Brainstem Respiratory Oscillators Develop Independently of Neuronal Migration Defects in the Wnt/PCP Mouse Mutant looptail

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Abstract

The proper development and maturation of neuronal circuits require precise migration of component neurons from their birthplace (germinal zone) to their final positions. Little is known about the effects of aberrant neuronal position on the functioning of organized neuronal groups, especially in mammals. Here, we investigated the formation and properties of brainstem respiratory neurons in looptail (Lp) mutant mice in which facial motor neurons closely apposed to some respiratory neurons fail to migrate due to loss of function of the Wnt/Planar Cell Polarity (PCP) protein Vangl2. Using calcium imaging and immunostaining on embryonic hindbrain preparations, we found that respiratory neurons constituting the embryonic parafacial oscillator (e-pF) settled at the ventral surface of the medulla in Vangl2+/− and Vangl2−/− embryos despite the failure of tangential migration of its normally adjacent facial motor nucleus. Anatomically, the e-pF neurons were displaced medially in Lp/+ embryos and rostro-medially Lp/Lp embryos. Pharmacological treatments showed that the e-pF oscillator exhibited characteristic network properties in both Lp/+ and Lp/Lp embryos. Furthermore, using hindbrain slices, we found that the other respiratory oscillator, the preBozinger complex, was also anatomically and functionally established in Lp mutants. Importantly, the displaced e-pF oscillator established functional connections with the preBozC oscillator in Lp/+ mutants. Our data highlight the robustness of the developmental processes that assemble the neuronal networks mediating an essential physiological function.

Citation: Thoby-Brisson M, Bouvier J, Glasco DM, Stewart ME, Dean C, et al. (2012) Brainstem Respiratory Oscillators Develop Independently of Neuronal Migration Defects in the Wnt/PCP Mouse Mutant looptail. PLoS ONE 7(2): e31140. doi:10.1371/journal.pone.0031140

Editor: Stefan Liebner, Institute of Neurology (Edinger-Institute), Germany

Received November 9, 2011; Accepted January 3, 2012; Published February 17, 2012

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Funding: This work was supported by the Centre National de la Recherche Scientifique, the Medical Research Council, Institut National de la Santé et de la Recherche Médicale (M.T.-B.), ANR grant ANR-07-Neuro-007-01 (G.F.), Research Leave (RL-08-013) and Research Council (URC-08-030) grants from the University of Missouri (A.C.), and a National Institutes of Health (NS04049) grant (A.C.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors declare that no competing interests exist.

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Introduction

The development of functional neuronal circuits requires appropriate migration of neurons from the germinal zone where they are born to their final position in the nervous tissue. Abnormal neuronal migration during development can cause neurological and cognitive impairments varying between mild to severe deficits [1–3]. Breathing is a spontaneous rhythmic behavior critical for life. However, mechanisms underlying the migration of neurons generating respiratory rhythm, and the consequences of their abnormal migration, have not been examined.

Respiratory rhythmogenesis relies on the activity of a brainstem respiratory rhythm generator located in the ventral medulla, and composed of two interacting oscillators: the preBozinger complex (preBozC) that drives inspiration [4] and the parafacial respiratory group (pFRG) controlling pre-inspiratory and expiratory activities [5,6]. It has been shown recently in rodents that respiratory oscillators emerge sequentially during development. At embryonic day (E) 14.5 in the mouse, rhythmic activity and chemosensitivity can be detected in the embryonic parafacial oscillator (e-pF) [7], while the preBozC oscillator activity appears one day later at E15.5 [8].

The final position of the e-pF is, ventrally, adjacent to the pial surface, and, dorsally, at the lateral edge of the facial motor nucleus (FMN) [7,9,10]. The e-pF and facial branchiomotor (FBM) neuron progenitors share expression of the visceral marker Phox2b [7,9], although they are located in completely different domains of the hindbrain ventricular zone, along both the anterior-posterior axis (in Egr2-positive and -negative rhombo-mers, respectively) and the dorsal-ventral axis (in dB2 and vMN domains, respectively). After exiting the cell cycle, migrating e-pF
and FBM neurons become adjacent at E11.5 [9]. Subsequently, the e-pF neurons migrate radially around the FMN to reach the ventral (pial) surface, a physiologically important location close to relevant chemosensory signals and cerebral vasculization in the adult [11,12]. Mechanisms underlying the migration of e-pF neurons are largely unknown. It is also not known whether defective e-pF migration/position can affect respiratory rhythm.

Here, we address these issues by testing whether a mutation that affects FBM neuron migration also affects e-pF neuron migration, and whether defects in e-pF positioning alter its respiratory-related functions. We used calcium imaging and pharmacological approaches to examine the functional and anatomical characteristics of the respiratory neuronal network in a mouse mutant where FBM neuron migration is abnormal. The transmembrane protein Van gogh-like 2 (Vangl2) is a component of the noncanonical Wnt/Planar Cell Polarity (PCP) signaling pathway [13,14] required for tangential migration of FBM neurons from rhombomere 4 (r4) to r3-r7 in the hindbrain [13,15,16]. In looptail (Lp) mutant (Vangl2−/−) mice, we found that the e-pF oscillator was rostro-medially displaced, but could still be detected at the ventral medullary surface, was bilaterally synchronized, and exhibited characteristic functional properties. The location and properties of the preBotC oscillator were unaffected in Lp mutants. Thus, hindbrain respiratory oscillators develop and establish function independently of mechanisms regulating FBM neuron position.

Methods

Ethics statement

Animal maintenance and experiments were performed in accordance with French National (JO 87-848) and European (86/609/CEE) legislation on animal experimentation, and following the guidelines of the Animals (Scientific Procedures) Act 1986 of the UK Government and the University of Missouri Animal Care and Use Committee (Animal Welfare Assurance Number A3394-01). The institutional animal care committees at CNRS, Gif-sur-Yvette and the University of Missouri specifically approved this study.

