the notched beam by a Hoxb1 null mutant mouse.
Figure 1
Figure 2
Figure 3
vestibulo-ocular ipsilateral

Figure 4
Figure 5
Figure 6
Figure 7

A) Time to turn (sec) 

B) Distance (cm) 

C) Total rearing events 

D) Unassisted rearing events 

E) Leanings events 

F) Time in the center (sec)
Figure 8

A. WT
B. Null

C. Synchronous strokes (nb)

D. Swim time (sec)

E. Time to cross (sec)

F. WT Direction

G. Null Direction

H. Number of slips

I. Number of hops

Figure 8
Figure 9