Animals

The looptail (Lp/Lp) inbred strain, which carries the Vangl2+ mutation, was originally obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The colony was maintained by brother-sister mating for over 100 generations, then bred to congenicity on the C3H/HeH background. Heterozygous animals were intercrossed to generate litters containing +/+; Lp/+ and Lp/Lp embryos, and mice were genotyped by pyrosequencing for the Vangl2 mutation as described previously [17]. The Lp mutation generates a potentially non-functional Vangl2 protein that localizes poorly to the plasma membrane [18,19].

Mice (Lp/+), were mated overnight; the day of finding the vaginal plug was considered as embryonic day (E) 0.5. Pregnant dams were shipped before E14.5 from MRC Harwell to CNRS, Gif-sur-Yvette in accordance with UK Home Office and EU guidelines. The size and gross anatomy of all embryos obtained from Lp/+ incrosses, as well as the spontaneous activity recorded from whole hindbrain preparations of all three genotypes (+/+; Lp/+; Lp/Lp) revealed that embryonic development in these litters (C3H/HeH background) was delayed by about one day compared to standard staging used in previous studies [C57BL/6, 129/Sv and OF1 backgrounds] [7]. Consequently, the e-pF, which emerges at E14.5 [7], was actually examined at E15.5 for +/+; Lp/+ and Lp/Lp embryos in the present study. Similarly, the pre-Boëttinger complex, which emerges one day later at E15.5 [8], was examined at E16.5 for all embryos. Moreover, since the looptail mutation is lethal at birth, it was not possible to further test the respiratory function at post-natal stages, and all experiments were performed at embryonic stages.

In vitro preparations

Pregnant females were sacrificed by cervical dislocation at the 14th, 15th or 16th day of gestation (E14.5, E15.5 or E16.5). Uterine horns were removed from the mother and embryos were excised from their individual bag and kept until the recording session in oxygenated artificial cerebrospinal fluid (a-CSF) at 25°C. The a-CSF composition (in mM) was: 120 NaCl, 8 KCl, 1.26 CaCl2, 1.5 MgCl2, 21 NaHCO3, 0.5 Na2HPO4, 30 glucose, pH 7.4. To induce acidification the pH of the a-CSF was lowered to 7.2 by decreasing the NaHCO3 concentration to 10.5 mM while adjusting the NaCl concentration at 130.5 mM. We used two different preparations to examine the two respiratory oscillators: isolated brainstems for the e-pF and transverse slices for the preBoëtC. Brainstem and slice preparations were dissected in the a-CSF solution at 4°C and obtained as described previously [7,8].

Briefly, a rostral section performed at the junction between the mesencephalon and the rhombencephalon and a caudal section performed below the fourth cervical roots allowed to isolate brainstems from the central nervous system (“whole hindbrain” preparation). After embedding brainstems in an agar block, transverse medullary slice preparations were obtained by serially sectioning the preparation in the transverse plane from rostral to caudal using a vibratome (Leica). A 450 μm thick slice isolating the preBotC oscillator was obtained with an anterior limit set 200–300 μm caudal to the posterior extremity of the facial motor nucleus (FMN). In the Lp/+ heterozygote, we had to adapt this procedure due to the mislocation of the FMN. Based on immunostainings (Figs. 1 and 2), we determined that the FMN is 200–300 μm more rostral than in a wild-type embryo. We therefore took the slice that was 500–600 μm more caudal than the posterior extremity of the FMN. In the homozygous Lp/Lp mutant that exhibits a completely open neural tube, anatomical landmarks normally used to obtain the transverse slice isolating the preBotC, such as the position of the FMN, the presence of the inferior olive, and the outline of the fourth ventricle, could not be detected visually in transmitted light. In addition after isolation, the malformed hindbrain was extremely fragile and had to be very carefully manipulated. Therefore, slices containing the preBotC oscillator in Lp/Lp embryos were obtained as follows: we made consecutive 450 μm thick slices over the entire length of the open neural tube and tested activity for all of them. After isolation, the preparations (isolated brainstems (e-pF) or transverse slices (preBotC)) were first incubated in the calcium indicator before being transferred into the recording chamber where they were continuously superfused with oxygenated a-CSF and maintained at 30°C. To allow imaging of neuronal activities in the recording chamber, isolated preparations were placed ventral (pial) side up for brainstems (e-pF) and rostral side up for transverse slices (preBotC).

Calcium imaging

Preparations were incubated for 40 min in oxygenated a-CSF containing the cell-permeable calcium indicator dye Calcium-Green 1AM (10 μM; Molecular Probes, Inc., Eugene, OR). After a 30 min recovery period in the recording chamber to wash out the excess dye and to allow spontaneous activities to emerge, an epifluorescent illumination system on an E-600-FN upright microscope (Nikon) equipped with a fluorescein filter block was used to excite the dye and capture the emitted light. Fluorescence
positive cells in the hindbrain of Figure 1. Location of the facial motor nucleus and Phox2b-Egr2 hybridization of illustrated in M and P, N and Q, O and R, correspond to adjacent slices sections (J–O) through E15.5 hindbrains of on whole hindbrain preparations (G–I, ventral view) and transverse G–O: Anti-Phox2b (red) and anti-Islet1,2 (green) immunofluorescence embryos. The black squares indicate the regions illustrated in G, H and I. + through E15.5 hindbrain preparations obtained at level 1 for a and anti-Islet1,2 (green) immunofluorescence on transverse sections corresponding to the sections illustrated in J–R. P–R: Anti-NK1R (red) dashed lines in G–I refer for each genotype to the axial level Phox2b-positive cells located ventral to the facial nucleus. Numbered dashed lines in G-I refer for each genotype to the axial level corresponding to the sections illustrated in J–R. P–R: Anti-NK1R (red) and anti-Islet1,2 (green) immunofluorescence on transverse sections through E15.5 hindbrain preparations obtained at level 1 for a +/+ embryo (P), and at level 2 for Lp/+ (Q) and Lp/Lp (R) embryos. Slices illustrated in M and P, N and Q, O and R, correspond to adjacent slices obtained from the same animal, thus showing possible co-expression of several markers in the same neuronal population. Immunostainings indicate the presence of Phox2b-positive or NK1R-positive cell groups present at the ventral surface of the hindbrain for all genotypes. D: dorsal, L: lateral, R: rostral.

doi:10.1371/journal.pone.0031140.g001

Pharmacological treatments

Drugs were obtained from Sigma (St Louis, MO, USA), dissolved in a-CSF and bath-applied for 5 to 10 minutes at the following concentrations: 0.1 μM Substance P (SP); 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); 10 μM Riluzole (Ril). Frequency measurements were performed during the last three minutes of drug application. Frequency values are given as means ± SEM, and statistical significance was tested using a Student’s t test or one-way ANOVA when appropriate. Differences were assumed to be statistically significant at p<0.05.

Electrophysiology

Phrenic nerve activity in whole hindbrain preparations was recorded from the C4 roots using glass micropipette suction electrodes (100 μm tip diameter). The micropipette was filled with a-CSF and connected to a high-gain AC amplifier (7P511; Grass Instruments) through silver wires. The collected signals were filtered (bandwidth, 3 Hz to 3 kHz), rectified and integrated using an electronic filter with a time constant of 100 ms (Neurolog System), then stored on a computer via a digitizing interface (Digidata 1322A; Molecular Devices) and analyzed with the PCan9 software (Molecular devices).

Immunostaining

Antibody staining was performed on frozen sections or whole hindbrain preparations. Brainstem preparations were fixed for 2 to 3 hours in 4% paraformaldehyde. For frozen sections, tissues were cryoprotected in 30% sucrose-PBS (phosphate-saline buffer) overnight, embedded in Tissue Tek (Leica), and cryo-sectioned at 20 μm. Preparations (slices and whole hindbrains) were incubated for 30 minutes in 1% fetal bovine serum (FBS) and 0.5% Triton X-100, and incubated overnight at 4°C with primary antibodies: mouse anti-islet1,2 (1/250, DSHB), rabbit anti-NK1R (1/5000, Sigma) and a rabbit anti-Phox2b (1/1500; Gift from C. Goridis, ENS, Paris, France). After several rinses, preparations were incubated for 1 hr with secondary antibodies: FITC-conjugated goat anti-mouse (1/1000, Abcam) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1/400, Invitrogen). Stained preparations were coverslipped and mounted in Vectashield medium (Vector Labs) for preserving fluorescence. Slides were scanned on an SP2 confocal microscope (Leica Microsystems). A contrast enhancement and a noise reduction filter were applied to the images using Adobe Photoshop. Control images were captured with a cooled CCD camera (Coolsnap HQ, Photometrics, Tucson, AZ) using an exposure time of 100 ms in overlapping mode (simultaneous exposure and readout) during periods of 30 to 120 sec and analyzed using Metamorph software (Universal Imaging Corporation, West Chester, PA). The average of intensity in a region of interest was calculated for each frame. Mean baseline fluorescence was measured over a period between two spontaneous events and was used to normalize the ΔF signal which was then displayed as (ΔF/F). The distributions of active cells obtained in these experiments depict only cells that took up the dye, and which could be detected with the imaging parameters used.

Figure 1. Location of the facial motor nucleus and Phox2b-positive cells in the hindbrain of Lp mutant embryos. A–C: In situ hybridization of Egr2 (black) and Hoxb1 (red) in E8.5 whole hindbrain preparations (lateral view) obtained from a +/+ (A), Lp/+ (B) and Lp/Lp (C) embryos. WT (+/+ ) embryo processed for Egr2 in situ only. Expression of Egr2 in rhombomere 3 (r3) and r5, and of Hoxb1 in r4 are unaffected in Lp/+ and Lp/Lp embryos. D–F: In situ hybridization of Tbx20 expression in E14.5 whole hindbrain preparations (ventral view) obtained from +/+ (D), Lp/+ (E) and Lp/Lp (F) embryos. Facial motoneurons failed to migrate properly both in Lp/+ and Lp/Lp embryos. The black squares indicate the regions illustrated in G, H and I. G–O: Anti-Phox2b (red) and anti-Islet1,2 (green) immunofluorescence on whole hindbrain preparations (G–I, ventral view) and transverse sections (J–O) through E15.5 hindbrains of +/+ (G, J, M), Lp/+ (H, K, N) and Lp/Lp (L, O, R) embryos. White arrowheads in G refer for each genotype to the axial level corresponding to the sections illustrated in J–R. P–R: Anti-NK1R (red) and anti-Islet1,2 (green) immunofluorescence on transverse sections through E15.5 hindbrain preparations obtained at level 1 for a +/+ embryo (P), and at level 2 for Lp/+ (Q) and Lp/Lp (R) embryos. Slices illustrated in M and P, N and Q, O and R, correspond to adjacent slices obtained from the same animal, thus showing possible co-expression of several markers in the same neuronal population. Immunostainings indicate the presence of Phox2b-positive or NK1R-positive cell groups present at the ventral surface of the hindbrain for all genotypes. D: dorsal, L: lateral, R: rostral. doi:10.1371/journal.pone.0031140.g001

Microsystems). A contrast enhancement and a noise reduction filter were applied to the images using Adobe Photoshop. Control
experiments in which the primary antibodies were replaced by normal serum showed no labeling.

In Situ Hybridization

Hindbrains were dissected from embryos, fixed overnight in 4% paraformaldehyde, and dehydrated in 100% MeOH before processing. Tissues were permeabilized with proteinase K, hybridized with digoxigenin-labeled Tbx20 riboprobe, incubated with anti-digoxigenin alkaline phosphatase-conjugated antibodies, and developed with NBT/BCIP substrates (Roche). For two-color in situ, tissues were hybridized with digoxigenin-labeled Egr2 and fluorescein-labeled Hoxb1 probes, and first incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody (NBT/BCIP substrate) to detect Egr2 expression. After extensive washing, the tissues were incubated with anti-fluorescein alkaline phosphatase-conjugated antibody (FastRed substrate, Sigma) to detect Hoxb1 expression. The hindbrains were cleared in glycerol and flat-mounted for photography. Images were acquired with an Olympus digital camera, and adjusted for brightness and contrast using Adobe Photoshop. Tbx20 cDNA was obtained from Dr. Michele Studer (University of Nice Sophia Antipolis, Nice, France). Egr2 and Hoxb1 cDNAs were generated from total RNA using specific primers.

Figure 2. Rostro-caudal distribution of facial motor neurons and respiratory neurons in the hindbrain of wild-type and Lp mutant embryos. Half slice drawings were obtained from images of brainstem slices labeled with anti-NK1R and Islet1,2 antibodies (dorsal up). The red dots indicate individual e-pF neurons, the gray areas indicate the FMN and the blue ovals indicate the preBOTC. The rostro-caudal extents of the preBOTC (blue), the e-pF (red) and the FMN (gray) are indicated by vertical colored bars. The numbers on the left of each drawing indicate the rostro-caudal position (in μm) of the slice relative to the preBOTC position, which is defined as zero (see the full scale on the left of +/+). Note that the e-pF is more rostrally and loosely distributed in the Lp/Lp preparation compared to the +/+ and Lp/+ preparations. Similar distributions were observed in a second preparation for each genotype. Xll: hypoglossal nucleus, nA: nucleus ambiguus, FMN: facial motor nucleus.

doi:10.1371/journal.pone.0031140.g002
Results
Phox2b-positive/Islet1,2-negative e-pF neurons are mislocated in looptail (Lp) mutants

Given that e-pF neurons are located normally in close proximity to the FMN [7,20], and that the FMN is misplaced in Lp/+ and Lp/Lp embryos [15,16], we used various e-pF molecular markers to examine its location in Lp mutants.

Since hindbrain morphology is largely disturbed in the Lp mutants due to the fully open neural tube, we first examined whether rhombomeric patterning in the hindbrain is preserved in Lp mutants. We performed wholemount in situ hybridization on E8.5 hindbrains with Eg2 and Hoxb1 probes, which are expressed in rhombomeres r3 and r5 [21], and in r4 [22], respectively. Eg2 and Hoxb1 were expressed normally in Lp/+ and Lp/Lp hindbrains, indicating that rhombomeric boundaries and identities were not affected (Fig. 1A–C).

Wholemount in situ hybridization for Thx20, a T-box transcription factor expressed by migratory viscero- and branchio-motor neurons of the hindbrain [23,24], was performed in E14.5 embryos, a stage at which the FMN has completely formed at the ventro-lateral (pial) surface of a brainstem area mostly derived from rhombomere 6 (r6; Fig. 1D). Although rhombomeres are not evident after E12.5, we will refer to positions of neurons in specific rhombomere-derived locations in older embryos based on the final positions of facial motor neurons in r6 in WT [25] and in r4 in Lp/Lp E12.5 embryos [16], and of glossopharyngeal and vagal motor neurons (nA) in r7/8 [26] in all genotypes. Accordingly, in Lp/+ embryos (n = 6), FMN neurons formed a nucleus (FMN) more rostrally than in wild-type embryos, approximately r5 (Fig. 1E). Moreover, in Lp/Lp embryos (n = 6), FMN neurons failed to move caudally, but migrated laterally within r4 to form an ectopic nucleus in the dorsal part of the hindbrain (Fig. 1F; see also Figs. 11, and 2) [16].

We next examined whether the distribution of e-pF neurons was spatially correlated with the position of the FMN in the different genotypes. The facial motor neurons and e-pF neurons can be spatially correlated with the position of the FMN in the different genotypes. The nucleus ambiguous (nA) served as a reference for rostro-caudal level. We found that the position of the preBotzinger complex (preBotC), relative to the nA, was not affected by the mutation. Therefore, the e-pF-like and FMN neurons were positioned relative to the location of the nA and the preBotC. This atlas clearly indicates that 1) the FMN is rostrally displaced in Lp/+ and Lp/Lp embryos, 2) the e-pF lays caudally but not laterally to the misplaced FMN in the mutants, and 3) in Lp/+ embryos, the e-pF is found in a similar rostro-caudal position to that of +/- embryos, whereas it is rostrally displaced and more dispersed in Lp/Lp mutants. Altogether, these data suggest that the looptail mutation affects the caudal, but not the ventral, migration of e-pF-like neurons.

Rhythmically active neurons are present at the ventral surface of the hindbrain in looptail mutants

Since cells expressing e-pF markers are found in Lp mutants, we used calcium imaging to examine whether cells exhibiting the functional characteristics of e-pF neurons were present in Lp mutants. After loading isolated hindbrain preparations obtained from E15.5 +/-, Lp/+ and Lp/Lp embryos with a calcium indicator (calcium green 1-AM), we searched for individual cells spontaneously generating rhythmically organized calcium transients (hereon referred to as active cells). Inspection of the entire ventral surface at high magnification revealed the presence of active cells for the three genotypes but in different locations. The FMN was visible in direct light on the ventral surface of +/- and Lp/+ hindbrain preparations as a dark region (see right side of preparations in Fig. 3A, B). In +/- embryos (n = 3), individual active cells were located in the parafacial region: in a stripe flanking the lateral part of the FMN, and sparsely dispersed over and extending caudally to the FMN (Fig. 3A, D; cumulative distribution in Fig. 3G) as previously described for e-pF neurons [7]. In Lp/+ embryos (n = 4), active cells were present in a large column starting from the caudal edge of the misplaced FMN, extending 300–400 microns more caudally and with a comparable medial position as the FMN (Fig. 3B, E; distribution in Fig. 3H). Few active cells were found lateral to the mis-located FMN. These data show that the rostro-caudal extension of the e-pF is preserved in Lp/+ embryos despite a medial displacement. In Lp/Lp embryos (n = 3), although the structure of the neural tube was dramatically affected due to failure of neural tube closure, a significant number of active cells could be detected at the ventral surface of the hindbrain in a rostro-medial position (Fig. 3C, F; distribution in Fig. 3I). For all genotypes, the map of active cells (Fig. 3D, E and I) coincides with the position of Phox2b+/Islet1,2+ expressing cells (Fig. 2). Together, these data suggest that individual cells sharing molecular or functional profiles with e-pF neurons are present at the ventral surface of the hindbrain in looptail mutants, even though they are displaced rostro-medially, concomitant with the failure of FMN neuron migration.

Next, we asked whether these active cells individually detected form together a functional network capable of generating a rhythmically organized activity. We examined, at low magnification, spontaneous population activities generated at the ventral location of the FMN does not impact the ability of Phox2b+/Islet1,2+/NK1R+ e-pF-like neurons to reach the ventral medullary surface, but at dorsal and medial positions compared to wild-type embryos.

Using immunostainings performed on transverse slices covering the entire rostro-caudal extension of the hindbrain, we constructed a map of the positions of the e-pF-like and FMN neurons for each genotype, as shown on Figure 2. The nucleus ambiguous (nA) served as a reference for rostro-caudal level. We found that the position of the preBotzinger complex (preBotC), relative to the nA, was not affected by the mutation. Therefore, the e-pF-like and FMN neurons were positioned relative to the location of the nA and the preBotC. This atlas clearly indicates that 1) the FMN is rostrally displaced in Lp/+ and Lp/Lp embryos, 2) the e-pF lays caudally but not laterally to the misplaced FMN in the mutants, and 3) in Lp/+ embryos, the e-pF is found in a similar rostro-caudal position to that of +/- embryos, whereas it is rostrally displaced and more dispersed in Lp/Lp mutants. Altogether, these data suggest that the looptail mutation affects the caudal, but not the ventral, migration of e-pF-like neurons.
detected at a high magnification for corresponding genotypes in the
region delimited by the white rectangles in A–C. Active cells located at
the ventral surface of the preparations are found for all genotypes. G–I:
Histograms of the rostro-caudal distribution of active cells relative to
the constant preBoTc position for 2 wild-type (G), 3 Lp/+ (H), and 3 Lp/Lp
(I) embryos. The red arrows indicate the rostral and the caudal extremity
of the FMN. The distribution of active cells shows a significant rostral
displacement in Lp/Lp embryos. J–L: Calcium transients illustrated as
relative fluorescent changes (ΔF/F) recorded in the region encompass-
ning the active cells (delimited by the blue rectangles in A–C). Relative
fluorescent changes intensity are color-coded, white corresponding to
the strongest activity (see the color scale at the bottom). The traces
below show the spontaneous calcium changes recorded over time in the
entire active region for each genotype. R: rostral.
doi:10.1371/journal.pone.0031140.g003

Figure 3. Altered distribution of active cells at the pial surface
of Lp mutant hindbrain preparations. A–C: Ventral view of whole
hindbrain preparations from E15.5 +/+ (A), Lp/+ (B) and Lp/Lp (C)
embryos loaded with Calcium Green 1-AM and observed in direct
fluorescence. Yellow ovals indicate the position of the facial motor
nucleus (FMN) that is clearly visible in direct light (see right side
of preparations in A and B). D–F: Maps for rhythmic active cells (red circles)
surface of isolated E15.5 brainstem preparations, including those
used to establish the maps of active cells. In +/+ preparations
(n = 10), rhythmic fluorescence changes, generated at a frequency
of 10.4±0.5 burst/min, were detected laterally to the FMN
(Fig. 3A), and were occasionally accompanied by a burst of activity
in the FMN (Fig. 3J). In Lp/+ preparations (n = 10), rhythmic
calcium changes, occurring at a frequency of 12.1±0.8 burst/min,
were observed caudal to the abnormally located FMN (Fig. 3B), in
a region encompassing the location where Phox2b+/Islet1,2
neurons were detected (Fig. 3K), but not in a
region immediately lateral to the mis-located FMN (the expected
location for the e-pF relative to the FMN). In Lp/Lp preparations
(n = 7), inspection of the entire ventral surface of the abnormally
structured neural tube (Fig. 3C) revealed one active region located
bilaterally at the rostral-most part of the preparation and in a
medial position, corresponding to the area where e-pF-like neurons were found (Fig. 3L). In this region, rhythmic fluorescence
changes were generated at a frequency of 10.5±0.9 burst/min.
The activity of the FMN could not be detected because of
its dorsal position. These data show that putative e-pF neurons in
Lp/+ and Lp/Lp embryos, despite being rostro-medially displaced,
migrated normally to the ventral surface of the hindbrain to form a
network that generates rhythmically organized spontaneous
activity, suggesting the presence of a functional e-pF oscillator in
these mutants.

The active network present in looptail mutant hindbrains
exhibits functional properties of the e-pF oscillator
Since active cells in Lp/+ and Lp/Lp embryos are mis-located
relative to +/+ embryos, it is possible that active neuronal groups
other than those constituting the e-pF oscillator could have
reached the ventral surface of the hindbrain and be the source of
the rhythmic calcium variations observed in these preparations.
Therefore, we characterized the active networks present in Lp/+ and
Lp/Lp embryos by testing their sensitivity to pharmacological
agents known to change e-pF activity, including their response to
acidity [7]. First, application of an acidic bathing medium
(pH 7.2) induced a significant increase in the frequency of the
network activity for the three genotypes (Fig. 4, second traces in A,
B and C; summarized in D). Second, blockade of glutamatergic
transmission with 10 μM CNQX did not prevent rhythmic activity
from being generated, and even induced an e-pF-characteristic
frequency increase in wild-type [7], as well as in Lp/+ and Lp/Lp
embryos (Fig. 4, third traces in A, B and C; summarized in D). Third,
blockade of the persistent sodium current with 10 μM
riluzole completely blocked rhythmogenesis for all genotypes
(Fig. 4, fourth traces in A, B and C; summarized in D). Finally,
frequency activity significantly increased for all genotypes in the
presence of 10−7 M Substance P, the preferred ligand for NK1R
Fig. 4, fifth traces in A, B and C; summarized in D), and consistent with the presence of NK1R-positive cells in this region (Fig. 1). These data indicate that the network generating rhythmic activity in the Lp/+ and Lp/Lp embryos exhibited characteristics of the e-pF described in +/+ preparations and in previous work [7]. Thus, despite a mis-location of the FMN, and a concomitant displacement of cells expressing e-pF markers, the e-pF oscillator forms and functions normally in Lp/+ and Lp/Lp embryos.

The e-pF oscillators are bilaterally distributed in the hindbrain and functionally synchronized through poorly characterized commissural connections [7,28]. We examined whether the displaced e-pF oscillators in Lp mutants were synchronously active by performing calcium imaging simultaneously in the two e-pF oscillators. In +/+ preparations, spontaneous fluorescent changes recorded at the ventral surface of hindbrain occurred simultaneously and synchronously in the two regions encompassing the e-pF oscillators (Fig. 5A). Similarly, in preparations obtained from Lp/+ (Fig. 5B) and Lp/Lp (Fig. 5C) embryos, rhythmically organized calcium transients were generated in phase in the two e-pF oscillators. Cross-correlograms in Figure 5 represent superimposed curves obtained from 4 different preparations for each genotype. In every preparation tested, rhythmic activity was generated synchronously in the left and the right e-pF oscillator. These data demonstrate that the bilateral synchrony of the e-pF oscillators is preserved in Lp/+ and Lp/Lp mutants, suggesting that commissural projections between the oscillators are established in the Lp mutants despite their abnormal positions.
The location and activity of the preBötzinger oscillator are not affected in \textit{looptail} mutants.

The second respiratory oscillator, the preBötzinger complex (preBo¨tC) is adjacent to motor neurons composing the nucleus ambiguus (nA). While the \textit{looptail} mutation affects the position of the FMN [16], its effects on the position of the nA motor neurons and the adjacent preBo¨tC interneurons have not been examined. We addressed this issue by confirming the anatomical data presented in Figure 2 using the nA as a landmark to check for the expression of a preBo¨tC marker (NK1R), combined with a functional analysis examining the generation of rhythmic activity in the preBo¨tC area. E16.5 hindbrains were transversally sectioned and processed for immunostaining with anti-NK1R antibody (preBo¨tC and nA marker) and Islet1,2 antibody to label the adjacent motor neurons of the nA. In \textit{+/+} embryos (n = 3; see also [8,28]), NK1R-positive cells were located ventral to the Islet1,2-positive cells of the nA, starting 250–300 \textmu m caudal to the posterior limit of the FMN (defining the “r6” location), and extending caudally over 400 \textmu m (Fig. 6A, A’; [28]). In \textit{Lp/+} embryos (n = 3), the NK1R- positive cells started 500–600 \textmu m caudal to the posterior limit of the FMN (defining the “r5” location), and extended caudally over 400 \textmu m (Fig. 6B, B’). In \textit{Lp/Lp} embryos (n = 2), NK1R-positive cells were located ventral to the nA, starting 1200–1400 \textmu m caudal to the posterior limit of the FMN (defining the “r4” location), and extending caudally over 300 \textmu m (Fig. 6C, C’).

Based on these anatomical data, transverse medullary slices were prepared from control and \textit{looptail} mutant embryos, and preBo¨tC rhythmic activity was recorded using calcium imaging. Spontaneous and rhythmically organized calcium transients were present in all slice preparations examined irrespective of the genotype (Fig. 7A, B, C, first traces). Activity frequencies did not differ significantly among genotypes (summarized in Fig. 7D). Mean frequency was 4.2±0.5 burst/min in \textit{+/+} slices, 3.4±0.4 burst/min in \textit{Lp/+} slices and 5.4±1 burst/min in \textit{Lp/Lp} slices. Exogenous application of SP (10^{-7} M) induced a significant frequency increase (≅1.5–2.2 fold) over baseline in all three genotypes (Fig. 7A, B, C, second traces; summarized in Fig. 7D). In contrast, 10 \textmu M riluzole did not change activity frequency, while 10 \textmu M CNQX blocked rhythm generation in all preparations and all genotypes (Fig. 7A, B, C, two bottom traces; summarized in Fig. 7D). These results demonstrate that rhythmically active preBo¨tC oscillators are present in \textit{looptail} mutants. Taken together with the immunostaining analysis, these data indicate that the preBo¨tC oscillator develops in the correct location and functions normally in \textit{Lp/+} and \textit{Lp/Lp} embryos.

Functional coupling between the e-pF and preBötzC oscillators in \textit{Lp/+} embryos

Finally, we investigated whether a functional coupling between the two respiratory oscillators was established in \textit{Lp} mutants. Hence we examined the consequences of a change in the pH of the bathing solution on the activity of the preBo¨tC through recording of the phrenic nerve (C4) root activity. We showed previously that the e-pF is intrinsically sensitive to acidification [7], whereas the preBo¨tC is not [9], and that the two oscillators are functionally coupled at E16.5 [7]. In control embryos (n = 6), nerve recordings from C4 roots revealed stable rhythmic bursting activity generated at a mean frequency of 7.3±0.5 burst/min. Lowering the pH from 7.4 to 7.2 induced a significant increase in C4 activity frequency up to 14.6±0.7 burst/min (Fig. 8A, C). Similarly, in preparations obtained from \textit{Lp/+} embryos (n = 6), rhythmic phrenic discharges, generated at a frequency of 6.5±0.5 burst/min at pH 7.4, increased in frequency to 14.9±0.6 burst/min at pH 7.2 (Fig. 8B, C). These results show that pH sensitivity is preserved in the \textit{Lp/+} mutant. In addition, there was no
statistical difference in C4 frequencies between the two genotypes in control conditions (pH 7.4), suggesting that the preBo¨tC receives an excitatory input from the displaced e-pF in Lp/+ heterozygotes. Together, these data strongly suggest that the e-pF and the preBo¨tC oscillators are functionally coupled, despite the abnormal position of the e-pF in the Lp/+ mutants. We could not examine functional coupling between the oscillators in Lp/Lp mutant embryos due to the absence of phrenic roots.

**Discussion**

Using calcium imaging and immunostaining on isolated hindbrain preparations from the Wnt/PCP Vangl2 mutant looptail, we show here that neurons constituting the embryonic parafacial and preBo¨tC respiratory oscillators are specified correctly and form functional oscillators generating rhythmically organized activities even though the e-pF is mis-positioned in mutant embryos (Fig. 9). Significantly, in Lp/+ mutants, chemosensitive entrainment of the respiratory-like rhythm is conserved, indicating that inter-oscillator connectivity may also be preserved. These data indicate that robust developmental processes are involved in the establishment of the two distinct respiratory oscillators required for breathing regardless of a largely disturbed hindbrain anatomy.

**Failure of e-pF neuron caudal migration in Lp/Lp embryos**

In Lp/+ embryos, the e-pF neurons were displaced medially, but their rostro-caudal positions (defined by activity mapping and immunostaining) were less affected (Fig. 9). In addition, the preBo¨tC could be definitively identified in Lp/+ embryos based on the position of the nA. By contrast, the e-pF and preBo¨tC neurons (defined by activity mapping and pharmacology) were farther apart in Lp/Lp embryos compared to Lp/+ embryos (Fig. 3), consistent with a significant rostral displacement of the e-pF neurons. Given that the dimensions of the rhombomeres and their patterning are not affected in Lp/Lp embryos (Fig. 1), and that the rostro-caudal position of the preBo¨tC is similar between Lp/Lp and Lp/+ embryos (Fig. 2), the location of the FBM neurons in r4 in Lp/Lp embryos indicates that e-pF neurons in these embryos are mostly located in r5 rather than r6 (Fig. 9), reflecting in these neurons a failure of caudal migration out of r5.

**Establishment of functional respiratory oscillators in looptail mutants**

Proper development of and interconnection between the e-pF and preBo¨tC oscillators is essential for functional breathing. We provide several lines of evidence for an effective coupling between the e-pF and the preBo¨tC in looptail mutants. First, one function of the e-pF is to increase respiratory frequency through interconnections with the preBo¨tC. This rhythm-promoting function of the e-pF was preserved in Lp/+ embryos, since the preBo¨tC frequency was significantly slower in slices (lacking the e-pF) than in whole hindbrain preparations (including the e-pF). Second, blockade of glutamatergic connections with exogenous application of CNQX induced in all genotypes, an increase in e-pF activity frequency, likely resulting from the silencing of the preBo¨tC oscillator [7], and freeing the e-pF from the frequency constraint imposed by interconnections with the preBo¨tC. Third, the increased phrenic frequency in response to acidosis in Lp/+ mutants also indicates that the two respiratory oscillators are functionally connected. In addition, we have shown that the two e-pF oscillators are bilaterally synchronized. This demonstrates that commissural connections between the two e-pF oscillators are established both in Lp/+ and Lp/Lp mutants. Taken together, multiple aspects of the circuitry underlying the function of the respiratory rhythm generator are maintained in the looptail mutants regardless the altered anatomical position of one of its two key components.

Another function of the e-pF/pFRG is to mediate the central response to acidosis, even at embryonic stages [9,29,30]. Gourine et al. (2010) [11] identified astrocytes as an important component for chemoreception in adults, since they are associated with blood vessels at the ventral surface of the hindbrain, and have the ability to modulate breathing pattern by responding to pH changes. The ventral position of e-pF neurons in the embryo may be a pre-
requisite for a functional interaction with these astrocytes. Our observation that a change in the pH of the bathing medium induces a change in the frequency of e-pF activity in Lp/+ and Lp/Lp embryos, and is able to affect phrenic nerve activity in Lp/+ embryos, indicates that the neuronal response to acidosis is preserved in Lp/Lp mutants. Thus, although many e-pF neurons fail to

Figure 8. Response to a low pH challenge is unaffected in the Lp/+ mutant. Integrated phrenic discharge (Int C4) at pH 7.4 (upper trace) and pH 7.2 (bottom traces) for control (A) and Lp/+ (B) embryos at E16.5. C: Quantification of burst frequencies for control (left) and heterozygous (right) embryos at pH 7.4 (white bars) and pH 7.2 (gray bars). Numbers of hindbrain preparations analyzed are indicated on the bars. The motor output of the respiratory network recorded from C4 nerve roots is comparable in Lp/+ and wild type preparations in control conditions, and the response to low pH is preserved in the Lp/+ mutant.

doi:10.1371/journal.pone.0031140.g008
their putative birthplace in r5. Since this failure to migrate mirrors
may result from failure of the neurons to migrate caudally out of
region, and form an active network generating rhythmically
Lp/– mutants [9], although their
migration. This mis-location
resulting in the complete absence of FB
neurons in Iles1cre/+; Phox2blox/lox
mutants [9], although their
ability to constitute a functional oscillator remains to be established
in this context. Consistent with these observations, our data show
that irrespective of the position of the FMN (ventral, rostral or
even dorsal) in Lp/+ and Lp/Lp embryos, e-pF neurons migrate
radially in a normal fashion to the ventral surface in the r5–r6
region, and form an active network generating rhythmically
organized activity. Interestingly, many e-pF neurons are located
rostrally, in an r5 position, in Lp/Lp mutants. This mis-location
may result from failure of the neurons to migrate caudally out of
their putative birthplace in r5. Since this failure to migrate mirrors
the failure of FM neurons to migrate out of r4 in Lp/Lp mutants,
Vangl2 may be independently required for the caudal migration of
FM neurons and e-pF neurons. Vangl2 is likely to regulate these
neuronal migrations by functioning in other cell types since the
gene functions non cell-autonomously to regulate facial motor
neuron migration in zebrafish [13], and is not expressed in e-pF
neurons at all stages tested (E12.5–14.5), spanning their migratory
phase (DMG and AC, unpublished data). Unlike FM neurons,
which migrate to form a compact nucleus (FMN), e-pF neurons
typically show a broad rostro-caudal distribution such that
significant numbers of e-pF neurons are found hundreds of
microns caudal to the FMN. As a result, even in Lp/Lp embryos,
some e-pF neurons are located in the r6 region (Fig. 2), even
though most of them fail to migrate out of the r5 region. This
suggests that other mechanisms, in addition to Vangl2-mediated
signaling, may also regulate e-pF neuron migration.

Consequences of defective neuronal positioning on
physiological function

The link between defective neuronal patterning and altered
physiology is supported by studies of mouse cerebellum mutants
where neural circuits regulating motor coordination are affected
[33], and of hindbrain segmentation mutants where neural
circuits regulating respiration are defective [7,34]. Therefore,
it is notable that the e-pF and preBotC oscillators exhibit normal
physiological characteristics in Lp/+ and Lp/Lp embryos despite
mis-positioning of e-pF neurons.

Lp/+ embryos survive to adulthood and reproduce, indicating
that breathing and jaw-associated chewing behaviors are func-
tional even though the e-pF neurons and mainly the FM
neurons, which respectively control these behaviors, are
mis-positioned in these animals. These observations demonstrate that
neural circuits can tolerate changes in the positioning of
cell-autonomous component of the central chemoreception.

Mechanisms regulating e-pF neuron migration

The radial migratory pathways of the FB and e-pF neurons
overlap in space and time. Following caudal migration from r4
into r6 between E11.5–13.5, FB neurons migrate radially
through the pial layer to form the FMN [23,31,32]. Similarly,
after caudal migration into r6 between E12–14, e-pF neurons
migrate radially past the FMN to form the e-pF at the pial surface,
ventral to the FMN [33]. Since the closely-apposed FB and e-pF
neurons take similar migratory pathways toward the ventral
surface in r6, it is possible that 1) the e-pF neurons are dependent
on FB neurons for migratory cues, or 2) the e-pF and FB
neurons migrate independently of each other using common or
distinct environmental cues. The latter possibility is reasonable,
given that both e-pF and FB neurons express Phox2b, which
could regulate expression of migration-associated genes. However,
numerous studies now indicate that e-pF and FB neurons migrate
independently of each other. First, in Eg2 knockout and
Phox2b-conditional mutant embryos, FB neurons migrate
naturally even though the e-pF neurons are
anatomically and functionally absent [7,9,20]. Importantly, e-pF
neurons migrate normally in the complete absence of FB
neurons in Iles1cre/+, Phox2blox/lox mutants [9], although their
ability to constitute a functional oscillator remains to be established
in this context. Consistent with these observations, our data show
that irrespective of the position of the FMN (ventral, rostral or
even dorsal) in Lp/+ and Lp/Lp embryos, e-pF neurons migrate
radially in a normal fashion to the ventral surface in the r5–r6
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neurons, which respectively control these behaviors, are
mis-positioned in these animals. These observations demonstrate that
neural circuits can tolerate changes in the positioning of
cell-autonomous component of the central chemoreception.
ing the neural network that regulates breathing. Importantly, we show that a functional respiratory circuit can be established inspite of profound neuronal migration and neural tube closure defects in the hindbrain, thus highlighting the robustness of developmental events participating in the formation of neuronal oscillators mediating an essential physiological function.

Acknowledgments

We are indebted to Roland Quinney, Jackie Harrison and colleagues at MRC Harwell for their help in coordinating shipments of looptail mice to France for experiments. These studies were initiated by A.C. during a research leave from the University of Missouri to CNRS, Gif-sur-Yvette. A.C. would like to thank the members of the Fortin and Champagnat labs for their hospitality during his stay.

Author Contributions

Conceived and designed the experiments: MT-B JC GF AC. Performed the experiments: MT-B JB DMG GF. Analyzed the data: MT-B JB AC. Contributed reagents/materials/analysis tools: MES CD JNM. Wrote the paper: MT-B AC. Conceived of and coordinated all aspects of the project: AC.

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